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INTERACTIONS AMONG INVADING TICKS, WILDLIFE, AND ZOONOTIC PATHOGENS

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Sarah Anne Hamer

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INTERACTIONS AMONG INVADING TICKS, WILDLIFE, AND ZOONOTIC PATHOGENS

By

Sarah Anne Hamer

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ABSTRACT

INTERACTIONS AMONG INVADING TICKS, WILDLIFE,
AND ZOONOTIC PATHOGENS

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Emerging vector-borne zoonotic diseases arise from complex interactions among pathogens, bridge vectors, wildlife hosts, and humans. *Ixodes scapularis* - the blacklegged tick – hosts a suite of zoonotic pathogens it in the Midwestern United States, including the agents of Lyme disease, human granulocytic anaplasmosis, and babesiosis. Risk of these diseases is increasing due in part to spread of *I. scapularis*. Over six years, I have investigated hypothesized co-invasion of *I. scapularis* and pathogens in the Midwest by tracking the spatial and temporal dynamics of invasion using of wild bird, mammal, pet dog, and vegetation drag surveys, with subsequent genetic analyses of pathogens. My studies operated not only where *I. scapularis* is readily found, but also in zones beyond its detected distribution yet susceptible to establishment.

In Michigan, I have documented in real-time a northward invasion of blacklegged ticks from a focal area of recent blacklegged tick detection on the west coast. White-footed mouse surveillance was most sensitive method for detection of low-density tick populations due to their importance for feeding immature ticks as well as the ease with which large sample sizes are acquired. Invasion was not as apparent in the eastward, inland direction, and future studies will address ecological parameters that may explain the differential distribution. Compared to the wildlife studies, surveillance of pet dogs was less sensitive for detection of blacklegged tick distribution, likely because of the common practice of canine tick chemoprophylaxis.

While the detected distribution of blacklegged ticks was focal and expanding, I detected a wider distribution of the Lyme disease pathogen, *Borrelia burgdorferi,* in alternative tick species and wildlife hosts. This pattern was most evident in bird-associated ticks at a focal site 90 km to the east of *I. scapularis* invasion front, where *I. dentatus* ticks removed from birds harbored 3.5% infection prevalence with *B. burgdorferi*, and no *I. scapularis* ticks were found. The *B. burgdorferi* strains found in this scenario of 'cryptic' transmission were comprised of many novel types not previously described from Lyme disease endemic areas, and also at least three strains previously associated with disseminated human Lyme disease. I hypothesize that cryptic cycles reduce the time lag between *I. scapularis* invasion and the build-up of infection prevalence, and may result in the introduction of novel strains to human and canines.

Across five states of the Midwest that represent a continuum of establishment of *I. scapularis,* I hypothesized that patterns of diversity of pathogens within *I. scapularis* may be useful in elucidating the broad-scale tick invasion and subsequent disease emergence. Analysis of 1565 adult *I. scapularis* ticks from 13 sites across five Midwestern states revealed that tick density, infection prevalence with multiple microbial agents, co-infections, and strain diversity of *B. burgdorferi* were positively correlated with the duration of establishment of tick populations, though observed differences were subtle. Cumulatively, these data suggest that the invasion of ticks and emergence of various tick-borne diseases may be more complex than the traditional scenario whereby infected, invading ticks are the only means of introduction of pathogens to naïve communities.

To my mentors, colleagues, family and friends.

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CHAPTER 1

Introduction

"Nowadays we live in a very explosive world, and while we may not know where or when the next outburst will be, we might hope to find ways of stopping it or at any rate damping down its force. It is not just nuclear bombs and wars that threaten us, though these rank very high on the list at the moment: there are other sorts of explosions - ecological explosions. An ecological explosion means the enormous increase in numbers of some kind of living organism - it may be an infectious virus like influenza, or a bacterium like bubonic plague, or a fungus like that of the potato disease, a green plant like the prickly pear, or an animal like the grey squirrel ... they may develop slowly and they may die down slowly; but they can be very impressive in their effects, and many people have been ruined by them ..."

--Charles Elton, (1958)

My dissertation investigates interactions among invading blacklegged ticks, their wildlife hosts, and a suite of zoonotic pathogens that are vectored by blacklegged ticks across the Midwestern United States. I have studied the invasion and establishment of blacklegged ticks within a zone of active invasion in Michigan, and I compare these findings to the status of ticks in several endemic areas where they have been established for various amounts of time. I also present data on diversity and incidence of multiple zoonotic pathogens across the Midwest. Throughout this dissertation, my emphasis is on understanding the changing status of blacklegged ticks and the Lyme disease pathogen, which is the agent of the most significant vector-borne disease presently occurring in the United States (Bacon et al. 2008).

My dissertation begins with this introductory chapter (Chapter 1), which reviews important recent developments in the study of emerging and resurging vector-borne zoonotic diseases. This review emphasizes recent advances in molecular and geographical analysis technologies have advanced the field of vector-borne disease ecology. Lyme disease is introduced as one example in a series of four vignettes that

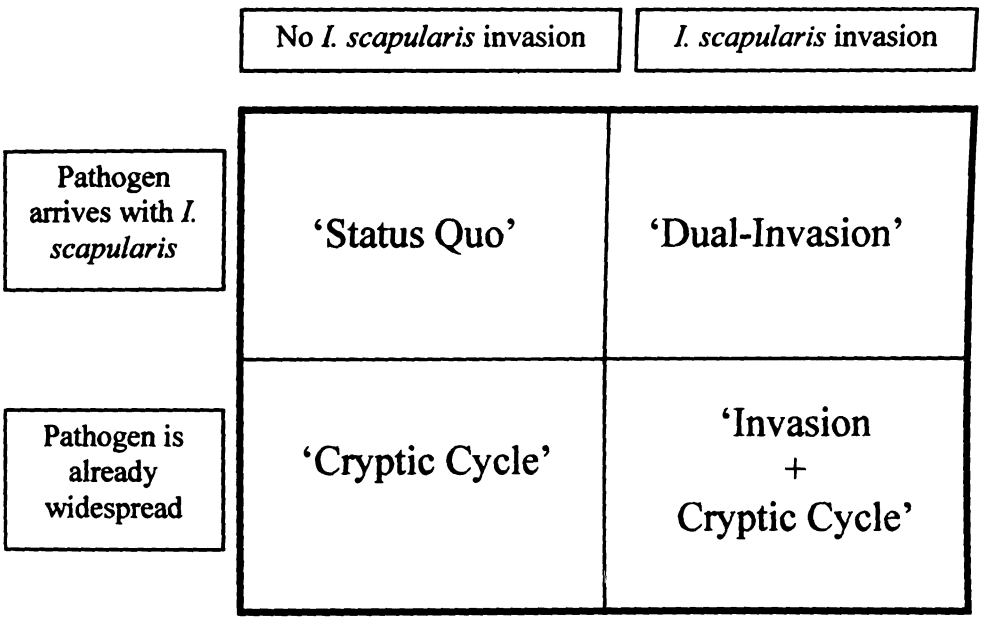
each illustrates contemporary problems in zoonotic disease emergence. The next five chapters (Chapters 2-6) each present original data collected in Michigan and the broader Midwestern US that have either been published within the scientific literature (Chapters 2-4) or is preparation for submission to a journal (Chapters 5-6). Each of these data chapters is formatted as a stand-alone manuscript. Chapter 7 provides a synthesis of key research findings and presents my ideas on future research directions.

The original research studies presented herein were sparked by findings of blacklegged ticks in previously uncolonized areas of southwestern Michigan (Foster 2004). Early in my program, I developed some competing hypotheses regarding the invading disease system, and organized them into a two-by-two table (Figure 1.1); these hypotheses are described further in Chapter 3. Research to address these hypotheses aimed to determine whether an invasion was indeed occurring, and if so, the extent to which the blacklegged tick and the Lyme disease pathogen were co-invading (the classical notion) versus independently invading or already established. Testing these hypotheses required me to sample for the Lyme disease pathogen in wildlife hosts and alternative tick species in areas outside of the detected distributional range of blacklegged ticks (Chapters 3 and 5). This is a contrast to many prior studies that only tested for the presence of the pathogen in areas with sympatric blacklegged ticks. Chapter 4 presents an assessment of the use of pet dogs as sentinels for tick invasion and disease emergence; this surveillance tool is well-utilized in Lyme disease endemic areas, but has not previously been studied in an area undergoing invasion by blacklegged ticks.

In order to more completely understand how and why the invasion of this disease system is occurring in Lower Michigan, I was interested in also studying the system

where it has been established for longer durations of time. The upper Midwestern United States (one of the two broad endemic foci for Lyme disease; the other being the Northeast) encompasses gradients of establishment of blacklegged ticks. An ecological principle is that the center of origin of a species is likely to also serve as the center of diversity for that species and related taxa (Cain 1944). I extended this notion to the blacklegged tick and its associated pathogens, for which I hypothesized that the areas where blacklegged ticks have been documented as established in the Midwest for the longest duration (Minnesota, Wisconsin, Michigan's Upper Peninsula) will harbor the greatest diversity of tick-borne pathogens, both at the inter- and intra-species levels, in comparison to areas where blacklegged ticks are currently invading (Chapters 2 and 6). This dissertation is therefore not only a means for better understanding the complex nature of biological invasions in general, but also for collecting distributional and infection prevalence data that may be useful to medical communities and outdoor recreationalists to reduce risk of tick-borne diseases.

Figure 1.1. Two-by-two table of hypotheses regarding the invasion of the blacklegged tick, *I. scapularis*, versus that of the Lyme disease pathogen, *B. burgdorferi*, based on observations of the distribution of each species. Hypotheses are described in detail in Chapter 3.



Revolutions in the study of emerging vector-borne zoonotic diseases

Abstract

Many vector-borne zoonotic diseases (VBZDs) are emerging into new zones or resurging from their past or current endemic ranges, and such spread is usually mediated by anthropogenic factors. Emerging VBZD systems involve invasive species, in which the invader is a vector, pathogen, reservoir host, human, or combination thereof, and VBZD emergence can be understood using the paradigms of classic invasive species biology. The advent of molecular and geographic analytical tools has revolutionized the study of VBZD. Through highlighting selected emerging VBZDs in a series of vignettes, I demonstrate the synergy of the molecular and geographic analytical tools that allow for a better understanding of disease across a heterogeneous landscape. -- a prerequisite for effective control.

Introduction

Vector-borne zoonotic diseases (VBZDs) are significant sources of human morbidity and mortality. VBZDs are a subset of zoonotic diseases, i.e., those disease systems in which pathogens of humans circulate in animal reservoirs. VBZDs arise from the interactions among four key players – pathogens, vectors, vertebrate reservoir hosts, and humans - all within a common environment that is conducive for disease transmission. The pathogens include viruses, rickettsiae, bacteria, protozoan parasites, flukes, and filarial nematodes, and encompass a diverse array of life cycles. The arthropod vectors involved in these disease systems typically are blood feeders in at least one, or sometimes all, life stages and include mites and ticks (Order Acari, Class

Chelicerata) and many groups within the Class Insecta. Vertebrate hosts include many species of mammals and birds.

Emergence is the process that results in a measurable increase in the prevalence of infection in humans, vectors, or reservoir hosts. Emergence is characterized by an expansion in geographic distribution of infection, and may involve infection of new host species. Zoonotic pathogens comprise over half (58%) of all human pathogen species (n = 1407 total), and nearly three-quarters (73%) of human pathogens that are classified as emerging or reemerging (Woolhouse and Gowtage-Sequeria 2005). Zoonotic pathogens are therefore more likely to show emergent properties compared to non-zoonotic pathogens, owing to the transmission probabilities inherent in the interfaces of humans, domesticated animals, and wildlife.

Many emerging VBZDs are receiving renewed attention given the availability of new tools and technologies which facilitate their detection and assessment of the health risks they pose across wide geographic areas. The purpose of this chapter is to review the status of emerging VBZDs with a particular perspective on two aspects: First, I identify parallels between emergence of VBZDs and invasion biology. Secondly, I highlight how major technological revolutions in biological sciences (specifically in molecular biology and quantitative geographic analyses) have spurred our understanding of emergence of VBZD, providing new conceptual frameworks to not only allow retrospective description but also prediction. These themes will be highlighted in a series of five vignettes.

VBZDs as multi-factorial systems. VBZDs represent complex ecological systems that involve multi-factorial abiotic and biotic components. Abiotic factors include rainfall,

temperature, soil, topography, hydrology, and climate. Biotic factors include the pathogen, the vector, and the suite of vertebrate reservoir hosts, each of which can be further subdivided. For example, pathogens typically circulate as various strains that have differing virulence properties; vectors have different life stages and pathogen infection may or may not be maintained during transition from one stage to the next; vertebrate reservoir host populations have age and sex structures that influence the ability of the host to be infected and to infect. Furthermore, population dynamic processes in vector and reservoir host populations influence pathogen transmission. For example, the annual phenological appearance of nymphal *Ixodes scapularis* ticks prior to larvae results in infection in white-footed mice, which then become an infective reservoir to larvae, permitting perpetuation of infection at foci in the absence of transovarial transmission.

Landscape epidemiology provides a useful conceptual framework for understanding the distribution of VBZDs. The field of landscape epidemiology was influenced by the founding studies of tick-borne viral infections conducted by Evgeny Pavlovsky in the former Soviet Union, where concepts of endemicity, focality, and nidality were formed (Pavlovsky 1966). Landscape epidemiology assesses the presence of appropriate abiotic and biotic factors which permit a pathogen to occur in a particular geographic area and to persist there over time. These factors comprise a biogeocenose, or aggregate of living and non-living environmental properties. Importantly, this aggregate can be quantified using modern tools of landscape ecology such as satellite imagery, and detailed databases of land cover and land use. This allows classification of the heterogeneous and patchy nature of the landscape, with only particular patches being suitable to support the disease system. These patches are called foci or nidi (see Table

1.1 for glossary of VBZD-related terms). The disease system is endemic when it exists at nidi with no need for external inputs. Landscape epidemiology permits development of risk maps, based upon models of environmental receptivity and landscape heterogeneity, that provide guidance for public health interventions to reduce disease risk (see below).

The basic reproductive rate of a pathogen, R_0, is a central concept in disease ecology, and it can be defined as the number of secondary infections that arise from a single infected host in a population (Anderson and May 1982). In the case of VBZD, the basic reproductive rate of the pathogen depends upon the infection prevalence of the vectors, their rate of transmission of the pathogen to reservoir hosts, and in turn, the rate at which reservoir hosts infect new vectors. When a pathogen that enters a new host or vector population, one of three outcomes may occur: it may become locally extinct ($R_0<1$); it may persist in steady-state ($R_0=1$); or it may increase in prevalence ($R_0>1$). The true reproductive rate of an invasive pathogen is not easily defined, as the number of unsuccessful invasion attempts is not possible to measure. Humans can influence R_0 through many different environmental perturbations, including intrusion into a landscape of endemic transmission and changes in land use and land cover to increase vector density, wildlife reservoir host density, and their interactions.

An important component of the biogeocenose that influences R_0 is the biodiversity of the community of vertebrate hosts. In VBZD systems in which the vector is a generalist and there exists a community of vertebrate host species that differ in their reservoir competence, the dilution model may operate (Ostfeld and Keesing 2000). According to this model, a vertebrate community with high species diversity contains

more reservoir-incompetent hosts than does a poorly-biodiverse community that may be dominated by only the most competent reservoirs. Thus, in the biodiverse community, the less-competent and incompetent hosts divert vectors from feeding on the competent hosts and thus dilute the infection prevalence and disease risk. In the context of VBZD, the dilution model has been demonstrated for Lyme disease (LoGiudice et al. 2003; LoGiudice et al. 2008), tick-borne encephalitis (Perkins et al. 2006), and West Nile virus (Swaddle and Calos 2008), but lack of effects have also been documented (Loss et al. 2009) and separation of density versus diversity effects is challenging (Begon 2008).

One of the fascinating aspects of emerging VBZDs is that a single vector and a few vertebrate hosts may support many pathogens within the same system at the same foci. For example, the blacklegged tick *Ixodes scapularis* and small rodent hosts maintain transmission of the agents of Lyme disease (bacterium), human granulocytic anaplasmosis (rickettsia), babesiosis (protist), and deer tick virus encephalitis (virus) in Northeastern and Midwestern United States.

Synthesis of invasion biology and emerging disease

Invasion biology is a branch of ecology which deals with changes in distribution of organisms with undesirable properties such as exotic species, pests and weeds. Good examples of recent note are the zebra mussel and the emerald ash borer. The process of biological invasion has four main components (With 2002): (1) a landscape exists that includes a receptive environment at some locations for the establishment of the invading organism, were it to be introduced; (2) introduction of the invasive species occurs ; (3) the invasive species establishes into an endemic status, then spreads locally and often

proliferates to the detriment of native species of similar biology; (4) the invasive species spreads to new regions from its newly established sites, permitting regional spread.

Parallels and analogous processes to invasive species are easily recognized with emerging VBZDs, in that both involve: (1) a process involving a receptive environment, an organism with invasive properties, and a route of introduction followed by establishment and spread; (2) retrospective analyses dominate; (3) an anthropocentric point of view dominates; (4) common methods of landscape ecology. Outstanding examples of importance currently include plague, Lyme disease, tick-borne encephalitis, American cutaneous leishmaniasis; each of these is discussed in detail below. The tempo of invasion-establishment-spread dynamics may be very fast (e.g., West Nile virus, transcontinental in a few years), decadal (e.g., Lyme disease and tick-borne encephalitis, regional emergence and spread over decades), or emergence over centuries (plague). The appearance of human infection may be the first signal that an invasion and establishment of a pathogen has already occurred, given that surveillance for invasive VBZD pathogens is not typically conducted among vectors, wildlife, or domesticated animals in the absence of human disease.

Mechanisms of invasion of pathogens associated with VBZDs are poorly known but clearly a common element driving most is anthropogenic change to the landscape. Emergence of many VBZD is supported by rapid movement of people, goods, and animals via commerce and other physical means. For example, the earliest records of introduction of yellow fever virus and its urban vector *Aedes aegypti* were with colonial and slave-trade shipping routes from the Old to the New Worlds. Modern transportation via aircraft and on large transport ships facilitates movement of pathogens either in

infected vectors (likely the case for West Nile virus), infected animals (rats and their fleas in the case of plague), or on living agents such as migrating birds or pet animals taken from one place to another. Climate change has been proposed as an element driving the emergence of some VBZDs, although strong tests of this relationship are lacking (Randolph 2004).

Revolutions in the study of VBZDs

The ability to study VBZDs has advanced markedly in the past 30 years with the advent of new tools for molecular and geographic analysis. Herein, I refer to these advances as revolutions, because they continue to be so substantial and allow for comprehensive analyses of VBZDs that were not before possible. These revolutions arose during the era (which continues into present day) of unprecedented increases in the rate of emergence of many VBZDs. Whereas historically most diseases are responded to in a retrospective fashion, the biological revolutions collectively afford the ability to predict when and where transmission will occur such that limited public health resources may effectively be targeted to slow species invasions and reduce human disease.

VBZD research has progressed where molecular tools meet spatial analysis. The distribution of infected bites of vectors across a landscape can be measured by molecular tools which are used to detect prevalence of infection with pathogens in vectors, which can then be mapped to show variation spatially. For example, Ruiz et al. (2010) used spatial and statistical modeling to analyze and predict mosquito infection with WNV at fine spatial and temporal scales around Chicago, IL. The authors found that increased air temperature was the strongest temporal predictor of mosquito infection, and some terrain

characteristics, including impervious surfaces and elevation in Chicago, also helped to predict infection. The origin of WNV that invaded New York in 1999 was determined through phylogenetic analysis with 33 other WNV strains and 8 related viruses from across the world, in which the New York strain most closely related to a WNV strain from a dead goose in Israel in the prior year (Lanciotti et al. 1999), suggesting a Mediterranean origin. Through a mosquito population genetic analyses based on microsatellite loci, the rapid westward spread of the virus across the country (in contrast to the predominant north-south patterns of migratory birds) has recently been attributed to dispersal mediated by the mosquito vector *Culex tarsalis* (Venkatesan and Rasgon 2010) The analysis of the recent outbreak of chikungunya virus in Italy in 2007, in which autochthonous transmission of a viral strain phylogenetically most similar to strains identified in an earlier outbreak in India (Rezza et al. 2007), provides yet another example in which molecular and spatial analytical tools provide a central foundation for outbreak investigations.

Prediction of emergence of a VBZD is possible only after understanding disease systems in their natural enzootic foci (Rogers and Randolph 2003). A first step in VBZD research is often to define the density and geographic distribution of one or more components of the disease system (the vectors, hosts, and pathogens). Typically, vectors are collected and their infection status is determined which allows computation of the 'entomological risk' (Mather et al. 1996), which is directly related to human disease risk. Quantifying infected vectors provides important information for public health agencies as a form of surveillance. Beyond surveillance of the current status, however, the tools of the biological revolutions allow the use of data to achieve the goal of prediction. To do

so, the following steps are generally followed (and will be expanded upon below): (1) Field specimens are collected from a place and during a time that is biologically meaningful. Field samples may be individuals of the vector species, or samples of tissue or blood from hosts including wildlife, domestic animals (including sentinel species), or humans. (2) Field specimens are assayed for infection with the pathogen or antibodies to the pathogen in the laboratory. Presence/absence, point prevalence, or prevalence across a time period may be determined. Further genetic characterization of the pathogen and strain-level associations with hosts may be made. (3) Infection data are modeled to predict landscape areas with high VBZD risk. A discussion of various aspects of VBZD research and advances in light of the biological revolutions follows.

Field collections

While the processing of samples in the laboratory and spatial analyses of data have become sophisticated with the tools of the biological revolutions, the methods of collecting field specimens have remained simple, effective, and relatively inexpensive. For example, the drag cloth is a tool used for collecting host-seeking ticks, and it is a one-meter-square piece of corduroy cloth that is light in color and is dragged over the low-lying vegetation behind the field worker as he/she hikes through the forest or field (Sonenshine 1993) (Figure 1.2). Host-seeking (questing) ticks respond to the cues of carbon dioxide, heat, and movement of the incoming host, and attach to the large surface area of the cloth upon contact. Ticks can easily be observed against the light cloth, and are plucked off and preserved for laboratory work. The most common mode of collecting mosquitoes is using a light trap. The original light trap design, the New Jersey Light

Trap, was made in 1942; in 1962, the design was revised and marketed as the Centers for Disease Control and Prevention miniature light trap and it has not changed substantially since then. This trap uses a battery to power a small fan which created a draft to blow mosquitoes into a mesh bag or container, and so long as the trap is running, the mosquitoes cannot escape. A small light bulb is used as an attractant to draw mosquitoes into the trap, and collections can be increased by 'baiting' the trap with dry ice which emits carbon dioxide to mimic host respiration (Newhouse 1966). The trap is hung in a tree at a height that is dictated by the research or surveillance questions (exposure of humans versus canopy-dwellers to mosquitoes; Figure 1.2).

Molecular Revolution

The gold standards for diagnosing infection in a sample have indeed changed since the germ theory of disease was described in 1860s and culture of organisms was the mainstay of pathogen testing. With the availability of new molecular detection tools, culture-based assays are now often replaced by direct detection of pathogen genetic sequences using polymerase chain reactions (PCR) and genetic characterization by sequencing which affords strain-level analysis. The need for culture to confirm laboratory findings is often still a prerequisite for meeting case definitions for states (i.e. Lyme disease diagnosis in non-endemic states requires culture of spirochetes from the expanding erythema migrans rash as well as documentation of an increase in antibody titer). Molecular techniques do not require that the pathogens be alive for detection and characterization, and thus such techniques are useful in human diagnosis even after prophylactic antibiotics have been given and organisms have been killed.

The molecular revolution in a broad sense began in 1953, when the structure of DNA was described by Watson and Crick, after which the field of molecular biology emerged. VBZD research is inextricably partnered to the molecular biology field for the diagnostic assays that are needed to detect and characterize pathogens. High-throughput and field-amenable assays have advanced VBZD surveillance and research, allowing for a high sensitivity of pathogen detection without the time and labor associated with culture and thus allowing for timely communication of epizootics and epidemics among public health agencies. Uncultivable VBZD pathogens have been identified using molecular tools (Relman 1990; Bhattacharya et al. 2002) Such molecular approaches in VBZD research, however, must be used in conjunction with ecological data on vector and host distribution (Chaves et al. 2007). The following review highlights selected tools of the molecular revolution that have particularly advanced VBZD research.

In the 1970s, the field of serology developed, providing a suite of assays that utilize the specificity of the binding between antigen and antibody for detection of antibodies to specific pathogens within blood. These immunoassays assess a patients exposure to a pathogen as opposed to detecting the pathogen itself. Serological assays include fluorescent antibody tests and enzyme linked immunosorbent assays (Engvall 1971) which involve the sequential layering of antigen, patient sera, and conjugated secondary antibodies in wells of a slide or microtiter plate with an absorbent surface. Presence of specific antibodies in an unknown sample is indicated by a color change upon addition of a substrate to the well which reacts with the conjugated secondary antibody, if bound to primary antibody. Serological assays are useful in VBZD surveillance because direct pathogen detection is limited by the usually short duration of

viremia, parasitemia, or bacteremia within a host, and by the seasonal activity of vectors, whereas antibodies against pathogens are generally long-lived and provide a more comprehensive assessment of pathogen presence in an area.

Polymerase chain reaction (PCR) was developed in the 1980s as a method for amplifying a particular segment of an organism's genome (Mullis 1987). In VBZD research, PCR is used to test a vector or host tissue or blood for the presence of pathogen DNA. Extracted genomic DNA from the organism is mixed with primers specific for a segment of the pathogen's genome, a DNA polymerase which synthesizes the region to be amplified, and deoxynucleotide triphosphates for building new DNA. PCR takes place in a thermocycler, in which fluctuating temperatures allow DNA denaturation, primer annealing to the single-stranded DNA, and synthesis of new DNA that is complementary to the template. A typical reaction of 40 amplification cycles can be completed within hours and results in exponential amplification. Primers can be chosen to be highly specific to a single strain or species of pathogen, to be general so as to amplify all bacteria (16S ribosomal RNA primers), or to amplify highly variable regions within a pathogens genome for further characterization. PCR products are visualized using gel electrophoresis which separates the amplified DNA based on fragment size. Successful amplification of the pathogen of interest will appear as a band at the expected fragment size upon comparison to a DNA ladder. Multiplex PCR employs multiple sets of primers within a single reaction for simultaneous detection or multiple pathogens.

Real-time/quantitative PCR (rt-PCR/qPCR) provides more information that traditional PCR through a real-time assessment of the amplification status of each sample. Developed in 1993 (Higuchi 1993), rt-PCR/qPCR involve the addition of a

fluorescent-labeled DNA probe to the template, primers, deoxynucleotide triphosphates, and DNA polymerase that are used in traditional PCR. During the annealing stage of the reaction, the probe and primers bind to the template, causing the probe to degrade and fluorescence to be emitted. The amount of bound probe is quantifiable based on the amount of fluorescence emitted after each amplification cycle. One can observe the amplification after each reaction cycle using a video camera in real-time, and the cycle number during which a sample amplifies above threshold to be considered positive can be determined. Thus, rt-PCR/qPCR provide an assessment of how positive a sample is. With a carefully made standard curve of known pathogen load, one can determine absolute quantities of pathogen within samples. The quantitative information provided in qPCR can be used to identify key host and vectors with high pathogen loads, as well as to explore effects of pathogen aggregation and 'super-spreaders'.

While pathogen detection may be adequately accomplished through amplification of one conserved gene target, research questions that require genetic characterization for strain differentiation may require amplification and sequencing of a gene target that is variable among strains (such as internally transcribed spacers; ITS). Better characterization yet may be gained in sequencing multiple genes, and this forms the basis of multilocus sequence typing (MLST), developed in the 1990s (Spratt 1999). MLST creates allelic profiles to distinguish bacterial genotypes through sequencing of seven house-keeping genes that have sufficient variation due to mutation or recombination to provide many alleles per locus. Sequences at these loci are concatenated and provide a unique allelic profile which can be compared to libraries of allelic profiles for pathogens available on the internet (www.pubmlst.org; www.mlst.net).

Microarrays, also known as gene chips, are a method for characterizing testing for the presence of multiple pathogens or pathogen strains simultaneously and can be designed for DNA, RNA, proteins, and antibodies, and have recently been used to characterize VBZD agents (Liang et al. 2002; Broekhuijsen et al. 2003). A DNA mircoarray is a slide or other substrate to which a collection of up to thousands of short segments of DNA (probes) is affixed. An amplified PCR product is applied to the array and DNA hybridization occurs to the individual probes with complementary bases. This specific binding can be observed through fluorophores. Similarly, an antibody microarray uses a collection of antibodies for antigen detection, and arrays are being developed for characterization of antibodies in reservoirs of VBZD agents.

Field-formatted assays are available for detection of the agents of visceral leishmaniasis (fluorogenic probe-based PCR assay; (Quispe-Tintaya et al. 2005), West Nile virus and St. Louis encephalitis (dipstick antigen detection assay; (Ryan et al. 2003), and malaria (dipstick antigen detection assay; (Sattabongkot et al. 2004). Field-formatted assays for detection of antibodies to VBZD agents are available for visceral leishmaniasis (Sundar 1998), and for Lyme disease, anaplasmosis, and ehrlichiosis (Stone et al. 2005). These tools often provide results within minutes and allow for public health or vector abatement districts to perform surveillance and respond immediately to increases in infection, without a time lag for sample processing.

While the tools of the molecular revolution allow for rapid sample processing and test results, careful interpretation of results and relation back to the ecology of the disease system allows advancement of the VBZD field. If a pathogen is detected in a vector or host using PCR, this does not necessarily imply that the pathogen is present in a sufficient

load to be infectious. Conversely, if a pathogen is not detected, it may be the case that the pathogen is truly absent, or, that the pathogen is present but in a load that is below the detection threshold of the assay. For proper interpretation, the sensitivity and specificity of the assays as well as the epidemiologically meaningful pathogen loads must be known.

A parallel technological advance has occurred to allow for the organization, analysis, and dissipation of results from the molecular advances. Termed bioinformatics, advances in computational and statistical methods have been made to allow for analysis and interpretation of biological data. Software and hardware have been developed that use mathematical techniques to manage the unprecedented amounts of molecular information that is generated with the tools of the molecular revolution, such as genome sequencing. Genome-level data offer the ability to better define vector-host-pathogen relationships, and to identify potential targets for control of pathogen transmission. The genomes of some vectors are now known, including *Anopheles gambiae* (Holt et al. 2002), the mosquito vector of the malaraia parasite, and *Aedes aegypti* (Nene et al. 2007), the mosquito vector of the yellow fever, chikungunya, and dengue viruses, and efforts are underway for the sequencing of the genome of *Ixodes scapularis* (Hill and Wikel 2005), the tick vector of the agents of Lyme disease, anaplasmosis, and babesiosis, and *Culex pipiens* (https://www.broadinstitute.org/annotation/genome/culex_pipiens.4/Home.html), the mosquito vector of West Nile virus. Of particular interest to VBZD researchers, bioinformatics tools allow one to search the published genomes of thousands of organisms, identify genes, and align similar sequences that may contain mutations, deletions, and insertions. Large databases of identified genetic sequences are available for searching such as GenBank, an open-access, web-based database of nucleotide

sequences hosted by the National Center for Biotechnology Information as a forum for deposition of annotated sequence information from laboratories across the world. GenBank is able to be searched using BLAST (the Basic Local Alignment Search Tool) to determine the similarity of unknown gene sequences to identified and published sequences (Altschul et al. 1990). Libraries of MLST allelic profiles are now available in similar open-access format (http://pubmlst.org).

In addition to the tools for managing sequence information, significant computer-based advances have been made for surveillance and reporting of VBZD. Web portals are increasingly used for consolidation of surveillance data from different abatement or public health districts, and this internet-based sharing of data allows for efficient response to changes in prevalence. ArboNET, hosted by the Centers for Disease Control, is a national electronic surveillance system to assist states in monitoring trends in mosquito-borne disease.

Tools of the molecular revolution at work: the bloodmeal analysis

Identification of key hosts in VBZD systems allows for targeting of management to reduce transmission. The bloodmeal analysis provides an example of a process in VBZD research that has become increasingly sophisticated and informative with the advent of the tools of the molecular revolution (see (Kent 2009) for review). Many vectors are catholic (ie generalists) in their feeding behavior; that is, they are able to successfully blood-feed on a variety of vertebrate host species, either opportunistically (based on host availability) or specific to host class. Bloodmeal analysis is the process by which the blood in a vector's abdomen (that has been consumed from a host during blood

feeding event) is identified to taxonomic group of the host. When used in conjunction with data on host availability, the bloodmeal analysis allows for assessments of vector-host relationships and host selectivity by vectors; when used in conjunction with infection data, routes of pathogen transmission may be identified. Many VBZD agents are transmitted from host to vector when a host is systemically infected and a vector bloodfeeds on the host, thus in-taking a bloodmeal containing pathogens. If the vector subsequently engages in a second bloodfeeding event on a different host, then the pathogens may be transmitted from vector to host. After each bloodfeeding event, the vector must rest to digest the meal; for example, mosquitoes rest and use blood nutrients to brood eggs; ticks use blood resources to molt into the next life stage.

In a primitive bloodmeal analysis, a bloodmeal may be dissected from the vector and viewed microscopically- the lack of nuclei in erythrocytes is indicative of a mammalian-derived bloodmeal whereas presence of nuclei indicates avian or reptilian-derived bloodmeal. This technique is quite limited in its utility and a high-quality blood smear from a freshly-engorged vector is required for cell visualization. In the mid 1970's, the science of bloodmeal analysis advanced to use serology-based tests (Tempelis 1975). Serology-based tests apply the vector bloodmeal against a library of polyclonal antibodies to candidate host species. If the bloodmeal host species was represented within the library, then antigen-antibody binding occurs and the host can be identified. However, these serology-based tests were often useful in identifying only to the taxonomic order of the host due to cross-reactivity; furthermore, one must create the library of all candidate hosts which may not be comprehensive. In the late 1990s, PCR-based bloodmeal analyses were developed which, used in conjunction with heteroduplex

analysis (PCR-HDA), allowed for genus-specific bloodmeal identification (Boakye et al. 1999). After DNA extraction of the bloodmeal, PCR is used to amplify a highly-conserved region of the mitochrodrial genome and the amplicon is mixed with a driver of known identity. The mixture is denatured and then cooled for heteroduplex formation. Samples are electrophoresed and relative mobility is compared to a set of known-identity standard samples. As with the serology-based bloodmeal analysis, the PCR-HDA approach also requires a library of standards whose acquisition from the field and preparation in the lab is labor-intensive, and HDA patterns that are not represented within the standards are not identifiable.

Modern bloodmeal analyses often use PCR of a mitochondrial DNA (e.g. the cytochrome b gene), which is more useful than genomic DNA due to the high copy number and the high level of divergence between species seen in mitochondrial genes. The high sensitivity of PCR-based assays allow for partially-digested mosquito bloodmeals to be used. PCR amplicons are then run against a series of DNA probes of known identity, or are sequenced and compared to published sequences of known identity. This approach to bloodmeal analysis has been employed to identify the host of ticks from a previous life stage, when only small amounts of the bloodmeal remain (Kirstein and Gray 1999; Pichon et al. 2003).

The Geographic Revolution

While the heterogeneous distribution of human diseases and their association with certain biotic and abiotic landscape features have long been noted, the ability to quantify such relations has advanced only recently with the new tools of the geographic

revolution. The science that characterizes the relationships between disease and geography is referred to as landscape epidemiology, spatial epidemiology, medical geographics, or health geographics. Efforts to detect clusters of human infection in relation to the environment date to the cholera outbreak at the Broad Street Pump in 1854, in which John Snow mapped the street address of infected humans to detect their concentric distribution about a water pump; infection ceased upon closure of the pump. Russian parasitologist Evangy Pavlovsky (1884-1965) is credited for bringing attention to the natural nidality of VBZDs, and notes the 'propensity of the infection for definite geographic landscapes' and the 'seasonality of the disease' (Pavlovsky 1966). Many VBZD systems involve a natural, sylvatic transmission of a pathogen among its reservoir hosts, and human disease results only after anthropogenic perturbation to the biogeoscenose, or translocation of the vectors, hosts, or pathogens to novel areas. Tools of the geographic revolution include global positioning systems (GPS), remote sensing (RS), geographic information systems (GIS), and spatial statistics (see review by Kitron (1998). These tools are used in a complementary fashion in VBZD research to collect, describe, analyze, and predict disease across a landscape.

GPS is the technology that allows researchers to determine the precise spatial location of an entity using a handheld unit that receives signals from multiple satellites of known locations orbiting the earth. In VBZD research, a GPS may be used on the ground, for example, to map the locations of abiotic environmental features associated with vector production such as storm water sewers which may be breeding sites for mosquitoes (Irwin et al. 2008).

RS utilizes satellites to measure the amounts of electromagnetic radiation reflected from different objects on the earth, and the output is a series of spatially-linked values. Different features on the landscape are associated with a unique radiation signal and so they may be identified based on their patterns after the satellite signals are calibrated in relation to ground-based variables. Remote sensing has made possible the creation of widely-available spatially-referenced datasets on landuse/landcover, and the most common application of RS to VBZD research is in the identification of particular landscape features and climactic features (Rogers and Randolph 2003). In many developing countries where epidemiological or survey data lack, satellite sensors are often used to gather environmental data that may be used to predict infection risk so that control efforts may be efficiently targeted (Brooker et al. 2002). Fourier time-series analysis is a technique for extracting cyclical temporal patterns from remotely-sensed environmental data through creating a set of Fourier variables that describe the space-time features of a landscape (Rogers and Randolph 2003).

GIS is the technology that forms the basis for the geographic information science discipline, and consists of computer programs originated in the 1960s for displaying layers of spatially-referenced data for map-making or more advanced statistical analyses of spatial patterns. GIS is increasingly important in the epidemiology field (see review by Clarke et al. (1996)). The overlay process is inherent to data processing in a GIS, in which a series of individual sets of spatial data and attributes are matched based on location, and these layers are then overlaid so they may be simultaneously visualized. For example, range maps of reservoir hosts may be overlaid with those of a vector for a given VBZD, and areas of overlap may be at risk of pathogen transmission. Whereas the

classic epidemiologic focus is on the non-spatial characteristics of disease (age, sex, time), GIS allows for investigation of the spatial characteristics of disease (Pfeiffer and Hugh-Jones 2002). Within a GIS framework, one can identify the biotic and abiotic environmental features that are associated with vector, host, or pathogen presence within endemic areas. Modeling then allows for these same features to be identified in non-endemic areas, and this allows assessment of the available suitable habitat in which invasion of the disease system is likely.

Spatial statistics can be used in conjunction with the above tools to describe and predict the distribution of vectors, hosts, infection, and suitable habitats. Statistical analyses of spatial patterns often include measures of spatial autocorrelation. Autocorrelation occurs when a value at a specific location is dependant upon the values of neighboring locations such that neighboring locations are most similar to each other. Kriging is a technique in which values for unsampled sites are generated based on interpolation of values from the sampled sites. Two statistical methods that are commonly employed to predict disease in relation to landscape features are logistic regression and discriminant analysis, followed by an evaluation of the models performance through comparison of the agreement between model predictions and actual field observations (Brooker et al. 2002). These methods will be illustrated below in the description of steps in the risk-mapping process.

Tools of the geographic revolution at work: risk modeling

The risk modeling process serves as an example in VBZD research of the application of the tools of the geographic revolution to obtain a product that is useful to

understand disease risk. Risk models may be used to illustrate disease locations for alerting medical communities and the public to risky areas, test hypotheses, identify knowledge gaps, or prioritize and evaluate surveillance and control efforts (Kitron 2000). The following steps are those that are generally followed for developing a VBZD risk model, once the purpose of the model is defined. (*1*) *Data acquisition.* Datasets to be modeled must be collected- usually through a combination of field surveys, compilation of human case reports, and downloading of remotely-sensed images. These spatial data are then overlaid within a GIS environment. (*2*) *Modeling.* Logistic regression modeling is used to predict group membership; in the case of risk modeling, logistic regression is used to evaluate the association between the probability that an area is at high risk for a disease and various landscape features. The landscape features serve as the independent variables, and the dependent variable of the model is discrete (such as high risk or low risk). Multiple models are constructed that use different combinations of landscape features to predict risk, and the most parsimonious model is selected using Akaike's Information Crietrion (AIC; (Akaike 1974). It is important to consider the next necessary step- model validation- before making the model, validation efforts may require a subset of data to be reserved (not used in model creation) for use in validation. For example, Eisen et al. (2007) used 80% of available data to build their model of habitat suitablility for plague, and reserved the remaining 20% for model evaluation; Brooker (2002) based their model of schistosomiasis infection of a randomly-selected 50% subset of available data, and used the remaining 50% for ROC analysis. (*4*) *Model validation.* Receiver operating characteristic curves (ROCs) can be used to assess the ability of the model to discriminate between binary categories (ie high and low risk for human disease; high and

low habitat suitability for vector establishment) by plotting sensitivity (true positives; cases in which observed disease falls within an area predicted by the model to by high risk) against one minus specificity (false positives; cases in which the model predicts an area to be high risk from which no disease is observed). The area under this curve provides a measure of the accuracy of the model (see review by Brooker et al. (2002)). Comparing the area under the ROC that results from different thresholds for assigning predictions into the binary categories provides a basis for threshold selection and improves the predictive accuracy of the final model. One can use the ROC analysis not only to evaluate a model for the geographic region upon which it was developed, but also to test how universal the model is when extrapolated to a new geographic area.

Some caution must be taken when interpreting risk maps. For example, areas without data are not often apparent in a map and area boundaries are not often biologically meaningful (Kitron 2000). Addressing temporal change in a risk map is often difficult, and maps quickly become outdated when VBZD systems are expanding in range. A fundamental problem exists in using the above steps to create and validate a risk map for an emerging disease system (noted by (Eisen et al. 2007);(Ostfeld et al. 2005)): habitat patches without the disease system may be so because they are not suitable for establishment of the vector, hosts, or pathogen; alternately, patches without disease may be comprised of fully suitable habitat, but the disease system invasion has simply not yet reached this area. While the former scenario represents predictive power of the model, the later scenario may result in false positives. In the risk-mapping procedure, there is an inherent assumption that the vector or pathogen being modeled is distributed everywhere that is suitable. However when a disease system is invading, this

assumption is violated. To circumvent this, one may restrict ground-truthing efforts to the geographic range which is known to have already been invaded (see Foster (2004). Furthermore, suitable habitats may be undergoing active control measures, and thus seemingly suitable patches may not support the disease system due to the effect of the control. A selective review of research studies in which VBZD systems are mapped to explain current disease and predict future disease distribution is presented by Ostfeld et al. (2005).

VBZD Vignettes

Below, I present a review of four VBZD systems that are emerging over different time scales, and whose study employs the tools of the molecular and geographic revolutions to predict risk or elucidate new relationships which improve our understanding of transmission and emergence. Each vignette is not intended as a comprehensive review of disease etiology and transmission, but instead provides a brief background to the system and highlights selected contemporary ecological research programs that apply new molecular and geographic analytical tools yield novel insight.

Vignette #1: Plague

Plague is an ancient VBZD responsible for many past epidemics including three great pandemics: the Justinian Plague in A.D. 541-542, originating in Ethiopia or Egypt; the Black Death in 1347-1351 in Eurasia; and the Modern Pandemic that began in the China in 1855 (Perry and Fetherston 1997). Unique to the Modern Pandemic is the vast geographic range expansion of disease across all continents, afforded by the movement of

infected rats and fleas on trade ships. Human plague continues into present day in Africa, Asia, and the Americas, with an average of 2821 cases reported per year for the time period of 1990-1999 of which over 80% are from Africa (Weekly Epidemiological Record, WHO). While human plague is indeed an emerging VBZD, with emergence on a slower time scale compared to most emerging VBZDs, enzootic foci of plague in rodents and fleas have long been established and transmission occurs continuously at many foci that does not regularly spill over and cause human disease (Duplantier 2005). The disease is able to spread rapidly during epizootic periods- when fleas leave reservoir hosts and feed on incidental, amplifying hosts that are susceptible to disease (review by (Gage and Kosoy 2005).

The etiologic agent of plague is the gram-negative bacterium *Yersinis pestis* that is maintained in nature in transmission cycles among rodents, lagomorphs, and their fleas. There are three *Y. pestis* biotypes that correspond with the sites of origin of the three pandemics (Guiyoule et al. 1994). *Y. pestis* was introduced to North America in 1900, and the strains of *Y. pestis orientalis* in North America are a subset of those found in Asia- the putative source of invasion (Gage and Kosoy 2005). *Y. pestis* is now under watch as a bioterrorism agent, and has been used as such in the past through the deliberate exposure of infected human or animal carcasses, release of infected laboratory-reared fleas, or weaponized pneumonic plague.

Two mechanisms of *Y. pestis* transmission have been identified: flea-borne and aerosol droplet. In the former, infection of a susceptible host may result after a mechanical transmission from contaminated flea mouthparts or feces, direct ingestion of infected fleas, or, most commonly, regurgitation of bacteria by blocked fleas. Blocked

flea transmission occurs after a flea feeds on an infectious host and *Y. pestis* organisms amplify in the fleas midgut and form a colony sufficient in size to form a blockage in the mouthparts of infected fleas (Gage and Kosoy 2005). Subsequent bloodfeeding attempts by the flea are unsuccessful because the bacterial mass prevents the passage of blood from the proventriculus to the midgut, and the flea starves after repeated attempts. The increase in bloodfeeding attempts allow for *Y. pestis* transmission when the flea regurgitates the blood and *Y. pestis* organisms from the mass into the feeding site of the host. Transmission via blockage is possible only after a long extrinsic incubation time (5 days to weeks depending on flea species), which contrasts with the relatively rapid rate of epizootic spread observed in plague outbreaks. Early-phase transmission from fleas in the absence of block formation has thus recently been identified as a transmission mechanism (Eisen et al. 2006).

While many mammals may become infected with *Y. pestis* upon the bite of an infected flea, the contribution of each host to the maintenance and spread of the pathogen differs. Reservoir hosts are those hosts that maintain sylvatic foci of transmission at a baseline level with their fleas and are able to do so in the absence of other hosts. Reservoir hosts perpetuate the pathogen in inter-epizootic periods. Specific characteristics qualify a host as a reservoir, including the ability to circulate high bacterial titers in their blood; heavy infestations by flea vectors; and residence in burrows or nests with heave flea infestations (Gage and Kosoy 2005). In contrast to reservoir hosts, epizootic hosts are usually naïve to the pathogen, and suffer high mortality upon infection which may initially result from spill-over from the sylvatic cycles (shared fleas) and subsequently develop into an epizootic. In North America, reservoir hosts for *Y. pestis*

include *Peromyscus* and *Microtis* species, and epizootic hosts include prairie dogs, ground squirrels, chipmunks, and woodrats (Gage and Kosoy 2005). Epidemics result when humans contract infection from fleas after encroaching in sylvatic or epizootic transmission cycles- in the latter case, fleas may seek humans as a host when their epizootic hosts die. *Y. pestis* infection in humans manifests in three ways in humans: bubonic plague is characterized by swollen lymph nodes called buboes at the site of flea bite, and is the most common form of disease in humans. Pneumonic plague is characterized by *Y. pestis* infection of the lungs and person-to-person transmission is possible via respiratory droplet aerosols- pneumonic plague is likely to result if plague is weaponized and used in bioterrorism. Septicemic plague is characterized by *Y. pestis* presence in the blood and may be accompanied by gangrene of the extreminities. Both pneumonic and septicemic plague may develop from bubonic plague.

Reasons for the resurgence of human plague include the discontinued surveillance for enzootic transmission once human cases cease; political unrest and other socio-economic factors that result in poverty and unhygienic living; deforestation that puts humans in contact with sylvatic cycles or creates new habitats for enzootic hosts and their fleas; introduction of new reservoir hosts to enzootic cycles; and evolution of the pathogen to increase host range or transmissibility (see review by Duplantier et al. (2005)).

Tools of the molecular and geographic revolutions have together provided a better understanding of plague transmission cycles, leading to prediction of zones of increased risk of transmission. For example, Woods et al. (2009) developed a rapid bloodmeal analysis using multiplex PCR to identify the host species for fleas that is sensitive up to

72 hours after a flea has fed. Using this technique on fleas collected in a pilot study in peridomestic settings in eastern Africa, where a majority of human cases occur in present day, the authors identified humans as the most common host across five species of flea in the Congo, followed by chickens and cats, and many fleas contained mixed bloodmeals with two of these host species. Importantly, this technique allowed for identification of a rat bloodmeal in a flea species that is typically associated only with humans, which suggests a possible mechanism of zoonotic transmission that does not require the presence of infectious rat fleas for bridging the pathogen from rats to humans. Eisen et al. (2007) used the logistic regression model approach to predict that 14.4% of the four-corner region of the southwestern United States has elevated risk of human plague. Using the locations of human plague cases in from 1957-2004 within an endemic focus of disease as the dependent data in the model, high-risk plague habitat was identified based on the presence of key habitat types at specific elevations (southern Rocky Mountain pinon-juniper, Colorado plateau pinon-juniper, Rocky Mountain ponderosa pine, and southern Rocky Mountain juniper at elevations up to 2300m).

Vignette #2: Lyme disease in the American Midwest

Ixodes scapularis, the blacklegged or deer tick, is distributed discontinuously throughout the eastern half of the United States, and is the vector of a suite of zoonotic pathogens across its range, of which *Borrelia burgdorferi*, agent of Lyme disease, is the leading cause of reported vector-borne disease in the United States (23,305 cases reported in 2005; MMWR 2007). A majority of cases of human Lyme disease are reported from two endemic foci in the northeastern and upper Midwestern United States, but new

evidence from the field suggests that these ticks are invading new areas from endemic foci. The initial epidemiological investigation of Lyme disease occurred in the 1970s when a cluster of children were misdiagnosed with juvenile rheumatoid arthritis in Old Lyme, CT, later to be associated with tick exposure and the presence of the *B. burgdorferi* pathogen. This pathogen is now known to have occurred in the eastern United States since at least the late 19th century (Steere et al. 2004). A series of changes in the landscape occurred that were favorable to the Lyme disease emergence, and continue today, such that Lyme disease remains the most common VBZD in the United States. Lyme disease is a significant source of human morbidity throughout Europe as well, where the tick species and pathogen species, host associations, and disease manifestation are different.

I. scapularis exhibits a two-year life cycle across most of its range and develops through larval, nymphal, and adult life stages (Spielman et al. 1985). During each life stage, the tick engages in one bloodfeeding event on a host that may last from 3-8 days after which the tick undergoes an inactive period for weeks to months and molts into the next life stage. Different host individuals are used during each blood meal. During bloodfeeding, transmission of *B. burgdorferi* may occur from tick to host or from host to tick, with the exception that larval ticks are usually hatched uninfected, and thus are unable to infect during the larval bloodfeeding event.

Multiple hosts are involved in Lyme disease ecology, as *I. scapularis* is a generalist feeder, and *B. burgdorferi* is a generalist pathogen. White-footed mice, chipmunks, birds, and lizards commonly host larval and nymphal ticks, whereas white-tailed deer are the most important host for adult ticks. Each host species differs in

reservoir competency for *B. burgdorferi*. White-footed mice considered the most efficient reservoir host for the pathogen, and in xenodiagnostic studies in the laboratory demonstrate that infectious white-footed mice are able to infect 70-100% of susceptible ticks (Donahue et al. 1987). Conversely, white-tailed deer are unable to maintain infection by *B. burgdorferi* and contain proteins of their complement system that kill the bacterium in situ and can clear a feeding tick of its infection (Telford et al. 1988).

During European settlement of North America, deforestation and conversion of the eastern United States to agriculture occurred, and deer were nearly extirpated due to over-hunting. These two factors are likely responsible for removing ticks and Lyme disease risk from the landscape, where maintenance of *B. burgdorferi* is hypothesized to have occurred for millennia ((Barbour and Fish 1993; Steere et al. 2004)). Reforestation occurred in the early 20th century, and deer, blacklegged ticks, and *B. burgdorferi* expanded from refugia. As deer numbers continue to rise and support tick populations, human land use patterns of building houses in the woods support increased contact between humans and ticks. As ticks do not move large distances during off-host periods, tick invasion to new habitats is a function of host movement. Mechanisms of tick invasion may include movement of ticks by birds in their migrations from endemic sites, movement by deer with relatively large home ranges, and a slower movement of ticks by small mammals with adjacent home ranges.

Tools of the molecular and geographic analytical revolutions have lead to some changes in our understanding of Lyme disease invasion, distribution, and maintenance. For example, multilocus sequence typing has been used to infer a European origin of the Lyme disease pathogen *Borrelia burgdorferi* (Margos et al. 2008) whereas earlier

research suggested a North American origin (Foretz et al. 1997; Marti Ras et al. 1997). A national survey for nymphal *I. scapularis* was recently conducted across the range of *I. scapularis* in the eastern half of the United States (Diuk-Wasser et al. 2006). Data on the density and infection prevalence of nymphal ticks has been modeled to predict nymphal density using remotely-sensed data and interpolated weather station data, which generated a continuous probability surface of the risk of encountering nymphs in the U.S. This model will be useful in identifying high risk areas for human exposure to infected nymphs, which can aid in decisions about personnel protection measures, residential or community intervention, and for vaccination programs (Diuk-Wasser et al. 2010). Furthermore, new relationships in this VBZD system have been revealed by Gatewood et al. (2009), who analyzed the relationships between climate, seasonal activity of *I. scapularis*, and *B. burgdorferi* genotype frequency across the northeastern and Midwestern United States. The authors found that the degree of seasonal synchrony of the larvae and nymphs was predicted by the magnitude of the difference between summer and winter daily temperature maximums. This finding has potential consequences for human disease, because particular human-invasive pathogen strains were more common among tick populations characterized by low seasonal synchrony.

Guerra et al. (2002) modeled the habitat suitability for the Lyme disease vector *Ixodes scapularis* in the upper Midwestern United States. The authors used both discriminant function analysis and logistic regression to identify the environmental features that best characterized the difference between tick positive and tick negative field sites. Soil order and landcover were identified as the main contributors to tick presence; in particular, sandy to loamy sand soils with deciduous, dry-mesic forests were

significant. The authors then mapped where these significant habitat types occurred to create a map of habitat suitability for the tick, which was 86% correct in classifying tick status at sites. This model has been projected to new areas (lower Michigan) and has been shown to maintain high predictive capacity in an independent test (Foster 2004).

Bloodmeal analyses have been used to identify the hosts of the Lyme disease vector tick in Europe. Bloodmeal analysis of flat ticks to determine the host feed upon during the previous bloodmeal is particularly challenging because the duration of time since bloodfeeding is on the order of months, even up to a year, and the remaining host DNA is therefore degraded (Randolph 2009). Using PCR of the 12S rDNA mitochondrial gene (Humair et al. 2007), Cadenas et al. (2007) successfully identified the host of 43.6% of questing ticks, with a range in the identification rate of 20-93% depending on month of tick collection. Combining bloodmeal analysis with *Borrelia* detection, the authors confirm the associations of *B. afzelii* and *B. burgdorferi* s.s. with rodents, and *B. valaisiana* and *B. garinii* with birds.

Vignette 3: Tick-borne encephalitis in Europe

Tick-borne encephalitis (TBE) is caused by a flavivirus like dengue, West Nile, and Japanese encephalitis viruses. *Ixodes persulcatus* and *I. ricinus* are the zoonotic vectors for the more pathogenic Eastern and Western subtypes, respectively (Dumpis et al. 1999). Climates and landscapes that promote greater seasonal overlap of nymphal and larval host-seeking support more intense enzootic transmission and greater TBE risk (Randolph et al. 1999). This is because the maintenance of TBE virus requires non-systemic transmission from infected nymphs to larvae of the next cohort (Labuda et al. 1993a). There are no vertebrate species that can maintain TBE virus systemically.

Rodents (mainly *Apodemus* spp.), however, can support non-systemic TBE virus transmission, and because they feed both stages, they are critical for TBE virus maintenance in nature (Labuda et al. 1993b; Randolph et al. 1999). Thus, TBE virus prevalence in ticks is much lower (0.5-5%, (Dumpis et al. 1999) compared with that of *B. burgdorferi sensu lato* (the agent of Lyme borreliosis, 25-50% (Hubalek and Halouzka 1998), which systemically infects many host species throughout its range.

The incidence of TBE over the last two decades generally has increased in Europe both within its endemic regions in Central and Eastern Europe (Sumilo et al. 2007) as well as in Western Europe (Broker and Gniel 2003). In a series of papers, Randolph and colleagues have scrutinized the causes for this increased incidence as putative factors include i) warmer climate, ii) improved surveillance and diagnostic assays, and iii) political changes. Using satellite imagery and field data from seven countries in Europe, Randolph et al. (2000) examined the activity of immature stages of *I. ricinus* in relation to TBE incidence. The authors found that larvae consistently started feeding and questing earlier in the year at sites within TBEv foci than elsewhere, and the larval activity in the spring was coincident with nymphs. Analysis of satellite-derived indices of land surface temperature revealed that a rapid fall in temperature in the fall predicted this activity may cause unfed larvae to overwinter with a spring emergence that is synchronous with nymphs. However, while overall climate trends may have influenced TBE incidence in predictable ways, the spatial heterogeneity in TBE dynamics within similar climatic zones and ecological landscapes indicate that changing climate cannot solely explain the dynamics of TBE (Randolph 2004; Rogers and Randolph 2006; Sumilo et al. 2007).

Instead, flow-on effects resulting from the breakdown of Communist rule created landscapes more favorable for TBE enzootic transmission as well as changes in human behavior that increased the risk of contact with infected ticks. After the transition from Communist rule, at least two changes took place that affected the landscape to increase tick population. Collective farming collapsed in the Baltic countries of Lithuania, Latvia, and Estonia (Sumilo et al. 2006). Secondary succession replaced farmland and pasture and led to a proliferation of wildlife and subsequent increase in vector ticks. Concomitant industrial collapse resulted in warmer climates - 'brightening' - due to the reduction of air pollutant emissions. This regional warming improved climate both for tick survivorship and for human outdoors activities. Thus, these socio-economic changes created more a suitable landscape for vector populations. Education and the availability of an effective vaccine allowed people to modify their risk of TBE and seemed to explain the reduction of incidence in certain areas. Available vaccination and epidemiological data in Lithuania and Latvia show that decreased TBE incidence is significantly associated with increased vaccination rates two years prior and high TBE incidence three years prior (Sumilo et al. 2009).

Vignette 4: American cutaneous leishmaniasis

American cutaneous leishmaniasis (ACL) is a VBZD involving human infection with protozoan parasites in the genus *Leishmaniasis*, in particular *L. braziliensis*, *L. amazonensis*, *L. panamensis*, *L. mexicana*, and *L. guyanesis*. Infection in humans is multifarious, but is characterized by focal cutaneous lesions, progression to mucocutaneous infection, and in some cases development of diffuse cutaneous lesions

(Weigle and Saravia 1996). The course of infection is distinct from those *Leishmania* species that cause visceral leishmaniasis, although geographic distributions of the two types of infections may overlap. Infected individuals often self-cure without therapy and show a strong adaptive immune response involving T-cell recognition of *Leishmania* antigens (Carvalho et al. 1995), leading to long term immunity but typically with disfiguring scars at lesion sites. Enzootic transmission of these parasites occurs by bites of a systematically diverse and geographically widespread complex of species of blood feeding sand flies (subfamily Phlebotominae, family Psychodidae) in the New World genus *Lutzomyia*. Vertebrate hosts of the parasites are wild rodents and marsupials (opposums), edentates (sloths), and commensal rats. Some of these transmission cycles are vertically stratified by forest floor and forest canopy. Transmission solely between sand flies and humans has been suggested for ACL, but not definitively documented, although a clinical case investigation reported large numbers of amastigotes (the parasite stage infectious to sand flies) in skin lesions in a man with AIDS in Brazil, suggesting that it might be possible under conditions of immunosuppression and consequent parasite proliferation (Souza et al. 1998).

The pristine zoonotic cycles for the parasites, vectors, and reservoir hosts occur in widely distributed, undisturbed forests of Central and South America. Risk of infection in humans was primarily occupational amongst forest workers and others entering the forest environment, and was strongly skewed towards adult males (Rawlins et al. 2001). Deforestation was accordingly predicted to decrease incidence of infections, but the opposite has occurred; incidence of cases has been increasing steadily in Colombia, Venezuela, Brazil, French Guyana, and northwest Argentina, the ratio of infections in

human males and females has equalized, and infections in children are now common (Campbell-Lendrum et al. 2001). This changing epidemiological pattern (not one merely of human intrusion into pristine habitat, but environmental modification leading to habitat disturbance and destruction) is correlated with adaptation of the entire transmission cycle from the forest setting to a peridomestic setting localized in rural settlements. The environmental changes are due to human population expansion and development of agriculture; establishment of roads; gold mining; and military activities. The sand fly vectors of some species have shifted larval habitats from forest litter associated with trees, and sylvan rodent burrows; to human garbage and rodent burrows near houses; in these peridomestic environments, vertebrate hosts competent for Leishmania infection are available. Adult sand flies have adopted human dwellings for resting sites in lieu of humid, shady forest sites. Whereas in forested settings in Brazil, sand fly feeding was either primarily on sloths or rodents (Christensen et al. 1982), a bloodmeal analysis of sand flies in deforested areas in Manaus, Brazil, an area with very high ACL incidence, indicated that sand flies fed upon humans, rodents, sloths, dogs, and domesticated fowl (Nery et al. 2004). Layered over the long term environmental changes and correlated epidemiological consequences are strong space-time clusters of human infection, with epidemics postulated to occur at intervals related to waxing and waning of immunity in the human population even while overall incidence has increased; and geographic localizations of infection regionally. The anthropogenic environmental changes associated with emergence of ACL from pristine forest environments are consistent with predictions of "drivers" of emergence for vector-borne diseases in general identified by Harrus and Baneth (2005), as well as with processes of emergence associated with habitat

modification and enhanced transmission or "parasite flow" (Daszak et al. 2001; Polley 2005).

The revolutions reviewed above have contributed substantially to elucidation of the peridomestic emergence of ACL in South America. Parasite infection in sand flies, in sylvatic vertebrate hosts, and in humans has been facilitated by application of PCR-based methods for parasite detection (de Bruijn and Barker 1992; Rodrigues et al. 2002; Mendonca et al. 2004), and molecular-based methods have been widely applied to elucidate systematic relationships and parasite phylogenies, trace evolutionary histories, examine species and strain associations with disease manifestations (Cupolillo et al. 1994). While New World sand fly identification and systematic relationships have remained largely morphological (Young and Duncan 1994), new methods of ribosomal spacer region sequence similarity have clarified species boundaries within the context of geographic distributions (Beati et al. 2004).

Conclusion

VBZD research is united in its underlying motivation of reducing human morbidity and mortality. Since the 1970s, the VBZD research field has benefited from revolutions in advent of tools to allow for diagnostics and assessment of infection across landscapes. Molecular-based tools, especially PCR and gene sequencing technologies, allow for determination of infection prevalence and strain-typing of pathogens in vectors and hosts. Many such tools are now formatted for use in the field and are complemented by web-based portals for data-sharing to allow for rapid response to epidemics and epizootics. Geographic analytical tools, especially remote-sensing and GIS, provide

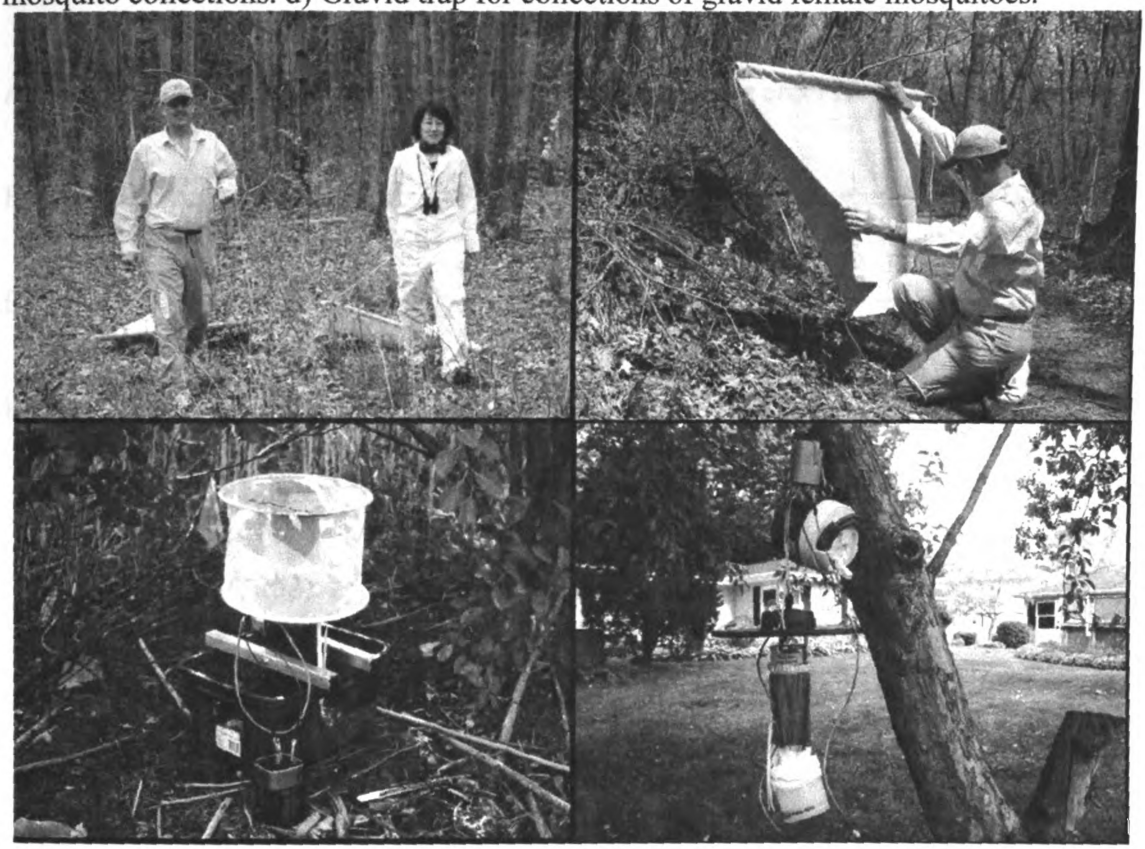
access to, and a platform for analysis of, spatial information across a spectrum of scales to analyze global as well as local patterns of disease.

With these tools, it is possible to not only describe disease retrospectively, but to predict disease across space and time. This has been done successfully for a number of VBZD systems, a subset of which has been outlined above. The final link in achieving the goal of reducing prevalence of human disease is to use such models to inform control practices.

Table 1.1. Glossary of vector-borne zoonotic disease related-terms

Biogeocenose	an association of animals and plants in an area of the earth's surface together with its climate and microclimate, geologic structure, soil, and water supply (Pavlovsky 1966).
Emergence	the process by which a pathogen or disease system becomes present in an area, which may be due to a range expansion of the pathogen or disease system from an endemic area to a new geographic area to which it is novel (invasion) or through evolution of novel pathogens and host associations within an endemic area.
Endemic	the quality of a pathogen or disease system that is native to a given area (indigenous), or has existed in an area for a relatively long time. Endemic is alternatively used to describe an organism that is uniquely found in a given area, and not found naturally anywhere else.
Enzootic	the quality of a disease system being maintained within animal reservoirs at a baseline level; disease of wildlife may or may not result.
Epidemic	an increase in incidence of disease of humans
Epizootic	an increase in incidence of disease of animals
Generalist	with regard to vector feeding, a generalist vector is a species that is able to successfully obtain a bloodmeal from a variety of host species. With regard to pathogens, a generalist pathogen is one that is able to infect a variety of host species.
Incidental host	An animal that may become infected with a pathogen but is not required for maintenance of the pathogen in nature. Incidental hosts are often referred to as 'dead-end' with respect to the pathogen.
Invasive	the quality of a pathogen, or disease system that is expanding in range into areas where it previously did not exist; invasive species typically cause damage to their new habitat.
Nidus	a geographic area in which a disease system is present.
Receptive	the quality of a habitat patch that contains the appropriate biotic and abiotic factors to support the establishment of an invading species; invasible.
Reservoir competence	the importance of a host species to pathogen transmission and the dynamics of infection. In VBZD studies, reservoir competence is often measured as the proportion of vectors that become infected after feeding on an infected host individual.
Reservoir host	An animal that maintains a pathogen in nature and can infect vectors upon blood-feeding.
Resurgence	the process by which a pathogen or disease system reappears in an area from which it has historically been transmitted followed by a period of local extirpation, or an increase in rate of transmission or virulence of a pathogen within an endemic area.
Sylvatic	descriptor of pathogen or disease system that is transmitted among wild animals
Zoonotic	a type of pathogen or disease system that is maintained in animal reservoirs and is transmissible to humans

Figure 1.2. Methods for field collections of vectors. a) Drag sampling for questing ticks; b) Checking drag cloth for tick presence. c) CDC light trap elevated in tree canopy for mosquito collections. d) Gravid trap for collections of gravid female mosquitoes.



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CHAPTER 2

Zoonotic pathogens in *Ixodes scapularis*, Michigan

Hamer SA, Hickling GJ, Roy P, Walker ED, Foster ES, Barber CC, and Tsao JI (2007). Zoonotic pathogens in *Ixodes scapularis*, Michigan. Emerging Infectious Diseases 7:1131-1133.

Abstract

Ixodes scapularis is endemic to a focal area of Michigan's Upper Peninsula and is currently invading the southwestern Lower Peninsula. Herein we report *Borrelia burgdorferi*, *Anaplasma phagocytophilum* and *Babesia odocoilei* infection within ticks from both foci. Infection prevalence with these pathogens was greater in ticks collected from endemic areas as compared to those collected from recently-invaded sites. These findings emphasize the need for heightened awareness by human and veterinary health professionals.

Introduction

Ixodes scapularis - the blacklegged tick - is currently the most infectious vector species to humans in the United States, transmitting the agents of Lyme borreliosis, human anaplasmosis (previously human granulocytic ehrlichiosis), and human babesiosis. Since Lyme borreliosis and human anaplasmosis became notifiable diseases in 1991 and 1999, respectively, annual incidence has been increasing with 19,804 and 537 reported cases in 2004 (Jajosky et al. 2006). The increase in *I. scapularis*-borne disease is due, in part, to geographic range expansion of *I. scapularis.* Despite the broad endemic areas to the west in Wisconsin and Minnesota and to the south in Indiana, active and passive surveillance suggested that the only populations of *I. scapularis* established

in Michigan prior to 2002 were in Menominee County in the Upper Peninsula (Walker et al. 1998). In 2002-2003, however, active wildlife sampling and tick dragging in southwestern Michigan indicated that invasion of *I. scapularis* was occurring (Foster 2004), with nearby tick populations in northwestern Indiana as the putative source. Here we describe a recent assay of *Borrelia spp.*, *Anaplasma phagocytophilum*, and *Babesia spp.* from a targeted sample of host-seeking ticks from an endemic area in Michigan's Upper Peninsula and from the Lower Michigan invasion zone, and conclude that infection prevalence with pathogens was greater within endemic ticks.

The Study

Over a 1.5 week period during the adult *I. scapularis* spring questing peak in April-May 2005, ticks were collected from two sites in Menominee County in Michigan's Upper Peninsula (endemic for *I. scapularis)* and three sites in southwestern Michigan (recently-invaded by *I. scapularis;* Figure 2.1) by dragging a 1-m^2 corduroy cloth over the forest floor for 500 m (Falco and Fish 1992). Drag cloths were checked at 20 m increments for ticks, which were removed and kept alive until identification. Tick densities were greater at endemic sites than recently-invaded sites, and within invaded sites, densities were greater at the sites closest to the putative source of invading ticks, indicative of duration of establishment. Host-seeking adults have had two previous bloodmeals, so we focused our efforts on adults to increase the probability of detecting pathogens. We collected in spring, because the adult *I. scapularis* questing peak in Michigan is greater then than in fall (Strand et al. 1992; Foster 2004). The endemic sites are comprised of upland mixed hardwoods (Site A in Figure 2.1) and conifer/shrub cover

(Site B) along the Menominee River, while the southern sites contain mixed deciduous forest overlying sand dunes along the eastern shores of Lake Michigan.

Ticks were aseptically dissected in half, with one half retained for a separate study. DNA was extracted from the other half, following overnight lysis, using DNeasy tissue kits (Qiagen, Valencia, CA) and used as template in three separate polymerase chain reactions to detect the 16s-23s rRNA intergenic spacer of *B. burgdorferi* or *B. miyamotoi* (Bunikis et al. 2004), the p44 gene of *A. phagocytophilum* (Zeidner et al. 2000), and the 18S rRNA gene of *Babesia*-genus organisms (Armstrong et al. 1998). *Babesia*-positive amplicons were purified and sequenced for species identification.

From endemic sites A and B, a total of 31 ticks were collected: 28 adult and 1 nymphal *I. scapularis*, and 2 adult *Dermacentor variabilis*. Of the adult *I. scapularis*, 17 (60.7%) were positive for *B. burgdorferi*, 4 (14.3%) were positive for *A. phagocytophilum*, and 2 (7.1%) were positive for *Babesia* spp. (later identified as *Ba. odocoilei* through sequence analysis; Table 2.1). Two adults were co-infected with *B. burgdorferi* and *A. phagocytophilum* and one adult was co-infected with *B. burgdorferi* and *Ba. odocoilei*. Rates of co-infection did not deviate from random expectation (Fisher's exact tests, $P = 0.518–0.640$). The single nymph was infected with *B. burgdorferi* only.

At newly-invaded sites C-E, a total of 105 ticks were collected: 91 adult and 10 nymphal *I. scapularis*, and 5 adult *D. variabilis*. Of the adult *I. scapularis*, 43 (47.3%) were positive for *B. burgdorferi*, 1 (1.1%) was positive for *A. phagocytophilum*, and 4 (4.4%) were positive for *Babesia* spp. (later identified as *Ba. odocoilei*). All 4 *Ba. odocoilei*-positive ticks were also infected with *B. burgdorferi*. This rate of co-infection

was significantly greater than random expectation (Fisher's exact test, $P = 0.046$). Of 10 nymphal *I. scapularis*, 2 (20.0%) were positive for *B. burgdorferi* and 2 (20.0%) were positive for *A. phagocytophilum*, including one co-infected tick (a nonsignificant co-infection rate, Fisher's exact test, $P = 0.378$).

Conclusions

These are the first records of *A. phagocytophilum* and *Ba. odocoilei* in ticks in Michigan, and it is clear that they are present among both endemic and recently-invaded *I. scapularis*. *B. burgdorferi* infection in *I. scapularis* of Michigan has been reported previously (Walker et al. 1994; Walker et al. 1998; Foster 2004; Diuk-Wasser et al. 2006), and two culture-confirmed cases of human Lyme borreliosis in Michigan have been reported, both contracted in Menominee County (Stobierski et al. 1994; Golde et al. 1998). Whereas *B. burgdorferi* and *A. phagocytophilum* are both zoonotic pathogens of humans, *Ba. odocoilei* is an intraerythrocytic protozoan parasite maintained in transmission cycles between *I. scapularis* and white-tailed deer and is not known to be pathogenic to humans (Armstrong et al. 1998). We did not detect *I. scapularis* infected with either *Ba. microti* or the WA1 piroplasm, the etiologic agents of human babesiosis in North America (Homer et al. 2000), nor with *B. miyamotoi*.

A comparison of the *B. burgdorferi*-infection rate in adult ticks collected herein from the endemic sites (60.7%) with equivalent records from the same county in 1992 (31.3%)(Walker et al. 1994) indicate that infection prevalence in the endemic focus has increased over time (Fisher's exact test, $P < 0.001$). *B. burgdorferi* infection prevalence of adult *I. scapularis* has also increased within the invasion zone of southwest Michigan,

from 37.0% in 2002-2003 (Foster 2004)(collection site 5km south of the southernmost site in 2006) to 47.3% in 2006, although this difference is only marginally statistically significant due to small 2006 sample size (one-tailed Fisher's exact test, $P = 0.046$).

In Indiana, the putative source of invading *I. scapularis* into lower Michigan, *I. scapularis* and *B. burgdorferi* infected-*I. scapularis* were first documented in 1987 and 1991, respectively (Pinger et al. 1996). *A. phagocytophilum* and *Ba. odocoilei* have been recently reported in Indiana (Steiner et al. 2006), with infection prevalences higher than those reported herein for Michigan (11.8% and 10.3% in Indiana; 1.1 and 4.4% in Michigan, respectively), which is suggestive of more recent establishment in Michigan.

Our findings of *B. burgdorferi, A. phagocytophilum*, and *Ba. odocoilei* infections in adult and nymphal *I. scapularis* in endemic and recently-invaded areas of Michigan provide evidence of the establishment of pathogen maintenance cycles within these zones. Furthermore, infection prevalence was greater in ticks collected from endemic foci as compared to those collected from the invasion front, though difference were not always statistically significant. These data imply a risk of human Lyme borreliosis in areas both endemic for and recently-invaded by *I. scapularis*. Indeed, since rising levels of tick infection and co-infection can be expected with increasing time since tick establishment, medical practitioners in Michigan should be including disease resulting from these pathogens within their index of suspicion. Co-infections within the adult ticks of our study were more common than expected based on individual pathogen infection rates, suggesting that these adults were infected with different pathogens during their larval and nymphal bloodmeals, that individual wildlife hosts may be infecting ticks with

multiple pathogens during a single bloodmeal, and/or that infection with one pathogen facilitates subsequent infection with others.

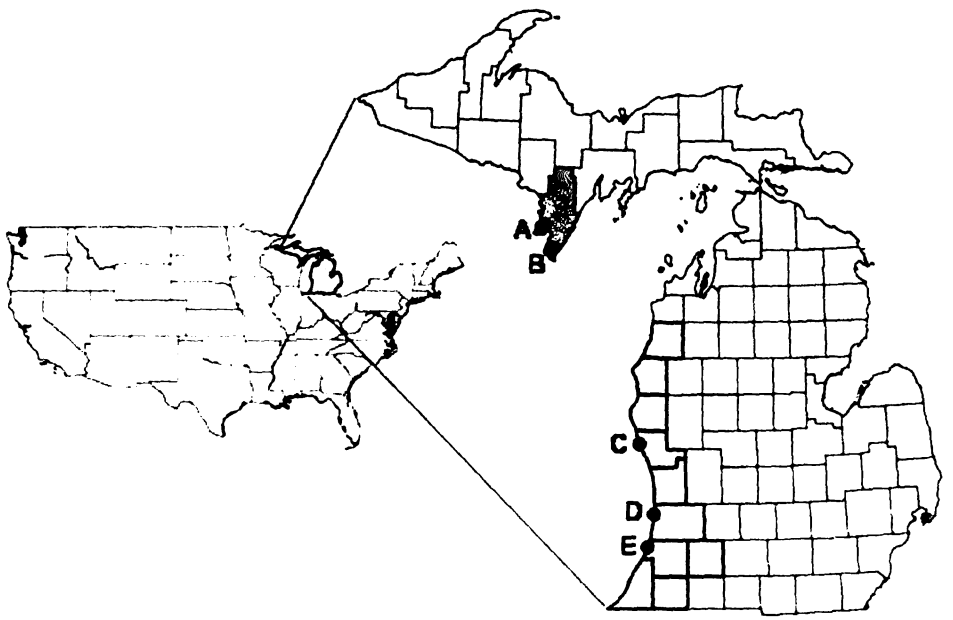

Figure 2.1. Tick dragging field sites within Michigan. A= Menominee North; B= Menominee South; C= Duck Lake State Park; D= Saugatuck Dunes State Park; E= Van Buren State Park. Gray shaded counties are those in which endemic (Upper Peninsula) and recently-invaded (Lower Peninsula) *I. scapularis* are known to occur.

Table 2.1. Michigan site-specific prevalences of adult and nymphal *I. scapularis* infection with three pathogens. Site names correspond to the Figure 2.1. Life stage: A= adult; N = nymph.

			No.	No. infected (%)			No. co-infected (%)	
Site	**Status**	**Stage**	***I. scapularis***	***B. burgdorferi***	***A. phagocytophilum***	***Ba. odocoilei***	***B.b. + A.p.***	***B.b. + B.o.***
A	Endemic	A	16	9 (56.3)	1 (6.3)	1 (6.3)	1 (6.3)	0
B	Endemic	A	12	8 (66.7)	3 (25.0)	1 (8.3)	1 (8.3)	1 (8.3)
		N	1	1 (100.0)	0	0	0	0
C	Invaded	A	4	2 (50.0)	0	0	0	0
		N	2	0	1 (50.0)	0	0	0
D	Invaded	A	18	9 (50.0)	0	1 (5.6)	0	1 (5.6)
		N	8	2 (25.0)	1 (12.5)	0	1 (12.5)	0
E	Invaded	A	69	32 (46.4)	1 (1.4)	3 (4.3)	0	3 (4.3)
Overall	Endemic	A	28	17 (60.7)	4 (14.3)	2 (7.1)	2 (7.1)	1 (3.6)
		N	1	1 (100)	0	0	0	0
	Invaded	A	91	43 (47.3)	1 (1.1)	4 (4.4)	0	4[a] (4.4)
		N	10	2 (20.0)	2 (20.0)	0	1 (10.0)	0

[a]Significant level of co-infection, Fisher's exact test, P = 0.046.

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CHAPTER 3

Invasion of the Lyme disease vector *Ixodes scapularis*: implications for *Borrelia burgdorferi* endemicity

Hamer SA, Tsao JI, Walker ED, Hickling GJ. 2010. Invasion of the Lyme disease vector *Ixodes scapularis*: implications for *Borrelia burgdorferi* endemicity. EcoHealth. doi: 10.1007/s10393-010-0287-0

Abstract

Lyme disease risk is increasing in the United States due in part to spread of *Ixodes scapularis*, the principal vector of the spirochetal pathogen *Borrelia burgdorferi*. A five-year study was undertaken to investigate a hypothesized co-invasion of *I. scapularis* and *B. burgdorferi* in lower Michigan. We tracked the spatial and temporal dynamics of the blacklegged tick and the spirochete using mammal, bird, and vegetation drag sampling at eight field sites along coastal and inland transects originating in a zone of recent *I. scapularis* establishment. We document northward invasion of ticks along Michigan's west coast over the study period; this pattern was most evident in ticks removed from rodents. *B. burgdorferi* infection prevalences in *I. scapularis* sampled from vegetation in the invasion zone were 9.3 and 36.6% in nymphs and adults, respectively. There was no evidence of *I. scapularis* invasion along the inland transect, however low-prevalence *B. burgdorferi* infection was detected in other tick species and in wildlife at inland sites, as well as at northern coastal sites in years prior to the arrival of *I. scapularis*. These infections suggest that cryptic *B. burgdorferi* transmission by other vector-competent tick species may be occurring in the absence of *I. scapularis*. Other *Borrelia* spirochetes, including those that group with *B. miyamotoi* and *B. andersonii*, were present at a low prevalence within invading ticks and local wildlife. This rapid

blacklegged tick invasion – measurable within five years – in combination with cryptic pathogen maintenance suggests a complex ecology of Lyme disease emergence in which wildlife sentinels can provide an early warning of disease emergence.

Introduction

Lyme disease accounts for over 90% of all reported vector-borne disease in the United States with more than 20,000 cases reported annually; its current invasive spread from endemic foci constitutes a major public health concern (Bacon et al. 2007). In the United States, Lyme disease is caused by the bacterium *Borrelia burgdorferi* and the blacklegged tick *Ixodes scapularis* serves as the vector for a majority of cases. Patterns of human disease mirror the geographic distribution of *B. burgdorferi*-infected *I. scapularis*, which is characterized by high-density endemic foci in the Northeast and upper Midwestern United States. Increasing incidence is associated with both biological and non-biological factors, including enhanced surveillance and awareness among medical professionals, human encroachment into tick habitat, creation of peridomestic habitats that attract wildlife hosts of ticks and pathogens, and increases in abundance and range expansions of wildlife. Blacklegged ticks were likely widespread prior to the most recent glaciation event, during which relict populations remained in refuges, from which they and their vertebrate hosts later expanded (Steere et al. 2004). Over the past 50 years, white-tailed deer (*Odocoileus virginiaus*) populations have undergone explosive growth due to reversion of agricultural lands to forest and restrictions on hunting. This deer expansion has facilitated the recent blacklegged tick expansion and Lyme disease emergence throughout the Northeast and Midwest. *I. scapularis* continues to spread from

both the northeastern and Midwestern foci (Steere et al. 2004), but the mechanisms for this spread are not established. *I. scapularis* serves as the vector for multiple zoonotic pathogens – including the agents of Lyme disease, human anaplasmosis, babesiosis, and Powassan encephalitis – so its expansion constitutes increased risk of multiple diseases.

In 2002, a new population of *I. scapularis* was detected in the southwestern corner of Michigan's Lower Peninsula (Foster 2004). This represented a significant change from the State's previous reported distribution (Walker et al. 1998), which was characterized by a probable lack of established *I. scapularis* throughout the Lower Peninsula. Established populations in the state were recognized at that time only in Menominee County of the Upper Peninsula, adjacent to endemic foci in Wisconsin (Walker et al. 1998). Nevertheless, a Midwestern model of habitat suitability (Guerra et al. 2002) predicted that sandy oak upland forests provide highly suitable habitat for *I. scapularis,* and *I. scapularis* indeed were discovered in such areas(Foster 2004). Suitable habitat types are widespread throughout lower Michigan, so further invasion of *I. scapularis* is thus predicted.

Tick versus pathogen invasion?

B. burgdorferi and *A. phagocytophilum* have been found within Michigan's recently-invaded tick populations (Hamer et al. 2007). The early detection of low-density infected tick populations afforded us the rare opportunity to test, in real time, three non-exclusive scenarios by which invasion of the Lyme disease vector and/or pathogen into lower Michigan may be occurring: (*i*) In the 'tick first' scenario, new uninfected *I. scapularis* populations become established as a result of long distance dispersal of adult

ticks by white-tailed deer. This deer-mediated invasion would introduce replete uninfected adult ticks, but not *B. burgdorferi* (as deer are incompetent hosts for this pathogen; (Telford et al. 1988)). In this scenario, we propose that *B. burgdorferi* enters the system later, as result of a slower secondary invasion mediated by infected mammalian or avian hosts. (*ii*) In the 'dual-invasion' scenario, mammalian or avian hosts introduce infected *I. scapularis* to new areas in high enough numbers to allow both *I. scapularis* and *B. burgdorferi* to establish. In this scenario, *B. burgdorferi* should be detectable early in the invasion process, when *I. scapularis* densities are still low. (*iii*) In the 'spirochete-first' scenario, enzootic transmission of the pathogen is maintained by cryptic vectors and reservoir hosts. These vectors are generally wildlife host-specialists that, unlike *I. scapularis*, do not bite humans (Telford and Spielman 1989b, a) and thus these transmission cycles have no implications for human or canine disease risk. Invasion of *I. scapularis* (either infected or uninfected) into zones of cryptic pathogen maintenance will, however, create opportunities for bridging the pathogen to humans and canines.

We initiated comprehensive sampling to investigate the spatial and temporal dynamics of the Lyme disease system in and beyond the zone of hypothesized *I. scapularis* invasion in lower Michigan. To address invasion, we studied areas not only where all three parasitic life stages of *I. scapularis* were endemic (presence of all three stages and/or at least six individuals of a single stage constitute the Centers for Disease Control and Prevention (CDC) definition of an 'established' population; (Dennis et al. 1998), but also in areas beyond its detected distribution. The objectives of this study were to: (*i*) test whether the detected distribution of *I. scapularis* changed over the period 2004-2008; and (*ii*) assess the patterns of occurrence of *B. burgdorferi* in relation to that

of *I. scapularis*. To address the 'tick-first' hypothesis, each field site was sampled regularly for the presence of *I. scapularis* to detect new colonization and/or trends in tick abundance at each site. To address the 'dual-invasion' and 'spirochete-first' hypotheses, a diverse community of hosts and their attached ticks were assayed to assess the relationship between *I. scapularis* presence and *B. burgdorferi* infection. Thus, we include results from a diverse assemblage of ticks, including those with low or no vectorial capacity and no associated zoonotic risk (i.e., tick species that are incompetent for spirochete transmission and/or species that do not regularly feed on human hosts). Although unimportant for transmission, incompetent ticks may serve as bio-indicators of *B. burgdorferi* presence in a given area if they contain an infectious blood meal or transtadially passed spirochetes from a previous meal.

Materials and Methods

Site selection and sampling regime. From 2004-2008, we assessed on- and off-host ticks and wildlife along two sampling transects established at a spatial scale that extended beyond the known limit of the lower Michigan *I. scapularis* population (Figure 3.1). Both transects originated near the southwestern Michigan zone where *I. scapularis* was first detected in 2002(Foster 2004). From this origin, the coastal transect extended north along Lake Michigan (sites labeled C1-C4; 81-145 km between sites: Van Buren State Park, Van Buren Co.; Duck Lake State Park, Muskegon Co.; Orchard Beach State Park, Manistee Co.; Sleeping Bear Dunes National Lakeshore, Benzie Co.; respectively). The inland transect extended northeastward (sites labeled I1-I4; 33-76 km between sites: Fort Custer State Recreation Area, Kalamazoo Co.; Lux Arbor Reserve of Kellogg Biological

Station; Barry Co.; Ionia State Recreation Area, Ionia Co.; and Rose Lake Wildlife Research Area, Clinton Co.; respectively). Both transects traversed areas where established *I. scapularis* had not been previously detected (Walker et al. 1998; Foster 2004), or were previously uninvestigated. Sampling was conducted in oak-dominated, closed-canopy deciduous forest when available, as this habitat type is positively associated with *I. scapularis* presence in the endemic Midwest (Guerra et al. 2002). Otherwise, sampling was conducted in the dominant forest type at the site. All data presented here are for May and June, when *I. scapularis* larvae, nymphs, and adults are simultaneously active. Each site was sampled in these months in all five years of the study, except for site C4 (2007 and 2008 only) and site I1 (all years except 2006).

Mammal trapping. At each field site, small mammals were trapped along four to six transects of 25 Sherman live traps (H. B. Sherman Traps, Tallahassee, FL) spaced 10 m apart and baited with sunflower seed. Medium-sized mammals were trapped using 12-16 32x10x12-inch live traps (Tomahawk Live Traps, Tomahawk, WI) and wooden box traps baited with peanut butter, jelly, and cat food on tortillas. Small mammals were anesthetized using Isoflurane (IsoFlo, Abbot Laboratories, Abbott Park, IL); medium-sized mammals were anesthetized using ketamine hydrochloride (Ketaset; Fort Dodge, Overland Park, KS) and xylazine hydrochloride (Rompun; Bayer Health Care, Kansas City, KS) followed by reversal with administration of yohimbine hydrochloride (Antagonil; Wildlife Laboratories, Fort Collins, CO). Animals were identified to species and sex by inspection. Each animal was examined for ticks, biopsied in both ears using a 2-mm (small mammals) or 4-mm (medium mammals) biopsy punch (Miltex Insturments,

York, PA), and ear-tagged (National Band and Tag, Newport, KY). Ticks and ear biopsies were stored separately in 70% ethanol. Additional ear biopsies were obtained from animals recaptured after an interval of 2 or more weeks; recaptures at a shorter interval were simply checked again for ticks. All animals were released at the site of capture. Small mammal trapping success rate was discounted by the number of empty, tripped traps as follows: number mammal captures / (number traps set - 0.5*no. tripped traps); this expression assumes that on average tripped traps were open for half the night. Wildlife procedures were approved through Michigan State University's Institutional Animal Use and Care Committee permit #02-07-13-000.

Bird mist netting. At each site, six 12-m mist nets (Avinet, Dryden, NY) were used to capture birds in the same areas where mammals were trapped. Nets were run from 0600 – 1200 h on fair weather days and checked every 45 min. Birds were weighed, identified to species and sex, measured, searched for ticks, and leg-banded with federally-issued bands before release. Mist netting was performed under federal permit #02640.

Questing tick sampling. Each site was sampled for questing ticks by dragging a 1-m^2 white corduroy cloth (Falco and Fish 1992) over the forest floor along the same six transects that were used for mammal trapping. Drag-sampling was performed on rain-free days in the late morning or late afternoon so as to avoid the hottest and least-humid times of day (Schulze et al. 2001; Diuk-Wasser et al. 2006). At least 1000 m^2 of vegetation were sampled per visit. The cloth was inspected every 20 m, and attached ticks were stored in 70% ethanol. Drag-sampling was not performed on excessively hot or wet days.

Borrelia burgdorferi *detection*. All ticks were identified to species and stage (Keirans and Clifford 1978; Sonenshine 1979; Durden and Keirans 1996). Total DNA from ticks and ear biopsies was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) following the manufacturer's animal tissue protocol with the following modifications. Ticks were first bisected using a sterile scalpel or were pulverized in liquid nitrogen, followed by an overnight incubation in lysis buffer. DNA was eluted using 50 µl elution solution warmed to 70° C. Ear biopsies (one per animal), adult and nymphal ticks were extracted individually, and conspecific larvae from the same individual animal or drag transect were pooled for extraction. Given our specific questions, we implemented a protocol for sub-sampling of ticks to assay for infection in the occasional cases of heavily-parasitized hosts. In cases where more than three adult or nymphal ticks of the same species, life stage, and sex were removed from an individual host, three were randomly selected for testing (i.e., the maximum number of ticks tested from a host would be, for each tick species present, six adults (three female and three male), three nymphs, and a larval pool). By including a subset of ticks from many hosts, rather than all ticks from a smaller number of hosts (given limited resources), this sampling strategy allowed for improved coverage of the host population, and also reduced statistical concerns of non-independence that would arise if very large numbers of ticks were collected from a small number of highly-infested hosts. *B. burgdorferi* strain B31-infected nymphal *I. scapularis* acquired from the CDC and water served as the positive and negative extraction controls, respectively.

B. burgdorferi was detected using either *i*) a nested polymerase chain reaction (PCR) for the 16S – 23S rRNA intergenic spacer region (IGS) of *Borrelia* spp. (Bunikis

et al. 2004) followed by visualization with gel electrophoresis or *ii*) a quantitative PCR (qPCR) of a region of the 16S rRNA of *B. burgdorferi* (Tsao et al. 2004). PCR enzyme kits were used throughout (nested PCR: PCR Supermix, Invitrogen, Carlsbad, CA and FailSafe PCR System, Epicentre, Madison, WI; qPCR: Universal PCR Master Mix, Applied Biosystems, Foster City, CA). In addition to the positive and negative DNA extraction controls for each batch of samples extracted, DNA from *B. burgdorferi* strain B31-infected ticks from laboratory colonies at the CDC and water served as the positive and negative PCR controls. In qPCR, a six-point standard dilution series of DNA extracted from cultured spirochetes ($10^4 - 10^{-1}$ organisms per 3 µl reaction volume) served as positive controls (*B. burgdorferi* strain C336, a rRNA spacer type (RST) II strain). Reactions for qPCR were done with an ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). Preliminary experiments showed that both tests were able to detect positive samples containing a minimum 10^0 organisms.

Nucleotide sequencing. A subset of *B. burgdorferi*-positive ticks and ear biopsies was subjected to DNA sequencing to confirm pathogen identity. Samples included: *i*) a random subset of positive samples from sites where *I. scapularis* is common; *ii*) a majority of test-positive samples from sites with an apparent absence of *I. scapularis*; and *iii*) any IGS amplicons of approximately 500 bp, characteristic of the relapsing-fever spirochete *B. miyamotoi* (cf. the 987 bp fragment characteristic of *B. burgdorferi*). If the sample was determined to be positive using qPCR, then IGS PCR was performed to generate the template for sequencing. The IGS product was purified (Qiagen PCR Purification Kit; Qiagen, Valencia, CA) and sequences were determined in both

directions using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). Sequences were identified as either *B. burgdorferi* or *B. miyamotoi* based on comparisons to published sequences using the basic local alignment search tool in GenBank (Altschul et al. 1990). *B. burgdorferi* strains were assigned to IGS ribosomal spacer type (RST; (Liveris et al. 1995)) by alignment with the prototypical strains published in Bunikis et al. (2004) using the program MEGA (Tamura et al. 2007).

Statistics. Logistic regression was used to assess trends in wildlife infestation and tick infection over the 5-year sampling period. Fishers exact test was used to assess differences in infestation among sites. Linear regression was used to assess trends in nymphal densities within sites over time. Within-year comparisons between the coastal and inland transects were made by calculating the z-ratio and associated two-tail probabilities for the difference between two independent proportions. A minimum infection prevalence (MIP; i.e., assuming only one positive larva per pool) was used for tests done on pooled larvae. Statistics were performed using Statistix 8 (Analytical Software, Tallahassee, FL).

Results

Wildlife captures. We achieved a total of 1,667 mammal captures, comprised of 1,472 small and 195 medium mammal captures. Small mammals were captured in a total of 7,998 adjusted trap nights (18.4% overall capture rate). White-footed mouse was the most commonly caught species (84.9% of all small mammal captures) – their capture rates did not differ between coastal and inland transect sites ($P = 0.95$). Medium mammals were

captured in a total of 777 adjusted trap nights (25.1% overall capture rate), with similar rates along both transects (P = 0.93). Raccoon was the most abundant species (65.6% of all medium mammal captures). Despite escaped animals and the release of lethargic shrews, 99.8% of captured mammals were processed, 82.5% of which were first captures and 6.5% were recaptures after a minimum 2-week interval; these animals were processed in full and used for computing the infestation and infection prevalences reported below. The remaining 11% represent white-footed mice recaptured within 2 weeks; these animals were only checked for ticks and released. These latter mice are not considered further in infestation analyses; however ticks removed from these animals are considered in infection analyses. In total, 14 mammal species were parasitized by eight species of ticks (Table 3.1).

A total of 747 bird captures, comprised of 55 species, occurred in 2180 net hours for an average of 34.3 birds per 100 net hours. Due to escaped birds, 99.2% were processed, of which 91.6% represent first captures and 6.7% were bird recaptured after a minimum 2-week interval; these birds were processed in full and used in computing infestation and infection prevalences below. The remaining 1.6% represent birds recaptured within 2 weeks – none harbored ticks. Birds were parasitized by three species of ticks (Table 3.1; Supplement 1).

I. scapularis on wildlife. Along the inland transect, *I. scapularis* were rarely found on wildlife, with only 0.5% of 574 white-footed mice parasitized. Two of these were trapped at site I3 in 2007 (3.0% infestation), with one harboring a single larva and nymph, and one harboring four larvae. The other mouse was trapped at site I1 in 2008 (3.9%

infestation) and harbored a single nymph. None of the 73 eastern chipmunks, 133 individuals of other mammal species, or 504 birds on this transect harbored *I. scapularis*.

In contrast, a steep gradient of *I. scapularis* infestation of wildlife was evident along the coastal transect. In all years the level of white-footed mouse infestation was highest at the southernmost C1 site (75-100% with no significant difference among years; $P = .08$), and larval and nymphal burdens averaged 4.5 and 0.6 per mouse, and 5.6 and 2.3 per infested mouse (maxima of 37 and 11, respectively). Infestation at the more northern sites was much lower, but increased progressively during the study (Figure 3.2). At site C2, infested mice were trapped in all five years, with infestation increasing significantly over time ($P < 0.001$). At site C3, no *I. scapularis*-infested mice were observed until the third summer after which infestation increased ($P = 0.76$). Site C4 harbored a low proportion of infested mice in both 2007 and 2008 (9 and 10%, respectively); these were the only two years when May-June sampling was conducted at this site (in July 2005 none of 21 mice trapped at C4 harbored *I. scapularis*, whereas 60% of 110, 13% of 54, and 0% of 16 mice were infested at sites C1, C2, C3 respectively).

Overall, 48.2% of 81 eastern chipmunks harbored *I. scapularis* along coastal sites. Like mice, infestation was highest at site C1, with all chipmunks ($n = 15$) infested in all years. Although fewer chipmunks were infested at the three more northern sites (Figure 3.2; $P < 0.0001$), infestation levels increased over time ($P = 0.01$ for sites C2 and C3 pooled). *I. scapularis* burdens on chipmunks were greatest at site C1; larval burdens there were similar to those of mice (average 4.7 per chipmunk and 5.8 per larval-infested chipmunk, maximum of 20), but nymphal burdens were much higher (average 7.2 per chipmunk and 7.7 per nymphal-infested chipmunk, maximum of 36). Of 117 other

mammals caught on the coastal transect, 28.2% comprising ten species harbored *I. scapularis* (Table 3.1; most were from C1 (54.5%) or C2 (24.2%)). Of 237 bird captures, *I. scapularis* was found on 14, of which 13 were from C1 and one was from C2. These 14 birds comprised five species: American robin, chipping sparrow, Eastern towhee, indigo bunting, and Northern cardinal (Supplement 1).

Questing ticks. A total of 58,459 m^2 of drag sampling was conducted, with 26,800 and 31,650 m^2 along the inland and coastal transects, respectively. A total of 1,984 ticks were collected of which 87% were *I. scapularis.* Four other tick species were collected by drag cloth: *Dermacentor variabilis* (n = 252), *Amblyomma americanum* (n = 4), *Haemaphysalis leporispalustris* (n = 2), and *I. dentatus* (n = 5).

All but one *I. scapularis* were from coastal sites. Along the inland transect, a single larva was dragged (at I3 in 2007). Conversely, along the coastal transect, 86 adults, 246 nymphs and 1,388 larvae were collected, of which 95.3, 91.5, and 93.3% respectively were from site C1. A significant gradient of *I. scapularis* density was detected, with the greatest abundance at site C1, where all life stages were present in all five summers of sampling (peak May/June densities were 8.2 adults, 29.7 nymphs, and 90.8 larvae per 1000 m^2) and nymphal abundance did not change across the five years ($R^2 = 0.14$; P = 0.26). Many fewer, yet increasing, numbers of *I. scapularis* occurred at the two sites to the north (Figure 3.3): at site C2, no *I. scapularis* were dragged at the start of the study whereas all stages were present by the end with a significant increase in nymphal density ($R^2 = 0.78$; P = 0.02). At site C3, an increase in nymphal density occurred ($R^2 = 0.05$, P =

0.09), and this was the only life stage present on drag cloths. No questing *I. scapularis* were collected at site C4.

B. burgdorferi *infection in drag-sampled ticks*. Along the coastal transect, 36.6, 9.3, and a minimum of 0.1% of adult, nymphal, and larval *I. scapularis* respectively were infected with *B. burgdorferi*, with the majority of infected ticks (95.1%) collected at site C1 (Figure 3.4). There was no significant change in infection prevalence among years at site C1 ($P = 0.38 - 0.71$ for the three age classes); too few positive ticks were collected at other coastal sites to test for equivalent trends. Along the inland transect, the single *I. scapularis* larva that was dragged at site I3 in 2007 tested positive for *B. burgdorferi* (Table 3.2). Of all *D. variabilis* collected on drag cloth, *B. burgdorferi* was found in 8 of 147 adults, 0 of 4 nymphs, and a minimum of 1 of 59 larvae processed in 15 pools, with no apparent temporal trends. All infected drag-sampled *D. variabilis* came from site C1, with the exception of two infected adults from sites I1 and I2. *B. burgdorferi* detection in the additional drag-sampled tick species across the years was as follows: 0 of 4 *A. americanum*, 0 of 1 *A. maculatum*, 0 of 1 *I. cookei*, 2 of 2 *H. leporispalustris* (both nymphs from site C1), and 0 of 3 *I. dentatus*.

***Host infection with* B. burgdorferi.** Of the 1056 white-footed mouse ear biopsies that were collected from 2004-2008, only 2.1% of 576 tested positive from inland sites (I1: 0/71; I2: 9/213; I3: 3/169; I4: 0/121, with no apparent trend over time), whereas 11.9% of 480 coastal mice tested positive ($P < 0.0002$). Infection prevalence was high in all years at site C1 (21.6 - 80%), and low and inconsistent at sites further north (Figure 3.2). A

total of 148 chipmunk biopsies were assayed for infection, of which 4.2% of 72 were positive along the inland transect (I1: 0/9; I2: 0/20; I3: 0/4; I4: 3/39, and 6.6% of 76 (all at site C1) were positive along the coastal transect (Figure 3.2; no difference between transects, P = 0.72). A total of 240 ear biopsies from alternative mammal species were assayed for infection: 6.5% of 124 and 4.3% of 116 were positive along the inland and coastal transects, respectively (no difference between transects, P = 0.57). Positive alternative mammals comprised four species (raccoon, red squirrel, meadow jumping mouse, and Virginia opossum) at two inland sites (I3 and I4) and three species (eastern gray squirrel, raccoon, and meadow jumping mouse) at two coastal sites (C1 and C3).

B. burgdorferi *infection in I. scapularis removed from hosts*. Along the inland transect, all five larvae removed from two mice at site I3 in 2007 were negative, whereas the single nymph from the same site and year was positive (Table 3.2), and the single nymph from site I1 in 2008 was negative. Along the coastal transect, infection in *I. scapularis* removed from mice and chipmunks was highest at all years at site C1 and decreased at sites to the north, with no infection in the ticks from site C4 (Figure 3.2). Alternative mammalian hosts harboring *I. scapularis* were found at all coastal sites, but *B. burgdorferi* infection in these ticks was found only at sites C1 and C2 (Supplement 2). Birds harboring *I. scapularis* were found only at sites C1 and C2, and *B. burgdorferi* infection in bird-derived *I. scapularis* occurred only at site C1 (Supplement 2).

B. burgdorferi *infection in alternative tick species*. Assaying samples of the seven species of ticks other than *I. scapularis* removed from mammals and birds revealed

relatively high levels of *B. burgdorferi* infection in alternative ticks at sites with sympatric *I. scapularis* (Supplement 2). Additionally, some alternative tick species from inland sites in the apparent absence of *I. scapularis* were associated with low-level infection prevalence. Infected *D. variabilis* removed from mice were found at sites I2 and I3, where a total of 7.1% of 98 nymphs (positives from 6 animals) and a minimum of 0.7% of 2006 larvae (positives from 14 animals) collected at these two sites together were infected. Contrast these results with that at site C1, where sympatric *I. scapularis* occur in high density: 17.6% of 74 nymphal (positives from 12 animals) and a minimum of 2.5% of 672 larval (positives from 17 animals) *D. variabilis* removed from white-footed mice were infected.

Of the alternative tick species collected from vertebrates, *I. texanus* was the most abundant and was ubiquitous across our study sites, commonly found parasitizing raccoons (Table 3.1). Infected attached *I. texanus* were found not only at all coastal transect sites, but low-level infection was documented in all three life stages from inland sites (2.4-2.6%; Supplement 2) as well. In summary, infected *D. variabilis, I. texanus, I. cookei, I. dentatus, I. marxi* and *H. leporispalustris* were removed from coastal wildlife, and infected *D. variabilis, I. texanus*, and *I. dentatus* were removed from inland wildlife (Supplement 2).

Nucleotide sequencing. Pathogen identity was further confirmed by nucleotide sequencing of the 16S-23S rRNA IGS region of a subset of the PCR positive samples reported above (i.e., including infection in host ear tissue, *I. scapularis* ticks, and other tick species). We obtained sequence confirmation of 26 samples from coastal sites where

I. scapularis is abundant, and 28 samples from inland sites where *I. scapularis* is absent or at a very low density. Representatives of all three *B. burgdorferi* RST groups were found at sites characterized by both the presence and absence of *I. scapularis* (Table 3.2).

Opportunistic detection of other* Borrelia *species. Using IGS PCR followed by DNA sequencing, we detected spirochetes that group with *B. miyamotoi*, an organism genetically similar to relapsing fever group spirochetes, in ears from 12 white-footed mice at sites C2-C4. Two tick samples (a single nymph and a pool of 20 larval *I. scapularis*) removed from the same eastern chipmunk in 2005 at site C1 also tested positive for *B. miyamotoi*-like spirochetes. Representative *B. miyamotoi*-like sequences from these ears and ticks are deposited in Genbank (accession numbers GQ856588-GQ856589). We also detected spirochetes that group with *B. andersonii*, a Lyme borreliosis group spirochete associated with *I. dentatus* ticks, birds, and rabbits (Marconi et al. 1995), in one *I. dentatus* nymph removed from a song sparrow from site I3 in 2007 (GQ856590) and in ear tissue from one white-footed mouse from site C1 in 2006. As the IGS region of *B. andersonii* was not previously characterized in Genbank, we identified this species based on characterization of the 16S gene (data not shown).

Discussion

Lyme disease is emerging in the United States due largely to the spread of the bridging vector, *I. scapularis*. Both in the Northeast and the Upper Midwest, the spread of this tick has been documented at a gross scale, but few have studied this spread in real time (Falco et al. 1995; Schulze et al. 1998; Cortinas and Kitron 2006) are exceptions).

By monitoring ticks and hosts over five years, we assessed the temporal and spatial dynamics of *I. scapularis* populations along coastal and inland transects in western lower Michigan. Our aim was to investigate three non-mutually exclusive hypotheses to explain how the disease system is emerging across the landscape; we refer to these as the 'tick-first', 'dual-invasion', and 'spirochete-first' hypotheses. Our results suggest that there are distinct but overlapping processes that determine *I. scapularis/B. burgdorferi* dynamics. In some areas, observations of newly-arrived *I. scapularis* into a host/vector community where *B. burgdorferi* is absent support the 'tick-first' and 'dual-invasion' hypotheses. Conversely, in other areas, the detection of infected wildlife and alternative tick species preceded the arrival of *I. scapularis*, consistent with the 'spirochete-first' hypothesis. Here we summarize our key findings and consider their support for these hypotheses.

I. scapularis *invasion*. At a broad spatial scale, a continuum of endemicity of *I. scapularis* exists in the Midwest, whereby Wisconsin, Minnesota, and a focal area in Michigan's Upper Peninsula harbor long-established tick populations (Jackson and Defoliart 1970; Drew et al. 1988; Strand et al. 1992), Illinois and Indiana harbor ticks that colonized later (Pinger and Glancy 1989; Bouseman et al. 1990), and Michigan's Lower Peninsula represents the most recent invasion front (Foster 2004; Hamer et al. 2007) . We postulate that these Michigan ticks represent an expanding focus from recently-described populations in northern Indiana (these are adjacent to the Michigan border and near Lake Michigan; (Pinger et al. 1996). The density of questing nymphs at coastal site C1, where *I. scapularis* was most common, averaged 16.8/1000 m^2, which is higher than the average density of 6.6/1000 m^2 in *I. scapularis*-positive sites from throughout the eastern

U.S. (Diuk-Wasser et al. 2006). Densities of nymphs at the other positive sites in our study were much lower.

As in many other states that have witnessed an emergence of Lyme disease in the past four decades, white-tailed deer populations have increased sharply in Michigan's southern Lower Peninsula over the past 50 years, from near extirpation in the 1960s to nearly one million animals presently as a consequence of harvest restrictions and reforestation (MDNR 2002). Deer densities throughout the invasion zone have clearly passed the threshold necessary to support tick populations. The reforestation that supported the deer expansion also created an abundance of suitable habitat for other woodland hosts and *I. scapularis*. Thus the observed recent invasion may be a consequence of a sufficient number of introductions and large enough propagule size deposited to achieve a sufficient density to establish and disperse.

The progressive increase in tick density along the coastal sites from south to north among years is suggestive of rapid tick invasion and colonization northward along the coastal dune forests of Lake Michigan. *I. scapularis* could only be detected at the southernmost site at the start of the study, whereas all four sites harbored established populations by the end of the study period. Numerous authors have proposed that climate change may facilitate tick invasion of new areas due to a warming of the microclimate experienced by the ticks and shorter periods of freeze (e.g. (Githeko et al. 2000; Lindgren and Gustafson 2001). The prospects for such shifts are debatable (Randolph 2004), and *I. scapularis* in lower Michigan is well south of its distributional limits, so we presently have no evidence to suggest climate change as a driver for this invasion.

The disparity in tick distribution between our two transects suggests that there are important ecological differences between coastal and inland sites (Sonenshine et al. 1995), with the former allowing for more introductions of ticks, and/or higher success in establishment after introduction. Hosts with high tick burdens and large home ranges, such as deer and migratory birds, are critical for long distance dispersal of *I. scapularis*, whereas small mammals with small home ranges may limit invasion by diverting ticks from more vagile hosts (Madhav et al. 2004). Deer densities were not quantified in our study, but densities of other mammalian and non-migratory birds during the study period, as indexed by our trap and mist-net success, were similar between coastal and inland sites. Migratory birds tend to concentrate along shorelines (Alerstam 1978) and birds fly parallel to the long axis of Lake Michigan (Diehl et al. 2003), which may allow for more drop-offs of ticks from birds at coastal compared to inland sites.

B. burgdorferi *distribution, abundance, and diversity*. Overall infection prevalences in drag-sampled *I. scapularis* in the invasion zone were 8.6 and 36.6% in nymphs and adults, respectively; these infection prevalences are generally lower than those that characterize *I. scapularis* in the endemic Northeast (15% in nymphs (Gatewood et al. 2009); 49% in adults (Schulze et al. 2003)). The density of infected nymphs (DIN) has been shown to be correlated with the incidence of human Lyme disease in a given area (Stafford et al. 1998). Across our study, the DIN at site C1 was 1.5/1000 m^2, which is similar to DINs in endemic sites of the northeast (1.3/1000 m^2) and Midwest (1.0/1000 m^2) (Gatewood et al. 2009). The overall prevalence of infection of white-footed mice at

site C1 was 32.2%, which is lower than that reported from endemic foci (for example, 76% by the end of summer in Connecticut (Barbour et al. 2009). In addition to the lower nymphal infection prevalence as an explanation for lower prevalence of mouse infection, the difference may be due in part to sampling prior to the end of the transmission period.

We detected *B. burgdorferi* not only in sites where *I. scapularis* is established or apparently recently-invaded, but also within alternative tick species and wildlife hosts occurring at sites in the apparent absence of *I. scapularis*. Cryptic transmission of *B. burgdorferi* sensu lato has been documented in various host and tick species (Anderson et al. 1989; Telford and Spielman 1989b; Brown and Lane 1992; Maupin et al. 1994; Oliver et al. 2003), but has not been investigated in an area of *I. scapularis* invasion. The requirements for demonstration of a cryptic transmission cycle include the presence and interactions of i) *B. burgdorferi*, ii) a vector-competent tick species, and iii) reservoir-competent hosts. While our data are suggestive of a cryptic cycle and confirm the presence of many reservoir competent hosts, and infection with several different strains of *B. burgdorferi* in such hosts and in various tick species, it remains unclear which tick species are responsible for maintaining transmission of *B. burgdorferi* in the absence of *I. scapularis*. We documented infection in both questing and on-host *D. variabilis*, as did Walker et al. (1998) at a highly endemic area in Michigan's Upper Peninsula, yet this tick species is not a competent vector (Piesman and Sinsky 1988). *I. cookei* and *I. marxi* both had a low prevalence of infection in our study, but these infected ticks were removed from animals (southern flying squirrel, raccoon) at site C1, where *I. scapularis* and *B. burgdorferi* were at relatively high prevalence. Infections in these alternative tick species thus may represent spillover from feeding on hosts shared by

I. scapularis. Furthermore, *I. cookei* is an incompetent vector (Ryder et al. 1992). Two infected tick species removed from wildlife at inland sites included *I. texanus* and *I. dentatus*. Although the vector competency of *I. texanus* is unknown, its most important host- raccoons- are known to be competent reservoirs, though only 35% as efficient as white-footed mice at infecting xenodiagnostic larvae (Fish and Daniels 1990). Both *I. dentatus* and eastern cottontails are competent for transmission and maintenance of *B. burgdorferi* sensu lato (Telford and Spielman 1989a), however parasitism of *I. dentatus* on hosts other than birds and rabbits hosts was low during our study, and are not likely to explain infections in these hosts. Lord et al. (1994) documented a site in Pennsylvania at which isolations of *B. burgdorferi* from mice were common, yet *I. scapularis* was not found, and suggested that some other mechanism besides transmission by a tick vector was responsible.

We detected three different Borrelia species within ticks and hosts (*B. burgdorferi*, *B. miyamotoi,* and *B. andersonii*), including a diverse assemblage of *B. burgdorferi* strains. The diversity of *B. burgdorferi* RST types along the coast suggests high rates of pathogen flow and/or multiple introduction events, rather than isolated founder effects. Similarly, all three RST types were found in alternative tick vectors or ear biopsies from sites beyond the invasion front. Studies of the population genetic structure of *B. burgdorferi* will be useful for determining the dynamics of *B. burgdorferi* in the presence and absence of *I. scapularis,* and how cryptic cycles affect *B. burgdorferi* infection prevalence and strain types in the bridging vector *I. scapularis*.

Intriguingly, a single *I. scapularis* larva dragged at an inland site was infected with *B. burgdorferi*, and sequencing of the IGS region was used to identify this organism

as RST 2, which is the most common type in the Midwest. Evidence for transovarial passage of *B. burgdorferi*, which may result in infected questing *I. scapularis* larvae, is rare (but see Magnarelli et al. (1987) who found that transovarial transmission in *I. dammini* occurs at a highly variable rate) While the single infected questing larva we report herein is of little epidemiological significance, our finding was especially surprising considering the extremely low density of *I. scapularis* in the area. One possible explanation is this infection may represent spirochetes acquired during a partial larval bloodmeal from an infected host that terminated in the larva being groomed off.

Implications for hypotheses. Our detections of *B. burgdorferi* shed light on our hypotheses about the emergence of the lower Michigan *I. scapularis/B. burgdorferi* system. At four sites (C3, C4, I1, I3), we detected *I. scapularis* on mice after previous years of surveillance had failed to detect infested mice, and we interpret this as evidence for tick invasion into these sites. At site C4, no *B. burgdorferi* has yet been found, despite finding *I. scapularis* in both 2007 and 2008, supporting a tick-first process. At site C3, the first detection of *B. burgdorferi* coincided with that of *I. scapularis,* supporting a dual invasion process. In contrast, at sites I1 and I3, *B. burgdorferi* was found in other sample types prior to detecting *I. scapularis,* supporting a spirochete-first process. As described above, it may be that *B. burgdorferi* is being maintained in cryptic transmission cycles. It is also possible that a very low density *I. scapularis* population - below our detection threshold - exists at such sites. Such low density *I. scapularis* populations, however, would not generally be considered capable of maintaining *B. burgdorferi* (Madhav et al. 2004; Ogden et al. 2008).

Implications for detection of* I. scapularis/B. burgdorferi *invasion. Studies of invasion are most often initiated once the invader has reached densities that result in negative impacts, including disease, as resources for active surveillance and research are not often appropriated in the absence of a problem. Rarely are standardized investigations of wildlife undertaken in areas of no or low tick density that can provide a sensitive real-time warning system for invading ticks, spirochetes and impending Lyme disease risk. One exception is Schulze et al. (1998), who reported a significant increase in human Lyme disease cases in 1990-1995 in Hunterdon County, NJ, that occurred after a geographic expansion in ticks on deer in the county between 1981 and 1987. Falco et al. (1995) detected a 2.6-fold increase in questing *I. scapularis* nymphal density in endemic Westchester Co., NY, in 1991 versus 1984 when nymphal densities increased from 13 to 34 nymphs/1000 m^2, coincident with an increase in reported human cases of infection. In contrast, at our study site C2, where nymphal drag data demonstrate invasion most clearly, densities ranged from zero to 5.3 nymphs/1000m^2 in 2004-2008 (Figure 3.2.) Thus, we are assessing an invasion during its earliest stages, and human disease incidence is still very low (see below).

We found that prevalence of infestation of *I. scapularis* on white-footed mice provided a sensitive index of invasion (Lord et al. 1992), as detection of ticks on mice generally preceded detection of ticks on drag cloths, alternative mammal species, and birds. Mouse sampling was also more useful than two other methods of surveillance for ticks and Lyme disease foci. Assessment of ticks on hunter-harvested deer in November across the study area was hampered by low densities of adult ticks in this early-stage invasion, and by a mismatch in the timing of the fall hunting season in relation to adult

questing phenology in the invasion zone (their major activity peak is in the spring; S. Hamer, unpublished data). The efficacy of canine serosurveillance in the area was likely reduced by widespread use of anti-parasite prophylaxis on pet dogs living in the invasion zone (Hamer et al. 2009).

Similarly, alternative tick species and wildlife hosts enabled detection of *B. burgdorferi* in the apparent absence of *I. scapularis*. Detection of cryptically cycling *B. burgdorferi* decouples its invasion from that of *I. scapularis*, and may hasten *B. burgdorferi* transmission dynamics among an invading *I. scapularis* ticks. Cryptically cycling *B. burgdorferi* thus may decrease the time lag between first detection of *I. scapularis* and first detection of *B. burgdorferi*-infected *I. scapularis*.

Study limitations. The results we present are based on sampling within the May and June period only, which does not include all months of blacklegged tick activity. The most commonly cited *I. scapularis* phenology, based on studies in Westchester Co., NY (Fish 1993), is characterized by bimodal peaks in adult activity in the spring and fall, and nymphal activity in the early summer which precedes peak larval activity in the late summer. Gatewood et al. (2009) noted that while the Northeast is generally characterized by distinctly separate peaks in nymphal and larval activity in June and August, respectively, as indicated above, the Midwest is characterized by synchronous feeding of nymphs and larvae in June through July, with a much smaller late-season peak in larval activity. While we present five years of data from May and June only (early summer), all our sites were also sampled during July-August (late summer) in at least one year with similar rodent trapping and drag sampling effort (unpublished data). At all sites where we

found *I. scapularis* in the late summer, this species was also found in the early summer period reported here. Furthermore, in 2008-2009 we conducted a longitudinal study of *I. scapularis* phenology at monthly intervals at site C1 (unpublished data). Larval infestation of mice was greatest in June (83.8%), followed by a decline (44.1, 48, 24.5, and 19.4% monthly infestation prevalence in July-October, respectively), and nymphal infestation followed a similar trend (24.3, 8.5, 2.0, 2.0, and 0% in June-October, respectively). These patterns support the synchronized feeding reported by Gatewood et al. (2009) and are similar those reported by Godsey et al. (1987) in Wisconsin and Strand et al. (1992) in Michigan's Upper Peninsula. Thus, we conclude that our sampling focused on the period during which we had the best chance of detecting infested mice each year, and that this period was appropriate for answering our research questions.

A longer duration of sampling at all sites would have provided greater sensitivity of detection of stable *I. scapularis* populations. While neither our data nor that of Gatewood et al. (2009) show significant larval activity in the late summer, late summer larval activity of greater (Northeast) or equivalent (Midwest) magnitude to early summer activity has been reported (Kitron et al. 1991; Daniels et al. 1996; Jones and Kitron 2000). In the Northeast, the early peak in May-June represents the remainder of the cohort of larvae that hatched the previous July and successfully overwintered, whereas the second peak of higher magnitude in August represents the subsequent cohort of newly-hatched larvae from recently laid eggs by adults that were active in spring as well as the previous fall (after ovipositional diapause; (Daniels et al. 1996)). In the Midwest, the separate peaks observed by some researchers at some sites are posited to result from the separate oviposition periods of the spring and fall adults (Kitron et al. 1991; Daniels

et al. 1996; Jones and Kitron 2000), whereby the early larvae may result from oviposition by fall adults and the late summer larvae result from oviposition by spring adults. Furthermore, there has been a suggestion that this bimodality may be more pronounced in newly-establishing populations (Kitron et al. 1991; Daniels et al. 1996; Jones and Kitron 2000). Therefore, if blacklegged tick invasion in Michigan is driven by adult ticks moving in during the spring (e.g., on deer), our sampling may miss the resulting larvae emerging late that first summer.

Assaying ticks of all stages and species removed from hosts increased our capability to detect the presence of *B. burgdorferi* at a site compared to assaying *I. scapularis* or host tissue alone. Our protocol, however, did not maximally allow us to detect *B. burgdorferi* infection as we did not assay all ticks removed from hosts. We selected a random subset of three per species/stage/sex combination to assay for infection, sometimes resulting in over ten ticks tested from some hosts that were infested my multiple tick species and stages. Given limited resources, this protocol allows us to gather infection data from ticks removed from a greater number and diversity of hosts as well as to avoid over-representing heavily parasitized animals in the overall results of tick infection. Testing of all ticks from such animals would have increased the odds of both detecting a positive tick on a given animal and detecting different genospecies that co-infect the same vertebrate host (although that latter was not a main study objective). While comprehensive tick testing may have resulted in a finer temporal resolution of invasion dynamics, we do not believe it would have altered our qualitative conclusions.

Implications for disease and prevention. Data collected from careful study of the underlying ecology and transmission of zoonotic pathogens within animal reservoirs before and during invasion can provide an early warning of increasing disease risk to human and companion animals. Cryptic cycling of *B. burgdorferi* does not imply human risk of Lyme disease in these areas. They, however, may hasten the establishment of transmission dynamics of *I. scapularis*. They also may introduce different strains of *B. burgdorferi* to *I. scapularis*, which may result in different clinical manifestations (Wormser et al. 1999).

Indeed, a significant increase in confirmed cases of human Lyme disease (R^2 = 0.28; 1-tailed P = 0.03) has occurred within the zone of active *I. scapularis* invasion in southwest/western Michigan. From 1996 (when Lyme disease became reportable in Michigan) through 2008, incidence in the 14-county region reached 0.63 cases per 100,000 people in 2008 (E. Foster, Michigan Department of Community Health, pers. comm.). Incidence remains substantially less than the average annual incidence in the 10 endemic states from which >93% of human Lyme disease across the U.S. is reported (average of 29.2 cases per 100,000 population for 2003-2005; (Bacon et al. 2007). In contrast with the significant peridomestic exposures that occur in the Northeastern United States, recreational exposure is probably common in the Michigan invasion zone, which is dominated by recreational areas, camps, vacation homes, and rural communities. Recreational exposure may produce low reported disease incidence locally, as recreational visitors will often return home before developing symptoms that lead them to seek medical attention. Spatial epidemiology and trace-back of human Lyme disease cases reported elsewhere in lower Michigan could help shed light on the this possibility.

Supplementary material 1. Bird-tick associations and infestation prevalences on the inland and coastal transects in Lower Michigan, May-June, 2004-2008. Number of birds carrying larvae/nymphs/adults is indicated with the percent of birds infested with at least one tick of any life stage in parenthesis. All ticks were tested for *Borrelia burgdorferi*; those birds with infected ticks are indicated in Supplementary material 2.

	Total	Coastal			Inland		
Species	**no.**	**no.**	***I. scapularis***	***I. dentatus***	***H. lep.***	**no.**	***I. dentatus***	***H. lep.***
American goldfinch	65	22				43		
American redstart	22	22				0		
American robin	46	20	4/8/0 (40)	1/1/0 (10)	2/2/0 (15)	26	9/11/0 (53.8)	0/2/0 (7.7)
Baltimore oriole	9	0				9		
black-billed cuckoo	1	0				1		
black-capped chickadee	30	11				19	2/0/0 (10.5)	
black-throated green war	1	1				0		
blue jay	1	0				1		
blue-winged warbler	1	0				1		
brown thrasher	3	0				3	2/1/0 (66.7)	
brown-headed cowbird	8	2		1/1/0 (25)		6		
cedar waxwing	9	2				7		
chipping sparrow	40	33	0/2/0 (6.1)			7		
common grackle	2	1				1		
common yellowthroat	53	9		0/0/1 (11.1)		44	3/0/0 (6.8)	
Connecticut warbler	1	1				0		
downy woodpecker	6	1				5		
Eastern bluebird	6	3				3		
Eastern kingbird	2	1				1		
Eastern phoebe	4	3		1/0/0 (33.3)		1		
Eastern towhee	2	1	0/1/0 (100)			1		
Eastern wood-pewee	8	3				5		
Empidonax flycatchers	14	6				8		
European starling	1	0				1		
field sparrow	8	2				6	1/0/0 (16.7)	
gray catbird	89	13				76	4/3/0 (6.6)	
gray-cheeked thrush	1	1				0		
great crested flycatcher	1	0				1		
hairy woodpecker	1	1				0		
house finch	3	3				0		
house wren	13	9		0/1/0 (11.1)		4	2/0/0 (50)	
indigo bunting	22	21	0/1/0 (4.8)			1		
mourning dove	1	0				1		
Northern cardinal	16	7	1/1/0 (14.3)	0/0/1 (14.3)		9	2/0/0 (22.2)	
Northern waterthrush	1	0				1		
orchard oriole	2	0				2		
ovenbird	1	1				0		
pileated woodpecker	1	1				0		
red-eyed vireo	15	15				0		
red-winged blackbird	80	0				80	13/0/0 (16.3)	0/1/0 (1.3)
rose-breasted grosbeak	7	2				5	1/0/0 (20)	
song sparrow	52	9		1/1/0 (11.1)		43	13/6/0 (32.6)	1/2/0 (7.0)
swamp sparrow	4	0				4	1/0/0 (25)	
Tennessee warbler	1	1				0		
Trail's flycatcher	4	0				4		
tree swallow	3	0				3		
tufted titmouse	2	2				0		
unknown	2	0				2		
warbling vireo	2	0				2		
white-crowned sparrow	2	0				2		
Wilson's warbler	2	0				2		
wood thrush	2	0				2	2/1/0 (100)	
yellow warbler	65	7				58	1/1/0 (3.4)	
yellow-breasted chat	1	0				1	1/0/0 (100)	
yellow-sided flycatcher	1	0				1		
yellow-throated vireo	1	0				1		
All species	**741**	**237**	**5/13/0 (5.5)**	**4/4/1 (3.4)**	**2/2/0 (1.3)**	**504**	**57/23/0 (12.9)**	**1/5/0 (1.2)**

Supplementary material 2. Infection prevalence of each species of tick removed from mammalian and avian hosts in lower Michigan, May-June, 2004-2008. Infection prevalence is presented as the percent of ticks that tested positive with the sample size of ticks tested is in parenthesis. Larval infection is reported as the minimum infection prevalence. LL = larvae; NN = nymph; AA = adult; misc. = miscellaneous tick species indicated in column; *A. amer.* = *A. americanum*; *H. lep.* = *H. leporispalustris*; . Regarding birds, only those species with positive ticks are listed below; a complete list of all 56 bird species and associated ticks is provided in Supplementary material 1.

Host species	Transect	Stage	*D. variabilis*	*I. scapularis*	*I. texanus*	*I. cookei*	*I. dentatus*	misc.
Northern short-tailed shrew	Coastal	LL		0 (1)				
		LL				0 (2)		
		NN			0 (1)	0 (1)		
	Inland	AA	1.1 (91)		0 (1)	0 (1)		
		LL		0 (52)				
		NN		6.9 (29)		0 (5)		
Virginia opossum	Coastal	AA						
		LL	0 (3)		100 (1)			
Southern flying squirrel		NN		0 (2)				*I. marxi*
	Coastal	AA	0 (3)					50 (2)
		LL				0 (19)		
		NN		0 (3)		0 (1)		
Striped skunk	Coastal	AA				0 (2)		
Meadow vole	Coastal	LL	0 (1)					
Long-tailed weasel		LL	0 (1)			0 (1)		
	Inland	NN				0 (2)		
		LL	0.7 (2116)	0 (5)			0 (1)	
	Inland	NN	6.6 (106)	50 (2)				
White-footed mouse		LL	2.4 (716)	5.4 (705)	0 (1)			
	Coastal	NN	17.6 (74)	37.8 (82)	0 (1)			
		LL	0 (1)		2.4 (41)	0 (1)		
		NN	0 (7)		2.6 (78)	0 (6)		
	Inland	AA	1.4 (209)		2.4 (83)	0 (2)		
		LL	0 (1)	0 (5)	0 (158)			
		NN	0 (1)	9.1 (22)	0 (94)			*A. amer.*
Raccoon	Coastal	AA	2.4 (164)	0 (2)	2.3 (88)	11.1 (9)		0 (2)
		LL	0 (2)	25 (4)				
Eastern gray squirrel		NN		40 (5)				
	Coastal	AA	0 (6)					
		LL		0 (2)				
Fox squirrel	Coastal	NN		0 (1)				*H. lep.*
	Inland	AA	0 (3)				12.9 (31)	0 (2)
		LL		66.7 (3)				
Eastern cottontail		NN	0 (1)					0 (3)
	Coastal	AA					0 (2)	0 (6)
		LL	5 (20)					
	Inland	NN	0 (2)					
Eastern chipmunk		LL	0 (2)	9.8 (82)	0 (1)			
	Coastal	NN		26.2 (65)				*I. marxi*
		NN	0 (1)					0 (1)
	Inland	AA			0 (1)			0 (3)
		LL		0 (1)				
Red squirrel	Coastal	AA			0 (3)			
		LL	0 (12)					
	Inland	NN	0 (1)				0 (1)	
		LL		0 (1)				
Meadow jumping mouse		NN		0 (2)				
	Coastal	AA					0 (1)	
	Inland	LL					2.9 (35)	
		LL		7.7 (13)				*H. lep.*
American Robin	Coastal	NN		37.5 (16)				50 (2)
		LL		100 (1)				
Northern cardinal	Coastal	NN		100 (1)				
Brown-headed cowbird	Coastal	LL					100 (1)	
		LL					5.1 (39)	
Song sparrow	Inland	NN					12.5 (8)	
Red-winged blackbird	Inland	LL					2.1 (47)	

Table 3.1. Tick-host associations and infestation prevalence on the inland and coastal transects in Lower Michigan, May-June, 2004-2008. L = larva; N = nymph; A = adult.

Host species	Transect	No. captures	No. animals infested with L/N/A (% infested with any stage)						
			D. variabilis	*I. scapularis*	*I. texanus*	*I. cookei*	*I. dentatus*	*I. marxi*	*H. leporispalustris*
Northern short-tailed shrew	Inland	10							
	Coastal	3							
Virginia opossum	Inland	22	0/0/15 (68.2)						
	Coastal	13	0/0/6 (46.2)	6/8/2 (61.5)		1/1/1 (9.1)			
Southern flying squirrel	Inland	1				0/3/0 (23.1)			
	Coastal	13	1/0/2 (23.1)	0/2/0 (15.4)	1/0/0 (7.7)				
Striped skunk	Coastal	2						0/0/1 (7.7)	
Meadow vole	Coastal	1		0/1/0 (50)		1/1/1 (100)			
Long-tailed weasel				1/0/0 (100)					
White-footed mouse	Inland	2	1/0/0 (50)						
	Inland	665	293/58/0 (46.2)	2/2/0 (0.5)		1/1/0 (50)			
	Coastal	570	106/42/0 (20.3)	175/53/0 (33.5)	1/1/0 (0.4)				
Raccoon[2]	Inland	64	1/4/37 (59.4)		6/26/32 (68.8)	1/6/1 (10.9)			
Eastern gray squirrel	Coastal	62	2/1/39 (62.9)	3/11/2 (21.0)	10/34/34 (72.6)	0/5/2 (9.7)	0/1/0 (1.6)	0/0/1 (1.6)	
Fox squirrel	Coastal	5	1/0/1 (20)	2/4/0 (80)					
Eastern cottontail	Coastal	1		1/1/0 (100)					
	Inland	4	0/0/1 (25)						
Eastern chipmunk	Coastal	1					0/1/4 (100)		0/0/1 (25)
	Inland	74	3/2/0 (6.8)				0/0/1 (100)		0/1/1 (100)
Red squirrel	Coastal	84	1/0/0 (1.2)	16/36/0 (46.4)	1/0/0 (1.2)				
	Inland	16	0/1/0 (6.3)		0/0/1 (6.3)	0/1/0 (6.3)			
Meadow jumping mouse	Coastal	2			0/0/1 (50)				
	Inland	12	6/1/0 (50)	1/0/0 (50)				0/1/1 (12.5)	
	Coastal	16							
Birds[1]	Inland	504		1/2/0 (12.5)			4/0/0 (33.3)		
							0/0/1 (6.3)		
	Coastal	237		5/14/0 (5.9)			57/29/0 (12.9)		1/5/6 (1.2)
							4/4/2 (3.8)		2/2/0 (1.3)

[1] Species-specific bird captures and tick associations are presented in Supplement 1.

[2] One coastal raccoon was infested with a single *Amblyomma americanum* adult; 1.6% infestation prevalence.

Table 3.2. Sequence confirmation and accession numbers of *B. burgdorferi* infection in various sample types from coastal and inland sites in lower Michigan, 2004-2008. I = inland transect site; C = coastal transect site. RST = 16S – 23S rRNA spacer type of *B. burgdorferi*. AF = adult female; AM = adult male; N = nymph; L = larvae; WFMO = white-footed mouse; SFSQ = southern flying squirrel; EAGS = eastern gray squirrel; VIOP = Virginia opossum; EACH – eastern chipmunk, NOCA = Northern cardinal; AMRO = American robin, SOSP = song sparrow; COON = raccoon, MJMO = meadow jumping mouse; EACO = eastern cottontail.

Site	Year	Sample Type	Host Species or Drag	RST (1, 2, 3)	Acc. No.
C1	2004	*D. variabilis* AF	Drag	2	GQ856591
		ear biopsy	WFMO	1	GQ856630
		ear biopsy	WFMO	2	GQ856631
		I. marxi AF	SFSQ	1	GQ856615
		I. scapularis L	WFMO	2	GQ856598
		I. scapularis N	EAGS	2	GQ856614
	2005	*D. variabilis* L	Drag	1	GQ856596
		D. variabilis L	WFMO	3	GQ856600
		I. scapularis N	VIOP	2	GQ856594
		I. scapularis N	WFMO	3	GQ856595
		I. texanus L	SFSQ	2	GQ856601
	2006	*D. variabilis* N	WFMO	2	GQ856639
		I. scapularis N	Drag	2	GQ856592
		I. scapularis N	EACH	1	GQ856612
		I. scapularis N	WFMO	2	GQ856611
	2007	*D. variabilis* N	WFMO	3	GQ856643
		ear biopsy	EACH	3	GQ856632
		H. leporispalustris N	Drag	3	GQ856609
		I. scapularis L	NOCA	3	GQ856619
		I. scapularis N	AMRO	3	GQ856616
C2	2005	ear biopsy	WFMO	2	GQ856620
	2006	*I. scapularis* L	WFMO	2	GQ856593
	2008	ear biopsy	WFMO	2	GQ856610
C3	2006	ear biopsy	WFMO	3	GQ856623
		ear biopsy	WFMO	3	GQ856622
		ear biopsy	WFMO	3	GQ856621
I2	2004	*D. variabilis* L	WFMO	1	GQ856638
	2005	*D. variabilis* L	WFMO	1	GQ856602
	2006	ear biopsy	WFMO	2	GQ856628
		ear biopsy	WFMO	2	GQ856629
		ear biopsy	WFMO	2	GQ856627
		ear biopsy	WFMO	2	GQ856626
		ear biopsy	WFMO	2	GQ856625
		ear biopsy	WFMO	2	GQ856624
	2007	*D. variabilis* L	WFMO	3	GQ856603
I3	2004	*D. variabilis* L	WFMO	2	GQ856599
		D. variabilis L	WFMO	2	GQ856597
		I. texanus N	COON	3	GQ856640
	2006	ear biopsy	WFMO	2	GQ856642
		ear biopsy	MJMO	2	GQ856641
	2007	*I. scapularis* N	WFMO	2	GQ856606
		I. scapularis L	Drag	2	GU190359
		D. variabilis L	WFMO	2	GQ856608
		D. variabilis L	WFMO	2	GQ856607
		D. variabilis L	WFMO	2	GQ856605
		D. variabilis L	WFMO	2	GQ856604
		ear biopsy	VIOP	2	GQ856636
		ear biopsy	COON	2	GQ856634
		ear biopsy	COON	2	GQ856635
		ear biopsy	WFMO	2	GQ856633
	2008	*I. dentatus* AF	EACO	2	GQ856618
		I. dentatus AM	EACO	2	GQ856617
I4	2004	*I. texanus* N	COON	1	GQ856613
	2006	ear biopsy	EACH	2	GQ856637

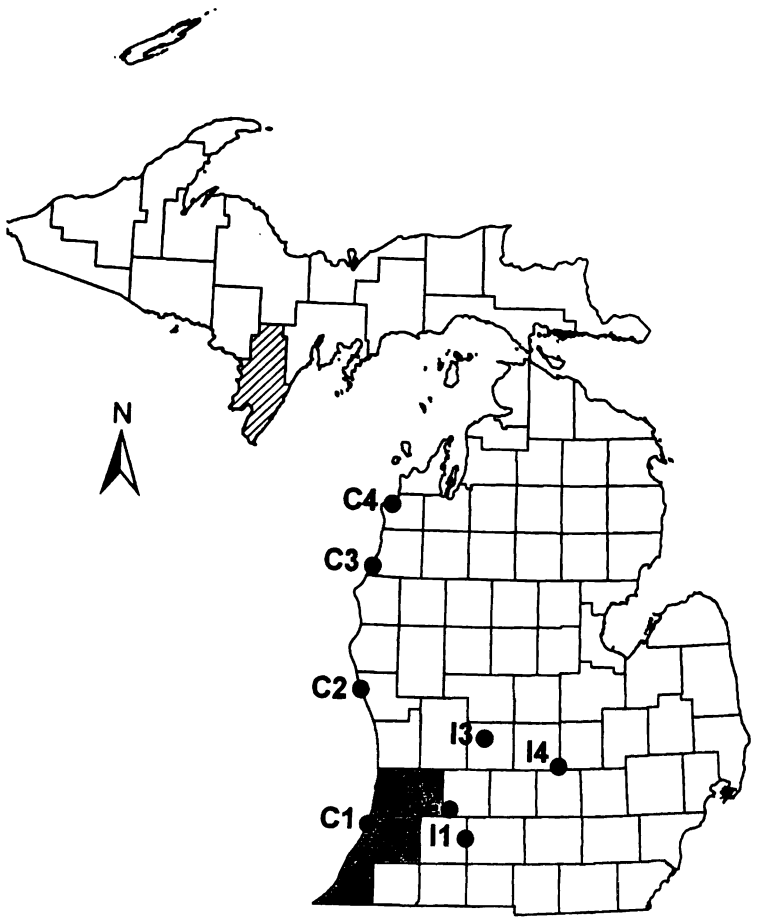

Figure. 3.1. Locations of study sites in Lower Michigan, 2004-2008. The four sites along Michigan's west coast comprise the coastal transect (from south to north, C1-C4), and the four inland sites comprise the inland transect (from southwest to northeast, I1-I4). Shading in the Lower Peninsula represents the three-county region where *I. scapularis* were detected on small mammals in 2002-2003(Foster 2004). The cross-hatched county in the Upper Peninsula is Menominee County, Michigan's longstanding endemic focus of *I. scapularis* and *B. burgdorferi*.

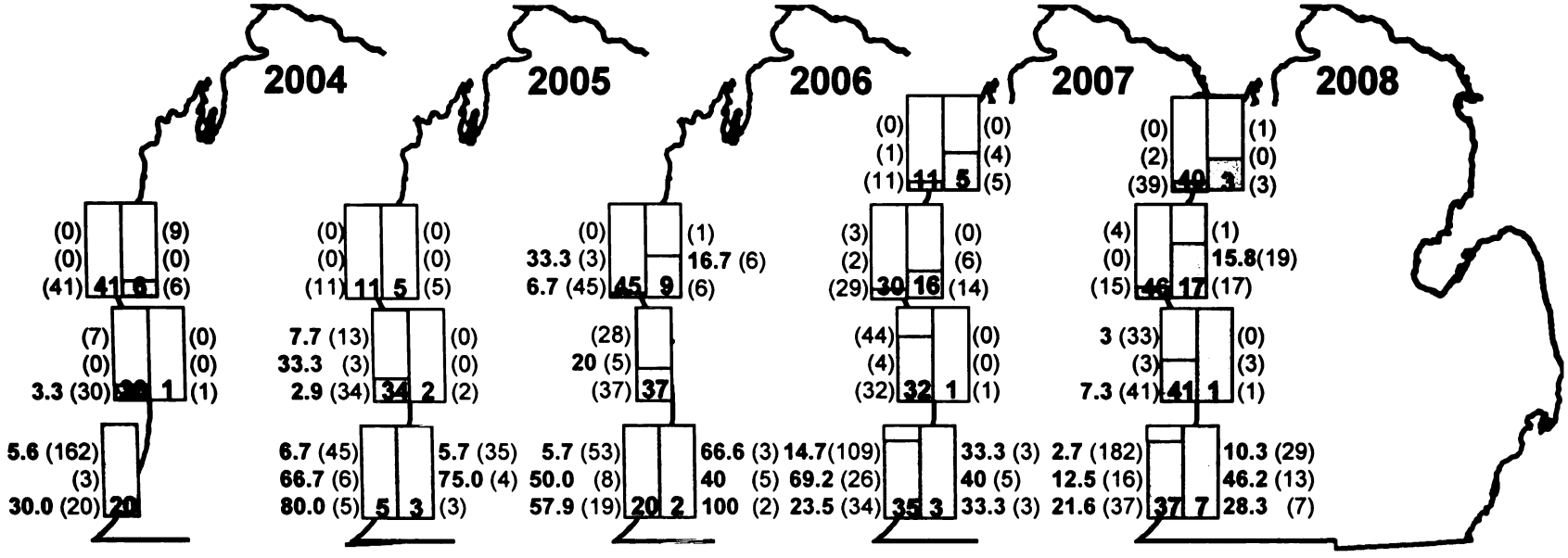

Figure. 3.2. Infestation of white-footed mouse (left rectangle) and eastern chipmunk (right rectangle) with *I. scapularis* at the four sites of the coastal transect, May-June, 2004-2008. Level of shading indicates the proportion of animals infested, with the number of animals screened for ticks inside the symbol. Adjacent to each symbol, *B. burgdorferi* infection prevalence (%) in (*i*) *I. scapularis* larvae (minimum infection prevalence), (*ii*) nymphs removed from hosts, and (*iii*) host ear biopsies are listed from top to bottom, each followed by sample size in parenthesis. At site C4, no traps were set in 2004-2006. At site C1 in 2004 and C2 in 2006, no chipmunks were captured despite traps being set. Information on the inland transect sites, where infestation and infection rates were close to zero, appears in the text.

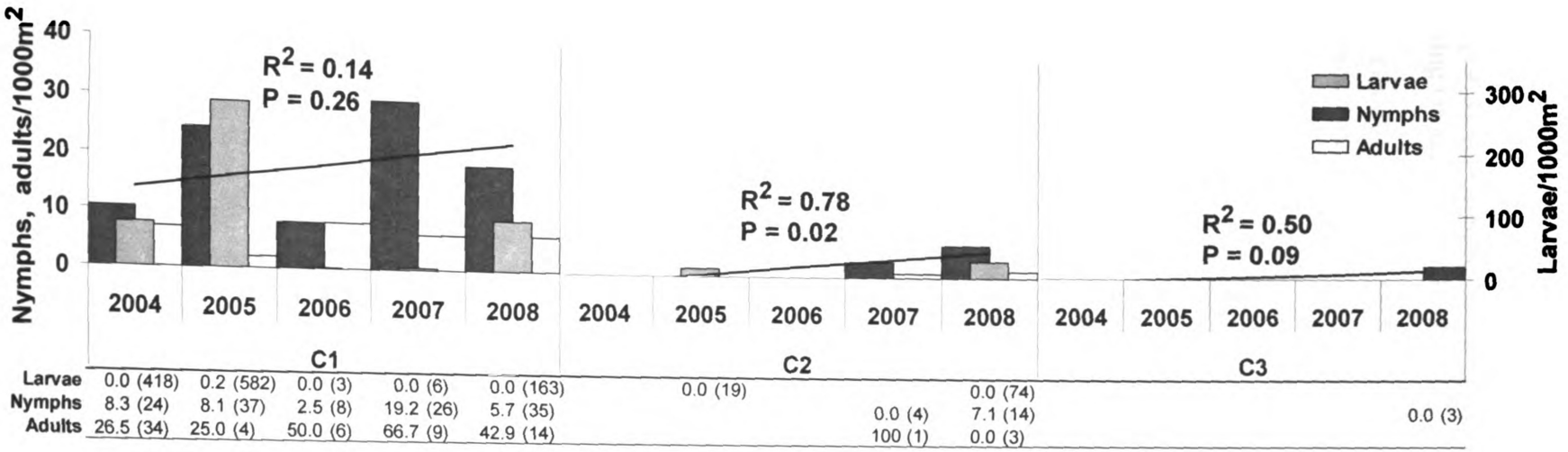

Figure. 3.3. Average densities and *B. burgdorferi* infection prevalences of drag-sampled *I. scapularis* at the three sites on the coastal transect in May and June, 2004-2008. No *I. scapularis* were dragged at site C4. Regression lines and coefficients for nymphal densities are shown. Infection prevalence for each life stage (minimum infection prevalence for larvae) is expressed as the percent positive, with the total number tested in parenthesis below each year. C = coastal

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CHAPTER 4

Canines as sentinels for emerging *Ixodes scapularis*-borne zoonoses risk

Hamer SA, Tsao JI, Walker ED, Mansfield LS, Foster ES, Hickling GJ. 2009. Canines as sentinels for emerging *Ixodes scapularis*-borne zoonoses risk. *American Journal of Veterinary Research* 70:49-56.

Abstract

To evaluate companion animal dogs as sentinels for emergence of *Ixodes scapularis* and associated canine and zoonotic pathogens in a region undergoing invasion by these ticks. Dogs brought to clinics located along a gradient of *I. scapularis* abundance in Lower Michigan in 2001-2002 (pilot study), and July-August, 2005. Sera were evaluated for *Borrelia burgdorferi*-specific antibodies using an indirect fluorescent antibody test, western blot, and rapid enzyme-linked immunosorbent assay. Ticks from dogs were subjected to PCR and DNA sequencing for identification of *Borrelia* and *Babesia* species and *Anaplasma phagocytophilum.* Of 353 canine sera from 18 counties, only two (0.6%) contained western blot-confirmed *B. burgdorferi* antibodies from naturally-acquired infections; this low seroprevalence rate did not differ from the early invasion study. Seventy-eight ticks of three species were collected. Ten of 13 dogs presenting with *I. scapularis* were from clinics within or immediately adjacent to the known tick invasion zone. Six of 18 (33%) *I. scapularis* and 12 of 60 (20%) non-competent vectors were positive for *B. burgdorferi.* No ticks were positive for *A. phagocytophilum*, and three tested positive for *Babesia* spp. Seroprevalence has remained very low throughout five years of *I. scapularis* invasion of the study area, so a canine serosurvey has been relatively ineffective in tracking early invasion dynamics. Furthermore, the common practice of canine tick chemoprophylaxis likely reduces

sensitivity of the canine serosurvey. Ticks testing positive for *B. burgdorferi* were more common and widely dispersed than were the very small number of seropositive dogs. We conclude therefore that in areas of low tick density, the use of canine patients as a source of ticks for species identification and pathogen detection is preferable to canine serosurvey as a method of surveillance. By retaining ticks from their canine patients for later identification and pathogen testing, companion animal veterinarians can play an important role in early detection in areas with increasing incidence of Lyme disease.

Introduction

Ixodes scapularis, the blacklegged tick, is abundant in parts of the northeastern and upper Midwestern United States, and is expanding geographically into previously-uninfested areas. Invasion of *I. scapularis* results in increased risk of disease to canines and humans from pathogens vectored by this tick, including *Borrelia burgdorferi*, *Anaplasma phagocytophilum*, and *Babesia* species, the agents of Lyme disease, granulocytic anaplasmosis, and babesiosis, respectively. Lyme disease is the most prevalent vector-borne disease of humans in the United States and Europe (Steere et al. 2004) and a common cause of acute arthritis and arthralgia in dogs (Appel et al. 1993).

One area of recent *I. scapularis* invasion is the Lower Peninsula of Michigan(Foster 2004; Hamer et al. 2007), where *I. scapularis* presence and incidence of *B. burgdorferi* infection in humans, canines, and wildlife have been increasing since the early 2000s. The putative sources of invasion are recently-established populations of *I. scapularis* immediately to the south, in northern Indiana(Pinger et al. 1996) and initial intensive surveys of *B. burgdorferi*-infected ticks and wildlife in Lower Michigan

demonstrate a gradient of *I. scapularis* density with the highest density in the southwestern corner of the state. Along Michigan's Lower Peninsula western lakeshore in 2007, *I. scapularis* dragging densities ranged from 30 to 3 to 0 nymphs/1000 m^2 at sites in the south, central, and north, respectively, with *B. burgdorferi* found in all areas where *I. scapularis* was found; drag sampling over 5600 m^2 at inland sites over the same time period revealed no *I. scapularis* nymphs (S. Hamer, unpublished data). The infested zone is of considerable public health significance because the Lake Michigan coast is a popular tourist site and harbors many state parks and recreation areas. Hence, it would be useful to quantify and delineate geographically the current disease risk levels in order to inform public health recommendations in the Lower Peninsula.

Dogs as sentinels for disease emergence. Assessing risk in zones of emerging Lyme disease, as opposed to in endemic zones, is challenging. Lack of public and medical awareness often hinders accurate diagnosis. Human case prevalence is initially low, so the positive predictive value of any diagnostic test is similarly low. Field surveys of ticks, mice, deer and other vertebrates can reveal trends in Lyme disease risk in these areas, but such surveys are laborious and costly. Consequently, numerous researchers have proposed that companion animal dogs are a useful sentinel animal that can assist in assessing risk in areas endemic for Lyme disease (Falco et al. 1993; Merino et al. 2000; Guerra et al. 2001; Bhide et al. 2004). Companion dogs typically have a close association with their human owners, but are more active in habitats where they can come into close contact with infected ticks. Eng et al. (1988) estimated that pet dogs were on average six times more likely than their owners to be seropositive to *B. burgdorferi*, and

dog ownership is a significant risk factor for human tick-borne disease (Jones et al. 2002). Most canine serosurveys, however, have been conducted in areas endemic for *I. scapularis* where human risk is already appreciated. Exceptions include Rand and colleagues(Rand et al. 1991; Rand et al. 1996), who found in Maine that seropositive canines identified areas of human risk in advance of human cases, and Duncan and colleagues (Duncan et al. 2004), who deemed the canine an appropriate sentinel in North Carolina where canine seroprevalence was <1% and transmission to humans in that state was rare. Nevertheless, the utility of canine serosurveys in areas where risk is low but emerging remains unclear.

History of serodiagnosis of Lyme disease in canine patients. Diagnosis of Lyme disease in canine patients is typically based on serology alone, and as in humans, has been problematic since the emergence of the disease due to cross-reactivity to spirochetes including *Treponema denticola* and *Leptospira interrogans* and lack of standardization of laboratory protocols. Recommended practice has therefore been a two-step diagnostic algorithm for determining infection of dogs with *B. burgdorferi*, whereby indirect fluorescent antibody tests (IFA) or enzyme-linked immunosorbent assays (ELISA) is used to screen sera and western blotting (WB)(Lindenmayer et al. 1990; Gauthier and Mansfield 1999) is used to confirm suspect-positive samples. Recently, a *B. burgdorferi* peptide antigen, the C_6 region of the VlsE, has been deployed in a commercially-available ELISA kit that provides sensitivity and specificity equaling that of the western blot (Levy et al. 2003).

Canids as sentinels for Lyme disease risk in Lower Michigan. In 1992-1993, prior to the current *I. scapularis* invasion of southwestern Michigan, a canine serosurvey was undertaken in six Michigan counties, including Menominee County in the Upper Peninsula, the only Michigan county endemic for Lyme disease at that time (Walker et al. 1998). Using ELISA and IFA, 25 of 299 (8.4%) canids from Menominee County were seropositive by one or both screening methods, whereas only one of 919 (0.1%) canids from the Lower Peninsula tested positive by IFA (and was negative by ELISA). This serosurvey thus establishes the almost complete absence of *B. burgdorferi*-exposed canids in the Lower Peninsula in the early 1990s.

In response to initial findings of invading ticks on wildlife (Foster 2004), we initiated a preliminary survey of canine exposure to Lyme disease ticks and pathogen in the presumed focus of invasion of southwestern Michigan. In 2005, we undertook a new serosurvey and tick survey of canine patients at veterinary clinics in Lower Michigan to define the zones of disease risk in an area undergoing tick invasion beyond the focus. Furthermore, we compare the results given the conventional two-tiered approach versus the new C_6 ELISA.

In designing the study, we hypothesized that *B. burgdorferi* seropositive canids would be found throughout the known geographic distribution of *I. scapularis*, and similarly, that *I. scapularis*-borne pathogens would be restricted to that same geographic range. To test these hypotheses, our objectives were four-fold: *i*) to determine rates of canid exposure to *B. burgdorferi* in an area undergoing active invasion by *I. scapularis*; *ii*) to determine rates of canid infestation by *I. scapularis* and other ticks; *iii*) to determine the prevalence of *B. burgdorferi*, *A. phagocytophilum* and *Babesia* species in

ticks from canine patients, and *iv*) to assess patterns of seroprevalence, vaccination, *I. scapularis* presence and tick infection in relation to the zone of *I. scapularis* invasion in southwestern MI. Specifically, we predicted that all these measures would be highest at clinics in the southwestern corner of the state – the putative site of earliest invading ticks – and would decline with increasing distance from this focus.

Materials and Methods

Sample Acquisition. In spring 2005, 18 veterinary clinics consented to participate in our canine tick and serosurvey; these clinics were selected along three transects that originated in the recently-invaded southwestern corner of Michigan and extended radially to the north, northeast, and east. These transects had been established previously for ongoing wildlife and tick sampling, and traversed the known geographic boundary of the invading ticks (S. Hamer unpublished data). An initial 133 clinics operating within the area of interest were identified from online directories and geo-coded using ArcGIS 8.0 (ESRI, Redlands, CA). Six clinics distributed along each transect were then randomly selected and contacted by phone to solicit participation in the study. For analysis, the clinics were grouped into three zones based on their proximity to the recently-established *I. scapularis* populations: Zone 1 included all clinics in counties where *I. scapularis* was known to be established (n = 6; establishment defined as documented presence of all three life stages of *I. scapularis* on drag cloth, wildlife, and/or humans); Zone 2 included all clinics in counties that bordered Zone 1 (n = 4). Zone 3 included all clinics in counties outside of Zones 1 and 2 (n = 8; Figure 4.1).

Clinics were asked to retain 30 serum samples from dogs acquired in the course of routine blood-sampling from July 15 – August 15. We requested this number based on the calculation that we would have a >95% chance of detecting at least one seropositive dog, assuming a 50% response rate by the clinics and an invasion area prevalence of 1% (cf. average canine seroprevalence for endemic sites in the northeastern U.S. is 30% (Guerra et al. 2001). In recently-invaded southwest Michigan, *I. scapularis* adult and nymphal host-seeking peaks in April and June, respectively (Foster 2004); the collection period chosen was the time of year when dogs would have likely seroconverted (i.e., >4-6 weeks after exposure (Appel et al. 1993) from infection by either nymphs or adults. Clinicians were instructed to store sera at -20 ° C until pick-up.

The clinic veterinarians were also asked to collect all ticks seen on canine patients during the same July-August period. Canines presenting ticks were not necessarily the same canines from which blood was collected. All ticks from each dog were stored in vials of 70% ethanol, and the veterinarian completed a short questionnaire to indicate the dog's breed, sex, age, zip code, travel history, Lyme disease vaccination and diagnosis status. After pick-up from clinics, sera were stored at -80 ° C before processing at the Michigan State University Medical Entomology Laboratory.

Indirect Fluorescent Antibody (IFA). Sera were thawed and centrifuged for 10 min at 13,000 rpm, then serially diluted with sterile 1X phosphate buffered saline (PBS) to 1:320 and 1:640. Both dilutions were screened for antibody presence using 12-well IFA slides fixed with whole cell *B. burgdorferi* organisms (low passage mixture of isolates B31 and 297; Fuller Laboratories, Fullerton, CA). Slides were warmed to room

temperature and each well was loaded with 20 µl of sample or control. One positive and one negative control (VMRD, Pullman, WA) were included on each slide. Slides were incubated in a humidifying chamber for 30 min at 37 ° F. Samples were washed from slides by rinsing with PBS three times after which excess PBS was tapped off onto a paper towel. While wells were still moist, 20 µl of stained fluorescein isothiocyanate (FITC)-conjugated anti-dog IgG antibody was added to the wells. This conjugate was prepared using sterile 0.1% Evans Blue solution in PBS to make a 1:100 dilution FITC-conjugated anti-dog IgG (Kirkegaard and Perry Laboratories, Gaithersburg, MD). The conjugated secondary antibody was incubated on the slide for 30 min at 37 ° F in the humidifying chamber and washed as above.

For visualization, a cover slip was placed onto the moist slide, which was then viewed under UV illumination at 400X (10X ocular, 40X objective) using fluorescent microscopy (Eclipse E800, Nikon, Melville, New York). All slides were read by one observer (SAH) who was blinded to the identity of the slides. Roughly 15 sec were spent scanning each of 10 fields within each well, with both the intensity and abundance of fluorescing spirochetes compared to the negative control. Wells with well-resolved and consistently bright yellow-green spirochetes were considered positive for anti-*B. burgdorferi* antibodies at the tested dilution. Samples with no fluorescing spirochetes at 1:640 were considered negative. All samples positive at 1:640 were tested in a series of two-fold dilutions until an endpoint titer was found. Samples positive at or above 1:640 dilutions were considered suspect-positive and were subjected to confirmatory testing using western blot.

Western blot (WB). Western blot analysis was used as a confirmatory test for all IFA suspect-positive sera, all sera derived from canines that were reported as having been vaccinated regardless of endpoint titer, and a random sample of the IFA-negative sera. Western blots were performed using the QualiCode Canine Lyme Disease Kit (Immunetics, Cambridge, MA); blots were prepared by the manufacturer and contained 18 separated antigens of *B. burgdorferi* strain B31. We followed kit instructions and interpretive criteria were as follows: 2 or more bands in the *p30 – p14* region without both *p31 (Outer surface protein (Osp) A)* and *p34 (Osp B)* indicate a natural positive; 2 or more bands in the *p30 – p14* region plus both *p31* and *p34* were indicative of vaccination; and less than 2 bands in the *p30 – p14* region were considered negative for the presence of *B. burgdorferi*-specific antibodies. Overall seroprevalence was computed as: number of WB-positive samples / (total number of samples assayed by IFA – number of WB-confirmed vaccine-positive samples).

C_6 Enzyme-linked Immunosorbent Assay (ELISA). In addition to the two-step diagnostic procedure outlined above, we subjected selected sera to a newly-available commercial ELISA-based canine Lyme kit (SNAP 3Dx test, Idexx Laboratories, Westbrook, ME) to evaluate its utility in an area of invading *I. scapularis* and to compare it to the more laborious two-step process. The kit employs a synthetic peptide (C_6) with sequence homology to one of six invariable regions within the variable domain of VlsE (Vmp-like sequence, Expressed), a surface lipoprotein of *B. burgdorferi*. Antibodies to VlsE are found in field-infected dogs, but not in vaccinated dogs (Levy et al. 2003). In addition to assaying for the presence of antibodies to *B. burgdorferi*, this test

simultaneously assays for antibodies to *Ehrlichia canis* and antigen of *Dirofilaria immitis* adult worms. Manufacturer's instructions were followed to assay all sera with IFA endpoint titers ≥ 1:2560 (n = 22) plus seven randomly-selected samples with lower endpoint titers.

Tick Processing and Polymerase Chain Reactions (PCR). All ticks removed from dogs were identified to life stage and species using standard taxonomic keys (Sonenshine 1979; Durden and Keirans 1996). Total DNA was extracted from each tick using the animal tissue protocol of the DNeasy Tissue Prep extraction kit (Qiagen, Valencia, CA). Known-infected nymphs from a CDC laboratory colony served as positive controls and no-template wells served as negative controls for the extraction. Ticks were prepared for extraction by slicing through the tick exoskeleton and midgut using a scalpel in a dry microcentrifuge tube. Lysis buffers were added and the solution incubated overnight. In the case that an engorged tick clogged the spin column, a sterile pipette tip was used to dislodge the clog. DNA was eluted in a single 100µl elution. Five µl of DNA was used in each of three separate PCRs of 50µl volumes to assay for *B. burgdorferi*, *A. phagocytophilum*, and *Babesia* species. Gel electrophoresis was carried out using 10 µl of PCR product in a pre-cast 4% agarose gel (E-gel system, Invitrogen, Carlsbad, CA). The *rrs–rrlA* (16S – 23S rRNA) intergenic spacer (IGS) of *B. burgdorferi* was amplified in a nested assay producing a 978 bp fragment (Bunikis et al. 2004). DNA extracted from cultured spirochetes served as a positive control (spirochetes were cultured from field-collected adult ticks from a different study in BSK-H complete media (Sigma-Aldrich, St. Louis, MO) and incubated at 37° C). The *p44* gene of *A. phagocytophilum*

(Zeidner et al. 2000) was amplified using a touchdown PCR program described in Steiner et al. (2006) to produce a334-bp fragment. *Babesia* genus-specific PCR was performed using primers for the 18S rRNA gene to produce a fragment of variable size, including a 408bp fragment for *B. microti* or a 437-bp fragment for *B. odocoilei* (Armstrong et al. 1998). Commercially-available *B. microti* organism (ATCC, Manassas, VA) was extracted as above and used as a positive control. Ticks were removed from dogs at different levels of engorgement, so their extracted DNA varied in concentration. PCR optimization trials using serially-diluted templates suggested that overly-concentrated template (from engorged ticks) inhibited detection of pathogen (data not shown), so we tested all DNA samples at both full-strength and at a 1:10 dilution in the non-nested PCRs. In all PCRs, negative controls consisted of wells in which all reagents except a DNA template were added.

Sequence Analysis. We undertook DNA sequence analysis of PCR amplicons of *B. burgdorferi* (to assess strain-level variation) and *Babesia* spp. (to identify to the species level). The 40 µl of amplicon that remained after electrophoresis was purified (PCR Purification Kit, Qiagen, Valencia, CA) and used as a template for DNA sequencing at the Research Technology Support Facility at Michigan State University on an ABIPrism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). Sequences were compared to those published in the National Center for Biotechnology Information sequence database using the nucleotide-nucleotide basic local alignment search tool (BLASTn) to identify the pathogen strains with the greatest sequence homology.

Spatial Analyses. Generalized linear models were used to assess the relationship between tick/pathogen measures (i.e., seroprevalence, vaccination rate, IFA background reactivity (IFA suspect-positives that tested negative on WB), presence of *I. scapularis* on dogs, and presence of *B. burgdorferi* in ticks removed from dogs) and the geographic zone of each clinic. All statistical analyses were performed using R-project (Team 2005).

Early Invasion Survey. We include data from a pilot serosurvey that we undertook in 2001-2002, which used similar methodology to collect samples from 17 clinics dispersed throughout Zone 1. This survey was initiated in response to the first direct field evidence, from drag and wildlife sampling, of invading *I. scapularis* in southwestern Michigan (Foster 2004). Briefly, veterinarians were asked to store surplus blood samples and place ticks from dogs in one common vial from June of 2001 through December of 2002. Sera were processed as above, except that suspect positives were defined as having an IFA cutoff titer of ≥ 1:320 in 2001 and ≥ 1:640 in 2002 on slides read by a single observer (ESF). A subset of suspect positives was subjected to WB. Serology and tick collection results are presented as a means for comparison with the 2005 survey data.

Results

In 2005, a total of 353 serum samples were provided by the enrolled veterinary clinics (mean of 20 per clinic; range 3 -30) of which 174 (49.3%) were from female dogs and 179 (50.7%) were from male dogs. All American Kennel Club breed groups were represented (Figure 4.2). Twenty-one canids (5.9%) were reported as vaccinated, 113 (32.1%) were reported as not vaccinated, and 219 (62%) were of unknown vaccination

status. None were reported to have previously been diagnosed with Lyme disease nor to have traveled to any other Lyme disease endemic area, although 232 (66%) reported unknown travel history or did not answer this question.

IFA. Of 353 serum samples, 79 (22.4%) had endpoint titers ≥ 1:640 and thus were considered suspect-positive for *B. burgdorferi* exposure (Figure 4.3). The maximum endpoint titer detected was 1:81920 (Table 4.1).

WB. Western blot analysis was performed on 105 samples (79 suspect-positives, 3 reported vaccinated with IFA endpoint titers <1:640, and 23 randomly-selected sera with IFA endpoint titers < 1:640). Of the suspect-positives, 2 (2.5%) were WB positive due to natural exposure; 19 (24.0%) were vaccine-positive (including 3 from canines reported as not vaccinated and 4 from canines of unknown vaccine history), and 58 (73.4%) were negative. The highest IFA endpoint titer in WB-negative samples was 1:5120. Two of the low IFA-titer, reportedly-vaccinated dogs were confirmed as vaccine-positive whereas the other was negative. WB-confirmed vaccine-positive sera had IFA endpoint titers ranging from 1:320 – 1:20480 (median: 1:2560; Figure 4.3). All 23 of the randomly-selected IFA-negative sera tested WB-negative.

The overall natural seroprevalence detected in the study was 2 of 332 (0.6%; denominator discounted by number of vaccine-positives). The two natural-positive samples had IFA endpoint titers of 1:81920 and 1:10240 and were submitted from clinics N and M, respectively (both within the *I. scapularis* invasion zone; Figure 4.1). The overall WB-confirmed vaccine rate was 21 of 353 (5.9%; Table 4.1).

C_6 ELISA. Twenty-nine sera were assayed for *B. burgdorferi* and *E. canis* antibodies and *D. immitis* antigen using the SNAP 3Dx kit. Of the 22 samples with IFA endpoint titers ≥ 1:2560, two tested positive for *B. burgdorferi* antibodies (these being the natural-positives previously identified by WB). Of the 7 randomly-selected lower-titer sera, none tested positive for *B. burgdorferi* antibodies. The Snap test was thus 100% sensitive and 100% specific when compared to our WB. No samples tested positive for the presence of *E. canis* antibodies or *D. immitis* antigen.

Tick PCR. A total of 78 ticks from 55 individual dogs were submitted by the clinics (mean = 4.9 per clinic; range 0-14). Three dogs had reported travel to *I. scapularis*-endemic areas (two to Michigan's UP and one to the northeastern US) and 28 did not have responses to this question. Ticks comprised nymphs and adults of three species: *I. scapularis, I. cookei*, and *D. variabilis*. A total of 18 *I. scapularis* were removed from 13 individual dogs (including one dog from clinic B which was reported to have traveled to an endemic area; all other dogs with *I. scapularis* had no known travel to endemic areas). Of the dogs with *I. scapularis*, 1 was from Zone 1 (clinic E), 9 were from Zone 2 (clinic B), and 3 were from Zone 3 (clinics H and R; Figure 4.4). Fifty-two and eight *D. variabilis* and *I. cookei* were removed from 35 and six dogs, respectively. Of the 78 ticks submitted, 18 ticks removed from 15 dogs tested positive for *B. burgdorferi* including ticks of all three species (Table 4.2). The prevalence of *B. burgdorferi* infection in *I. scapularis* was 33% (6 of 18; nymphs and adults combined). The prevalence of *B. burgdorferi* infection in *D. variabilis* was 17% (9 of 52; nymphs and adults combined). The prevalence of *B. burgdorferi* infection in *I. cookei* was 38% (3 of 8;

nymphs and adults combined). Multiple infected ticks were removed from the same individual dogs (three infected *D. variabilis* from one dog at clinic O, and two infected *I. scapularis* from one dog at clinic N; both clinics are in Zone 1). *B. burgdorferi*-positive ticks were identified at 9 of the 18 clinics (Figure 4.4). Results of positive and negative extraction and PCR controls were as expected.

Three of 78 ticks (3.9%) were infected with *Babesia* spp; these comprised one adult *D. variabilis* from clinic L and two nymphal *I. cookei* from clinics I and N (Figure 4.4). One *Babesia*-positive nymphal *I. cookei* from clinic N was co-infected with *B. burgdorferi*. No ticks tested positive for *A. phagocytophilum*.

Sequence Analyses. All PCR amplicons for *B. burgdorferi* and *Babesia* spp.-positive ticks were purified and sequenced. Sequences were obtained for 13 *B. burgdorferi* amplicons with an average trimmed sequence length of 790 bases. These sequences were found to have ≥ 98% sequence identity with previously reported *B. burgdorferi* 16S – 23S rDNA gene sequences of the following strains: B515 (n = 4; accession number AF467860.1); CS2 (n = 2; accession number DQ437492.1); B356 (n = 2; accession number AF467861.1); Lenz (n = 1; accession number EF537391.1); RSP2 (n = 1; accession number EF649782.1); MI415 (n = 1; accession number EF537369.1); HB1 (n = 1; accession number EF537294.1); and IP3 (n = 2; accession number EF537392.1). Sequences were obtained for 2 *Babesia* amplicons (both from *I. cookei* nymphs) with an average trimmed sequence length of 316 bases. The sequence from the nymph from clinic N had 93% sequence identity with *Babesia odocoilei* Wisconsin 1 (accession number AY237638.1), whereas the sequence from the nymph from clinic I had 91%

sequence identity with *Babesia* sp. BiCM002 (accession number AB053216.2). These *Babesia* matches provide an indication of species similarity but are insufficient for species identification.

Spatial Analyses. Canine vaccination rates were significantly higher in Zone 1 (*I. scapularis* established) than in the other two zones (average rates for Zones 1, 2, 3 were 14.0, 1.7, 1.9%, respectively; $P = 0.008$). Seroprevalence, background IFA activity, *I. scapularis* presence and *B. burgdorferi* presence were all unrelated to clinic zone ($P = 0.169, 0.728, 0.738, 0.616$; respectively). The only clinics where multiple *B. burgdorferi* positive ticks were found were those closest to the 'endemic' southwestern corner of the state (i.e., clinics O and N) and just north of the invasion zone (clinic B). The only co-infected tick (carrying both *B. burgdorferi* and *Ba. odocoilei*) was from the southwestern-most clinic, N.

Early Invasion Survey. In 2001-02 a total of 2030 sera from 17 Zone 1 clinics were screened by IFA, with 235 samples identified as suspect-positive. WB was performed on 20 suspects (selected randomly), resulting in 2, 12, and 6 samples classified as positive due to natural exposure, vaccine positive, and negative; respectively. Extrapolating from this albeit very small sample size of western blotted samples, these data suggest overall seropositivity, vaccination, and background IFA activity rates of 1.2, 6.9, and 30%, respectively. A total of 345 ticks were removed from dogs of which 26 (7.5%) *were I. scapularis*; this species was recovered from 8 clinics and only adults were found. Ticks of other species included: *D. variabilis* (n = 298; 86.4%); *Rhipicephalus sanguineus* (n =

7; 2.0%); *I. cookei* (n = 11; 3.2%) and *Amblyomma americanum* (n = 3; 0.9%). No pathogen testing of these ticks was undertaken.

Discussion

Numerous researchers have proposed that companion animal dogs are a useful sentinel animal that can assist in assessing risk in areas endemic for Lyme disease (Falco et al. 1993; Merino et al. 2000; Guerra et al. 2001; Bhide et al. 2004). Most such serosurveys, however, have been conducted in areas already endemic for *I. scapularis*. Here we consider the efficacy of such surveys in areas where there is concern about low-prevalence emerging disease.

Despite evidence from tick and other wildlife surveys that *B. burgdorferi* is emerging in lower Michigan (Foster 2004), *B. burgdorferi* seroprevalence was extremely low (2 in 334; 0.6%) in canine patients from lower Michigan. The two naturally-exposed dogs detected in this study were from clinics within the *I. scapularis* invasion zone, where local exposure can be expected. Canine seroprevalence in this zone during the early invasion study in 2001-2002 was 1.2%, and neither the 2005 nor the 2001-2002 seropositivity rates reported herein are substantially greater than those found during the 1992-1993 Lower Peninsula serosurvey (0.1%; Walker et al. 1998). This leads us to conclude that canine serosurvey is an insensitive approach to detection of the invasion of *I. scapularis – B. burgdorferi* that has occurred over the timeframe bounded by these studies (as detected by the wildlife sampling and vegetation dragging that began during our 2001-02 pilot study).

In previous studies, canine seroprevalence has typically been reported to be 30-50% in *B. burgdorferi*-endemic areas, and 3-5% in areas such as Alabama where Lyme disease is considered largely absent (see (Bhide et al. 2004) for a review). Our low seroprevalence estimate is explained, in part, by many of the clinics operating in the outer zones where *I. scapularis* is apparently not yet established (Figure 4.1). Second, seroconversion may not yet have occurred for all exposed dogs. In laboratory studies, dogs exposed to infected adult ticks develop detectable antibodies 4-6 weeks after exposure; titers increase for an additional 6-8 weeks and remain high for at least one year. In contrast, 50% of dogs exposed to infected nymphs fail to seroconvert or convert only after repeated exposure (Appel et al. 1993); therefore, some dogs infected in the summer of 2005 may not have seroconverted by the July-August blood-sampling. Third, flea and tick chemoprophylaxis, which is increasingly being recommended by veterinarians in southwestern Michigan (see below), may be reducing levels of infection and subsequent seroconversion.

In our data, background IFA activity was high, with 73.4% of IFA suspect-positives (positive at 1:640 or higher dilution) not confirmed as natural or vaccine-positive by either WB or C_6 ELISA. The geographical distribution of these IFA high-titer sera was unrelated to the location of the Lyme disease hotspot, so these IFA 'false positives' may represent cross-reactivity with heterologous antibodies or subjectivity of IFA slide interpretation. This high background reactivity even on negative samples emphasizes the importance of using confirmatory testing such as the WB or rapid ELISA kits as we have done in the current study. Given their widespread use of the *Borrelia* IFA alone, considerable caution should be exercised in interpreting previous canine

serosurveys. We found the C_6 rapid ELISA kit to be 100% sensitive and 100% specific when compared to WB, confirming the value of this test for canine disease sentinel studies (see also (Levy et al. 2003; Duncan et al. 2004; Stone et al. 2005).

Canine Tick Survey as an Alternative to Canine Serosurvey. The majority of the 13 dogs that harbored *I. scapularis* during our study period were from clinics within the zone of established *I. scapularis* (Zone 1; 1 dog) and just north of this zone (Zone 2; 9 dogs), demonstrating that the dog, in harboring *I. scapularis*, serves as a useful sentinel for presence of this tick in areas undergoing invasion. Contrary to other studies (Hinrichsen et al. 2001), however, the geographic distribution of *I. scapularis* was not predictive of canine seropositivity. Six of 18 *I. scapularis* – from six different dogs – plus 12 of 60 ticks of other species – from nine different dogs- were found to be infected with *B. burgdorferi*. Thus it was much easier to find *B. burgdorferi* in ticks off the dogs than to find antibodies to *B. burgdorferi* in the dog's sera.

Of the three species of ticks that clinics submitted from canine patients, only *I. scapularis* is regarded as a competent vector for *B. burgdorferi* and *Babesia* spp. Yet in addition to the *I. scapularis* found infected with *B. burgdorferi*, we found 12 *B. burgdorferi* PCR-positive and three *Babesia* sp. PCR-positive non-vector ticks, including one co-infected *I. cookei* removed from a dog in the southwestern-most site in the study. We posit that a bloodfed non-vector tick could become infected via three means (not mutually exclusive). First, the non-vector tick may have been sampled during or soon after feeding on a spirochetemic dog, so that PCR detects the spirochetemic bloodmeal within the engorged tick. In our study, 39 individual dogs were parasitized by *D.*

variabilis or *I. cookei*, and *B. burgdorferi*-infected ticks of these species (n = 12) were removed from nine individual dogs. That two individual dogs produced more than one infected tick lends support to the spirochetemic dog scenario; however, no dogs in our study were reported to have been diagnosed with Lyme disease (74.5% veterinarian response rate) and canine seroprevalence was negligible. Second, we posit that transstadial passage of pathogen acquired in a previous infectious bloodmeal (most likely from a non-canine host) may result in an infected non-engorged tick, though the infected non-vector is unable to transmit the pathogen. Walker et al.(Walker et al. 1994) cultured *B. burgdorferi* from five questing *D. variabilis*, and found a 1.3% infection rate in this species. Similarly, during our ongoing study of *Borrelia* infection in questing adult ticks in the Midwestern states we found 6 of 59 (10.2%) *D. variabilis* to be PCR-positive *for B. burgdorferi* (S. Hamer, unpublished data). Studies in the Lyme disease-endemic area of Michigan's Upper Peninsula found six of 75 (8.0%) questing adult *D. variabilis* to be infected, albeit with a spirochete load much lower than that of infected *I. scapularis* collected simultaneously (P. Roy, pers. comm.). These reports of low-moderate prevalence, with very low spirochete loads, are consistent with transmission studies showing *D. variabilis* to be an incompetent vector for *B. burgdorferi* (Piesman and Sinsky 1988). Third, we note that non-vector ticks potentially could acquire spirochetes by co-feeding with vector ticks, even in the absence of systemic infection of the canine host. This mechanism, -- proposed for the maintenance of tick-borne encephalitis virus in Europe(Randolph et al. 1996) -- has not been tested for non-vector ticks and *B. burgdorferi*, and has only been demonstrated for *I. scapularis* under laboratory conditions with artificially high tick burdens(Piesman and Happ 2001). Nevertheless, while the

mechanism remains unclear, our findings do suggest that assays of *both* the vector *and* non-vector ticks found attached to canine patients can contribute to detection of pathogen presence in a given area - irrespective of the tick species' inability to transmit the pathogen of interest.

Relative to the low apparent exposure of canids to *B. burgdorferi* based on our serological findings, ticks testing positive for this pathogen were far more common than expected. We hypothesize *a posteriori* that our tick and serum samples came from different subpopulations of pet dogs: i.e., that serum samples came from a range of dogs that included those prophylactically vaccinated and/or chemically protected against tick bites; whereas tick samples mainly came from dogs not chemically protected from tick exposure. In 2008, we conducted a short phone survey of veterinarians and licensed veterinary technicians at nine of the participating veterinary clinics (3, 2, and 4 in Zones 1, 2, and 3; respectively) to assess whether anti-tick and anti-tick-borne pathogen measures are recommended at the clinics. In responding, the clinicians were asked to recall as best they could the situation in summer of 2005, when our sampling was underway. Clinicians estimated that 20 – 65% (average 50%) of the pet dogs attending their clinic were being actively protected against ticks during those summer months - with topical agents being the most common form of prophylaxis - and that 5-25% (average 12%) of dogs were being vaccinated against Lyme disease. These data suggest that acquisition of serum from pet dogs randomly selected at veterinary clinics (which is a common method of sampling in canine serosurveys; see current study, (Rand et al. 1996; Guerra et al. 2001; Hinrichsen et al. 2001)) will under sample the unprotected dogs that are likely the most effective sentinels for pathogen transmission in an area.

Since this serosurvey, ongoing surveillance (through wildlife trapping, drag sampling and human submissions) has documented continuing range expansion of *I. scapularis* in southwestern Michigan (Hamer unpublished). We anticipate that *I. scapularis* and associated pathogens will spread north and east from the present southwestern Michigan hotspot in coming years, so that both veterinary and human clinicians will see an increase in Lyme cases among their patients. Dogs are unlikely to be a sensitive sero-sentinel for changes in tick and pathogen activity in these areas of low tick density. We conclude that *I. scapularis* emergence, and consequent increase in canine and human disease risk, can be predicted more effectively by surveys of *I. scapularis* and other non-vector ticks removed from dogs than by serosurvey of the canines..

Figure 4.1. Locations of the 18 veterinary clinics (labeled A - R) that participated in the 2005 canine tick and serosurvey of Lower Michigan. Shading indicates counties in which *I. scapularis* has recently invaded (Lower Peninsula) or is endemic (Upper Peninsula); shaded counties are those within which all three life stages of *I. scapularis* have been documented (Foster 2004). Circles indicate the estimated Lyme disease vaccination rates (expressed as a proportion) for the dogs at each clinic. The locations of the two dogs seropositive for antibodies to *B. burgdorferi* from natural exposure are shown.

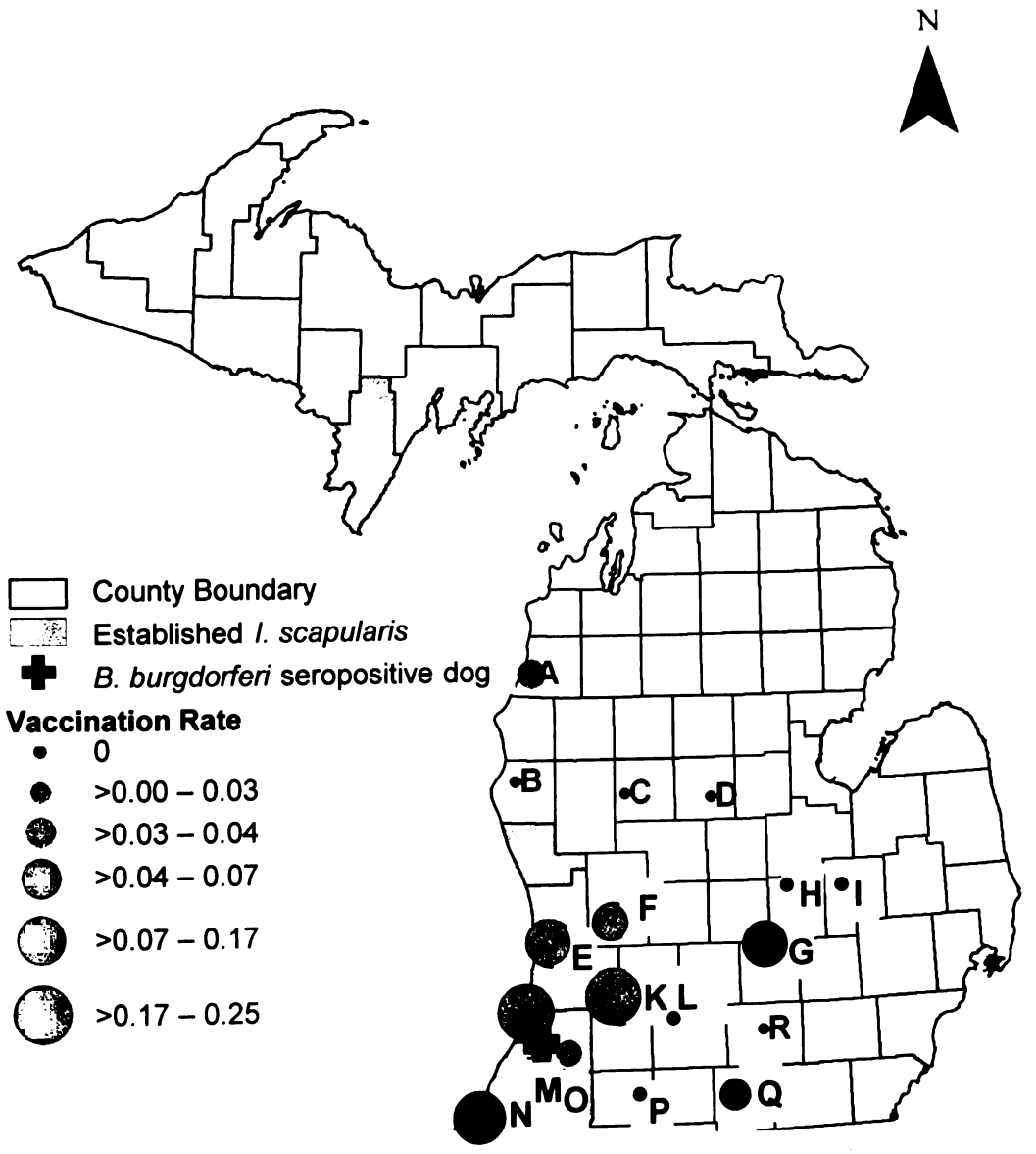

Figure 4.2. Proportions of the serum samples collected (n = 353) from individuals of the various American Kennel Club breed groups. Dogs reported as a specified breed mix are classified under that breed group (i.e. German Shepard mix = herding group) whereas reports of mixed breed with no breed specification are listed as 'Mix'.

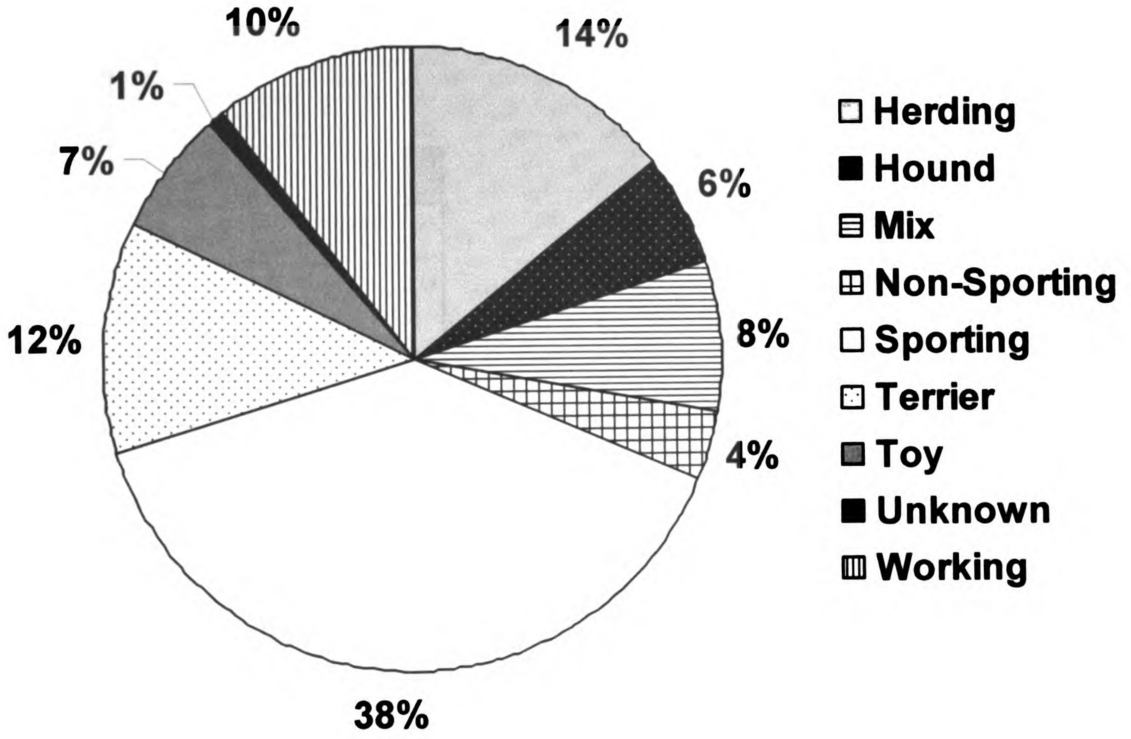

Figure 4.3. Frequency distribution of IFA endpoint titers for *B. burgdorferi* antibody detection in canine sera (n = 353). Sera with titers ≤ 1:320 were classified as negative for infection with *B. burgdorferi*. The remaining high-titer sera, plus sera from dogs reported as having been vaccinated, were classified as natural positive, vaccine positive, or negative based on western blot.

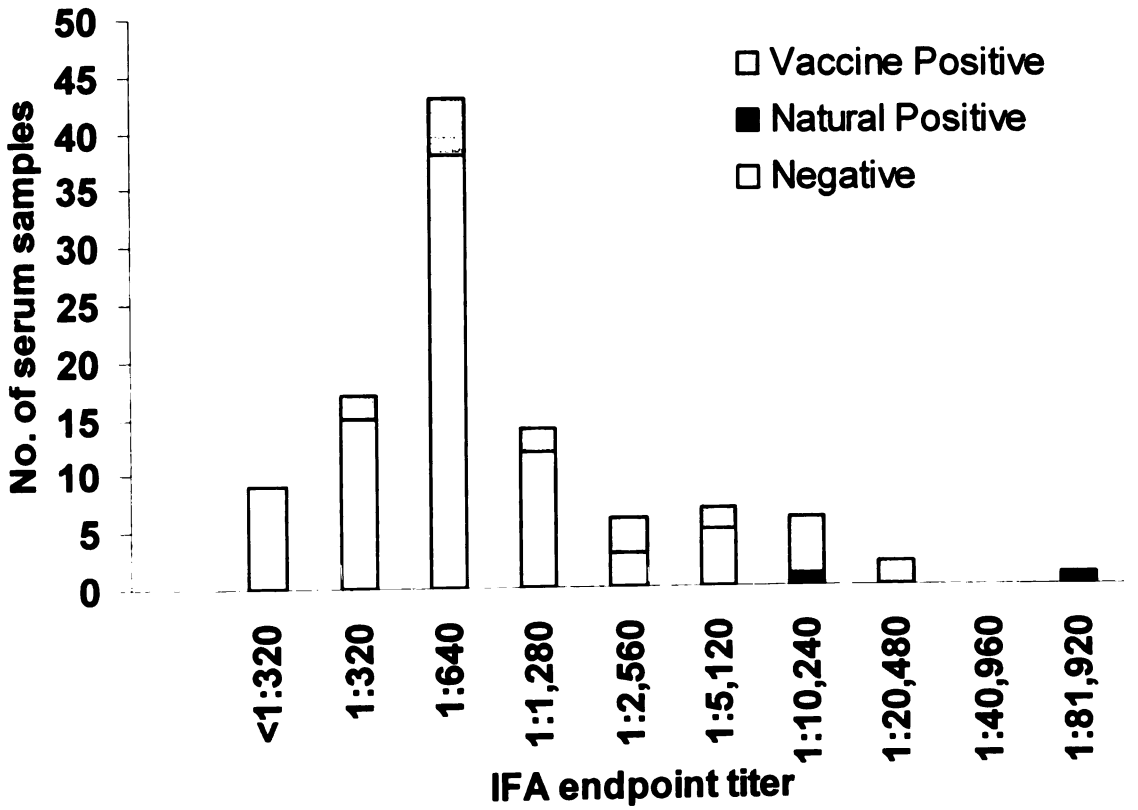

Figure 4.4. Distribution of clinics with canines harboring *I. scapularis*, *B. burgdorferi*-infected ticks of any species, and *Babesia*-infected ticks of any species. The shading indicates counties in which *I. scapularis* has recently invaded and is now established with documented presence of all three life stages (Foster 2004).

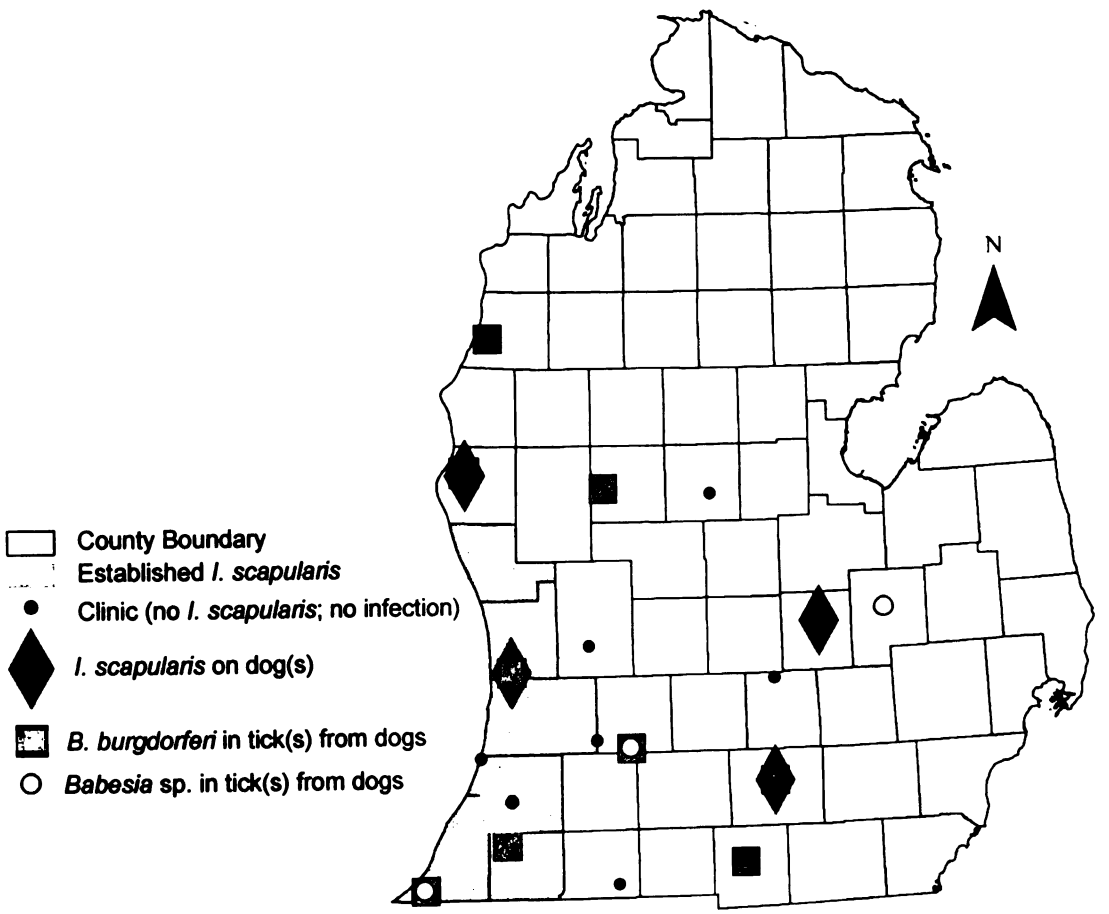

Table 4.1. Results of IFA and WB serological assays of canine serum samples from 18 clinics in lower Michigan. IFA endpoint titers are expressed as reciprocals. IFA suspect positives are those samples with endpoint titers of 1:640 or more dilute. All IFA suspect-positives, plus randomly-selected IFA negatives (number indicated above), were subject to confirmatory WB. Overall seroprevalence computed using denominator of (total samples – number of vaccine positives). In no case did an IFA negative sample test positive by WB. Zone assignment relates to proximity of clinic to established populations of *I. scapularis*. Neg = negative test result; Pos = positive test result; Vax = vaccine positive test result.

Clinic	Zone	No. samples	IFA Results		No. IFA negs tested by WB	WB results					Number (%)	
			Endpt. titer max	Suspect positive		IFA neg, WB neg	IFA neg, WB vax	IFA pos, WB neg	IFA pos, WB vax	IFA pos, WB pos	Vax	Pos
A	3	25	1280	4	1	1	0	3	1	0	1 (4.0)	0
B	2	27	5120	4	0	0	0	4	0	0	0	0
C	3	15	1280	3	2	2	0	3	0	0	0	0
D	3	15	640	2	1	1	0	2	0	0	0	0
E	1	24	20480	13	0	0	0	10	3	0	3 (12.5)	0
F	2	30	2560	6	1	1	0	4	2	0	2 (6.7)	0
G	3	14	640	3	1	1	0	2	1	0	1 (7.1)	0
H	3	18	640	1	1	1	0	1	0	0	0	0
I	3	18	2560	1	1	1	0	1	0	0	0	0
J	1	25	10240	10	5	3	2	6	4	0	6 (24.0)	0
K	1	4	5120	1	1	1	0	0	1	0	1 (25.0)	0
L	2	19	1280	5	1	1	0	5	0	0	0	0
M	1	3	10240	1	0	0	0	0	0	1	0	1 (33.3)
N	1	30	81920	9	3	3	0	3	5	1	5 (16.7)	1 (3.3)
O	1	30	1280	5	4	4	0	4	1	0	1 (3.0)	0
P	2	28	640	2	1	1	0	2	0	0	0	0
Q	3	24	10240	8	2	2	0	7	1	0	1 (4.2)	0
R	3	4	640	1	1	1	0	1	0	0	0	0
	Total	**349**	**81920**	**78**	**25**	**23**	**2**	**57**	**19**	**2**	**21 (5.9)**	**2 (0.6)**

Table 4.2. Tick species and life stage-specific results of PCR testing for *B. burgdorferi*, *A. phagocytophilum*, and *Babesia* species in ticks removed from canine patients at clinics throughout lower Michigan, 2005.

			Number PCR positive (%)		
Tick Species	Life Stage	Number Tested	*B. burgdorferi*	*A. phagocytophilum*	*Babesia* spp.*
D. variabilis	Adult	51	9 (17.6)	0	1 (2.0)
	Nymph	1	0	0	0
I. cookei	Adult	2	1 (50.0)	0	0
	Nymph	6	2 (33.3)	0	2 (33.3)
I. scapularis	Adult	16	5 (31.3)	0	0
	Nymph	2	1 (50)	0	0

* One *I. cookei* nymph was infected with *Ba. odocoilei*; identities of the remaining two sequences remain general to *Babesia* genus.

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CHAPTER 5

Cryptic transmission of *Borrelia burgdorferi*, *B. andersonii*, and *B. miyamotoi* by bird-derived ticks in the absence of the blacklegged tick

Abstract

Like other areas in the eastern United States, blacklegged ticks, *Ixodes scapularis*, and the Lyme disease pathogen, *Borrelia burgdorferi*, are invading lower Michigan. We asked the question whether *B. burgdorferi* may be maintained in cryptic enzootic cycles prior to the arrival of *I. scapularis*. In particular, we hypothesized that *B. burgdorferi* may be maintained within this landscape through *Ixodes dentatus*-mediated cryptic cycles. During the summer breeding and fall migratory seasons of 2004 –2008, we tested this hypothesis by mist-netting 19,631 wild birds of 105 species at a focal site in Lower Michigan, 90 km east of the zone of blacklegged tick invasion. Eleven percent of birds, comprising 56 species, were infested with ticks, and a total of 12,301 ticks were removed (86% were *I. dentatus*). No resident birds, rabbits or other small mammals sampled at our site harbored *I. scapularis*, whereas 72.8% of small mammals sampled in the tick invasion zone over the same period were infested. In bird-derived ticks, we identified low-prevalence infection of three *Borrelia* species, including a minimum prevalence of 3.5% with *B. burgdorferi*, 1.6% with *B. andersonii*, and 0.7% with *B. miyamotoi* (this constitutes the first report of *B. miyamotoi* in ticks from wild birds). At least 35% of rabbits had ear tissue or ticks infected with either *B. burgdorferi* or *B. andersonii*.

We found a total of 25 *B. burgdorferi* rDNA intergenic spacer strains at this cryptic site, the majority of which have not been previously reported from the Lyme

disease endemic northeastern or Midwestern US. Also present within cryptic transmission cycles were at least three strains of *B. burgdorferi* previously associated with disseminated Lyme disease in humans. This study represents the first time that significant levels of *B. burgdorferi* have been found concurrently in birds, rabbits, and their specialist ticks in the apparent absence of *I. scapularis*. Such cryptic cycles may reduce the time lag between *I. scapularis* invasion and the build-up of infection prevalence. Mobile infected birds and cryptic cycles together have the potential to introduce novel *B. burgdorferi* strains and accelerate the increase in risk to human and canines within *I. scapularis* invasion zones.

Introduction

The predominant maintenance cycle of the Lyme disease pathogen *Borrelia burgdorferi* in the eastern United States involves the blacklegged tick *Ixodes scapularis* - the main enzootic and zoonotic vector - and the white-footed mouse *Peromyscus leucopus* - the main reservoir host for the pathogen and preferred host for juvenile ticks (James and Oliver 1990). The generalist feeding capacity of *I. scapulairs* allows this vector to bridge the pathogen from wildlife reservoirs to humans and dogs- both which may become diseased upon infection. Lyme disease is the most frequent vector-borne disease in the northern hemisphere, with over 20,000 cases reported annually in the United States, and incidence is increasing annually (Bacon et al. 2007).

In 2002, a new population of *I. scapularis* was detected in the southwestern corner of Michigan's Lower Peninsula (Foster 2004), where these ticks were historically absent (Walker et al. 1998). A subsequent invasion process ensued as ticks colonized to

the north through coastal dune forests along the Lake Michigan lakeshore (Hamer et al. 2007; Hamer et al. 2010). In tracking this invasion, we documented the presence of *B. burgdorferi* not only in recently-invaded *I. scapularis* and their small rodent hosts, but also in alternative tick and wildlife species prior to the arrival of *I. scapularis*. Cryptic pathogen maintenance – that is, enzootic maintenance of *B. burgdorferi* in ticks that specialize on wildlife and do not commonly bite humans - was considered as one explanation for the incongruent distributions of *I. scapularis* and *B. burgdorferi*.

Cryptic transmission cycles of *B. burgdorferi* have been described previously, with the zoonotic potential of such cycles considered low because the vector species are host specialists that do not regularly bite humans or dogs. For example, *I. neotome/I. spinipalpis* (now synonymized) is a woodrat-specialist tick that rarely bites humans and yet is more important that *I. pacificus* (the human-biting equivalent of *I. scapularis* in the western United States) in maintaining *B. burgdorferi* (Brown and Lane 1992; Maupin et al. 1994). Similarly, in the southeastern United States, *I. affinis* and *I. major* – both rodent-feeding enzootic vectors – are locally more important that *I. scapularis* in maintaining *B. burgdorferi* (Oliver et al. 2003). *I. dentatus* feeds almost exclusively on birds and eastern cottontail rabbits and has been shown to maintain transmission of *B. burgdorferi* between birds and eastern cottontails (*Sylvilagus floridanus*) in Nantucket, MA, in a zone of sympatric *I. scapularis* and endemic Lyme disease (Telford and Spielman 1989b), where the authors concluded that infection may occasionally be exchanged between the *I. dentatus*- and *I. scapularis* cycles. In laboratory studies, *I. dentatus* has been confirmed as a competent vector of *B. burgdorferi*, although less so than *I. scapularis* (Telford and Spielman 1989a). A majority of North American bird

species (Brinkerhoff et al. 2010) as well as eastern cottontails (Telford and Spielman 1989b) are competent *Borrelia* reservoirs, although competence is generally less than that of the white-footed mouse. Additional evidence of *I. dentatus*-associated *B. burgdorferi* was found in Connecticut where sympatric *I. scapularis* also occur, when a spirochete indistinguishable from *B. burgdorferi* strain B31 was isolated (Anderson et al. 1990). Antigenic variants of *B. burgdorferi* have also been found in *I. dentatus* removed from rabbits (Anderson 1989). These variants were later characterized as being sufficiently distant genetically as to represent a new genospecies, *B. andersonii* (Marconi et al. 1995). Consequently, *I. dentatus* is associated with both *B. burgdorferi* and *B. andersonii* in some areas where it is sympatric with *I. scapularis*. The level of interaction between the *I. scapularis*- and *I. dentatus*-driven cycles and their respective spirochetes remains unclear, and such cycles have never before been investigated in an area of *I. scapularis* absence or an area undergoing invasion by *I. scapularis*.

The diversity of *B. burgdorferi* can be assessed at a number of loci, of which the 16S-23S rRNA intergenic spacer (IGS) is an ecologically, epidemiologically, and pathologically-informative target (Bunikis et al. 2004a). At least 24 IGS strains have been described, which can more broadly be delimited into three ribosomal spacer types (RST 1, 2, and 3; (Liveris et al. 1995). RST 1 strains are associated with a higher frequency of disseminated infection in humans and more invasive disease in experimental animals (Seinost et al. 1999; Wormser et al. 2008). Gatewood et al. (2009) found that infection in *I. scapularis* nymphs due to RST 1 strains was greater in the Northeastern as compared to Midwestern United States, where RST 2 and 3 strains were relatively more dominant. This pattern was associated with the degree of synchrony of feeding of the

immature stages of *I. scapularis*, in which there is a longer duration between nymphal and larval activity in Northeast, which may favor longer-persisting RST 1 strains. Similarly, within recently-invaded ticks in lower Michigan, Hamer et al. (2010) found that only 13% of strains were within the RST 1 group. In addition to geographic structure to *B. burgdorferi* genotypes, the influence of host species of pathogen genotype is also the subject of study. Whereas Brisson and Dykhuizen (2004) found host associations of different *B. burgdorferi* strains, Hanincova et al. (2006) found that most of the known *B. burgforgeri* genotypes can infect a range of hosts, and that cross-species transmission among various mammalian hosts is common.

We hypothesized that *B. burgdorferi* can be maintained by *Ixodes dentatus* and bird and eastern cottontail hosts in the absence of *I. scapularis*. Further, in consideration of the previous evidence for host association of strains, and the host specificity of *I. dentatus* on birds and rabbits (i.e., hosts not hosts used heavily in *I. scapularis*-driven scenarios of *B. burgdorferi* maintenance), we hypothesized that the pathogen strain diversity in bird-associated ticks differs from that of *I. scapularis*-mediated transmission at endemic sites. We addressed these hypotheses by i) characterizing the tick parasites of birds, their seasonal phenology, and their infection status with *Borrelia* spirochetes at a focal site in lower Michigan; ii) searching for *I. scapularis* on mice and chipmunks at the site; and iii) characterizing strain diversity of the *Borrelia* spp. spirochetes we detected.

Materials and methods

Bird mist netting. The Pitsfield Banding Station in Vicksburg, MI (Figure 5.1) is a late-succession forest on old agricultural fields and gravel pits that were farmed and mined

through the 1970s. Bird banding has occurred at Pitsfield since 1985. The banding area encompasses approximately 10 hectares and is used for collection of data on avian productivity and survival. In 2004-2007 we collaborated with bird banders to mist-net birds and collect tick samples for an average of 3 days per week during the bird breeding season of May-August and for an average of 5 days/week during the fall migratory season of September-November. We aimed to investigate birds in both the breeding and fall migratory seasons so as to assess both local and bird-imported ticks and *Borrelia* strains. Between 22 and 36, 12 m mist nets (Avinet, Dryden, NY) were used to capture birds. Nets were run from sunrise for approximately 6 hours on fair weather days and checked every hour. Birds were weighed, identified to species, sex, and age class (hatch year or after hatch year), and leg-banded with federally-issued bands before release. Recaptures of previously-banded birds were noted. All birds were checked for ticks using a magnifying head loupe and straws to blow feathers from ears. Searching was restricted to the neck, head, ears, and face. Ticks were plucked and preserved in 70% ethanol. Due to time constraints and bird safety, for a small number of birds, ticks were observed but not removed and these birds are considered only in the overall infestation estimates with ticks categorized as unidentified. For a different small number of birds, primarily those that were very heavily infested, not all ticks were removed- in these cases; we assume the removed ticks are representative of the tick species/stages that were present. Michigan State University Institutional Animal Use and Care Committee approved all research.

Mammal trapping. To increase our search intensity for *I. scapularis*, we sampled the small mammal community with a particular focus on white-footed mice and eastern

chipmunks, as these are the most sensitive indicators of low-density blacklegged ticks in lower Michigan (Hamer et al. 2010). In addition to trapping small mammals at Pitsfield, we also trapped at Van Buren State Park, 90 km due west of Pitsfield, where *I. scapularis* is known to have invaded within the past decade. This latter trapping was done to control for *I. scapularis* phenology; both areas were sampled in May and June, when *I. scapularis* larvae and nymphs are simultaneously active. Additionally, at Pitsfield we targeted the capture of eastern cottontails, the preferred host for adult *I. dentatus*. In 2004-2009, small mammals were trapped for an average of three nights per summer using an average of 100 Sherman live traps (H. B. Sherman Traps, Tallahassee, FL) spaced 10 m apart and baited with sunflower seed. Rabbits were trapped with wooden box traps baited with apples; these traps on occasion also captured other medium-sized mammals including raccoon (*Procyon lotor*), Virginia opossum (*Didelphis virginiaina*), and woodchuck (*Marmota monax*). Small mammals were anesthetized using Isoflurane (IsoFlo, Abbot Laboratories, Abbott Park, IL) and medium mammals were anesthetized using ketamine hydrochloride (Ketaset; Fort Dodge, Overland Park, KS) and xylazine hydrochloride (Rompun; Bayer Health Care, Kansas City, KS), with yohimbine hydrochloride (Antagonil; Wildlife Laboratories, Fort Collins, CO) used to antagonize the xylazine. Vital data were obtained by inspection. Each animal then was examined for ticks, biopsied from both ears using a 2-mm (small mammals) or 4-mm (medium mammals) biopsy punch (Miltex Insturments, York, PA), and finally marked with a uniquely numbered ear tag (National Band and Tag, Newport, KY). Ticks and ear biopsies were stored separately in 70% ethanol. Animals recaptured within the same site

visit (i.e. recaptured the day after initial processing) were simply checked again for ticks. All animals were released at the site of capture.

Tick and *Borrelia* spp. detection. Ticks were identified to species and stage (Keirans and Clifford 1978; Sonenshine 1979; Durden and Keirans 1996). Representative tick specimens are vouchered in the National Tick Collection at Georgia Southern University. Species identity for a subset of ticks was confirmed molecularly by amplifying and sequencing the tick 5.8S – 28S rDNA internally transcribed spacer (ITS-2) (Poucher et al. (1999). Total DNA from all ticks and ear biopsies was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) following the manufacturer's animal tissue protocol, but with the following modifications. Ticks were first bisected using a sterile scalpel or were pulverized in liquid nitrogen, followed by an overnight incubation in lysis buffer. DNA was eluted using 50 µl elution solution warmed to 70° C. Ear biopsies (one per animal), and adult and nymphal ticks, were extracted individually; same-species larvae from the same individual animal were pooled for extraction. *B. burgdorferi* strain B31-infected nymphal *I. scapularis* generously provided by the Centers for Disease Control and Prevention (CDC) served as the positive extraction control, and water as a negative extraction control.

All tick and ear biopsy DNA extracts were tested for the presence of *Borrelia* species using a nested polymerase chain reaction (PCR) for the 16S – 23S rRNA intergenic spacer region (IGS) of *Borrelia* spp. (Bunikis et al. 2004a) followed by visualization with gel electrophoresis. The outer forward primer is located at the 3' end of the 16S gene and the outer reverse primer is located in the coding sequence for *ileT* tRNA

in the spacer. Included in the amplicon is the *alaT* gene for tRNA and approximately 805 nucleotides of a total of 3052 nucleotides of the spacer, by *B. burgdorferi* B31 coordinates. PCR enzyme kits were used throughout (PCR Supermix, Invitrogen, Carlsbad, CA and FailSafe PCR System, Epicentre, Madison, WI). DNA from *B. burgdorferi* strain B31-infected ticks from the CDC served as a positive PCR control, and water served as a negative PCR control. Preliminary experiments showed that this nested assay remains sensitive in the range of 10^0 and 10^{-1} organisms. A subset of IGS-positive DNA samples was subjected to amplification of the 16S rDNA gene using both a real-time, quantitiative PCR (qPCR) with a probe specific to *B. burgdorferi* (Tsao et al. 2004) or an assay using primers RS11 and S5 as published in Rudenko et al. (2009).

Nucleotide sequencing. Species identification and strain typing of *Borrelia*-positive tick and ear tissue samples was attained through DNA sequencing. All IGS products were purified (Qiagen PCR Purification Kit; Qiagen, Valencia, CA) and sequences were determined using the inner primers on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). Sequences were identified as either *B. burgdorferi, B. andersonii,* or *B. miyamotoi* based on comparisons to published sequences using the basic local alignment search tool in GenBank (Altschul et al. 1990). For *B. burgdorferi* sequences, a 500 nucleotide segment of the IGS was aligned with the prototypical strains published in Bunikis et al. (2004a) using the ClustalW algorithims within the program Mega4 (Tamura et al. 2007). Analysis of this fragment size allowed for identification of the 10 main IGS groups (groups 1-10; the minimal matrix for differentiation of these 10 groups includes mutations which all occur within the first 309 nucleotides of the

fragment), and of 20 IGS subtypes (1A, 1B, 2A/C, 2B, 2D, 3A, 3B/C, 3D, 4A, 4B, 5, 6A, 6B, 6C, 7A/B, 8A/C, 8B, 8D, 9, 10), as presented in Bunikis et al. (2004a). Alignments were manually checked. Additionally, sequences were identified to broad ribosomal spacer type (RST 1, 2, or 3; (Liveris et al. 1995) based on clustering topology of the IGS phylogenetic trees. In the case that a sequence we derived did not identically match any published strain or any strain we previously found in *I. scapularis* across the Midwest, we classified it as novel IGS mutant. Sequence chromatographs were manually scrutinized for confidence in nucleotide assignments and evidence of mixed strain infections which were noted and removed from further analysis. For at least one representative of each detected strain, we also determined the sequence in the reverse direction and/or determined the sequence in the forward direction twice to validate the occurrence of unique mutations. A similar protocol was followed for *B. miyamotoi* sequences, for which prototypical IGS strains for use in alignments were obtained from Bunikis et al. (2004b). For *B. andersonii*, the IGS region was not previously published to Genbank and therefore not available for comparisons to our strains. We therefore sequenced the 16S gene of a subset of suspect-*B. andersonii* samples in both directions to allow for species identification using the Rudenko et al. (2009) primers above, as this gene target has previously been described for *B. andersonii* We found 100% 16S sequence homology to four published sequences, including those found in the kidney of an eastern cottontail in New York (Marconi et al. 1995), in *I. dentatus* larvae and nymphs from rabbits in Missouri, and in a drag-sampled nymph in Georgia (Lin et al. 2003), thereby facilitating future identification of *B. andersonii* based on our IGS sequences. A representative 16S sequence from our Michigan *B. andersonii* has been deposited in Genbank, accession no.

GU993310. Double-stranded sequencing of the ITS-2 region of the tick rDNA (to validate tick species identity) was performed using the primers used in the PCR assays. Resulting sequences were compared to published sequences in Genbank.

To evaluate phylogenetic relationships among *B. burgdorferi* haplotypes, we constructed unrooted neighbor-joining phylogenies and unrooted minimum spanning trees (MST) using Mega4 and TCS 1.21, (Clement et al. 2000; Tamura et al. 2007) respectively. Evolutionary distances were computed using the Kimura 2-parameter method and are in the units of number of base substitutions per site. Percentage support values for clades within the neighbor-joining tree were obtained from 1000 bootstrap iterations. The MST method determines the gene network in which the total length of the branches that connect haplotypes is minimized, and discrimination among equal-length MSTs required the assumption that older alleles are more common than recently derived alleles, and new mutations are more likely found in the same population as their ancestor.

Statistics. To determine mist netting efficiency, one net hour (NH) is defined as the equivalent of one 12m net run for one hour. Chi-squared tests for independence were used to assess coinfestations. Logistic regression was used to assess trends in tick infestation and tick/host infection over the 4-year sampling period. Comparisons between birds captured in the migratory versus breeding seasons were made by calculating the z-ratio and associated probabilities for the difference between two independent proportions. A minimum infection prevalence (MIP; i.e., assuming only one positive larva per pool) was used for tests done on pooled larvae. Statistics were performed using Statistix 8 (Analytical Software, Tallahassee, FL). The effect of sample size on strain richness was

assessed using a web-based 'rarefaction calculator' (University of Alberta, Edmonton, Canada; available at http://www.biology.ualberta.ca/jbrzusto/rarefact.php). Strain richness was estimated by using the nonparametric model of Chao, which considers the number of operational taxonomic units observed, and the frequency with which each was observed, to estimate total population strain richness (Chao and Tsung-Jen 2003). Evidence for gene conversion was examined using Sawyer's Test in GENECONV version 1.81 (http://www.math.wustl.edu/~sawyer/geneconv/), which tests the null hypothesis that nucleotide substitutions observed in a set of aligned sequences are randomly distributed (Sawyer 1989).

Results

Trapping success and infestation prevalence. A total 19,631 captures of birds, representing 105 species, occurred in 48,030 net hours for an overall netting efficiency of 40.9 captures per 100NH. Efficiency was significantly higher in October and November (53.3 birds/100NH) versus May through August (33.4 birds/100NH), reflecting the influx of migrants. Of all capture events, 19.5% were recaptures. Gray Catbird, Myrtle Warbler, American Goldfinch, and White-throated Sparrow were the most abundant species with a sample size of over 1000 captures of each species, together comprising 35.6% of all captures.

A total of 2074 bird captures (10.6% of all captures) were infested with ticks, of which 74.2% were hatch year, 22.5% were after hatch year, and 3.3% were of unknown age class. A total of 12,301 ticks were removed from birds, with a mean infestation burden of 5.9 ticks per infested bird, and hatch year birds had a significantly higher burden of ticks (6.2 per infested bird) versus after hatch year (5.2 per infested bird; P =

0.0003). Similar numbers of male and female birds were infested (366 males and 350 females), although the majority of birds from which ticks were removed were of unknown sex (n = 1,350 birds). Of all ticks, 86.4% were *I. dentatus* (10,363 larvae and 265 nymphs), 13.4% were *Haemaphysalis leporispalustris* (1535 larvae and 118 nymphs), 0.1% were *I. scapularis* (7 larvae and 6 nymphs), and 0.06% were *Dermacentor variabilis* (6 larvae). Voucher specimens of ticks were deposited with the National Tick Registry. Molecular identification of the tick ITS-2 region resulted in visible differences in the fragment size of *I. scapularis, I. dentatus*, and *H. leporispalustris* on agarose gel. Our *I. scapularis* sequences matched with 100% homology to published *I. scapularis* sequences. Because the ITS-2 regions of *I. dentatus* and *H. leporispalustris* were not previously deposited in Genbank, our samples for these two species matched poorly to published species (*I. dentatus* matched with 85% homology to *I. pacificus*; *H. leporispalustris* matched with 90% homology to *H. longicornis*). Two different sequences for *H. leporispalustris* were found. Representative ITS-2 sequences for all three tick species were deposited in Genbank (GU993304- GU993307).

The most commonly parasitized bird species, in which at least one-third of all individuals were infested, included Brown Thrasher, Carolina Wren, Eastern Towhee, White-throated Sparrow, Song Sparrow, Lincoln's Sparrow, Hermit Thrush, American Robin, and Yellow-breasted Chat (Table 5.1). In total, 285 birds were noted to harbor ticks and were released with no ticks removed. Of the remaining birds, 84.6, 23.5, 0.2 and 0.06% of parasitized birds harbored *I. dentatus, H. leporispalustris, I. scapularis* and *D. variabilis*, comprising 7.8, 2.2, 0.02 and 0.005% of all birds, respectively. Five of the top

ten hosts most commonly parasitized by *I. dentatus* were also among the top ten most parasitized by *H. leporispalustris*, including Brown Thrasher, Lincoln's Sparrow, Song Sparrow, Eastern Towhee, and Carolina Wren. Co-infestation of birds with both *I. dentatus* and *H. leporispalustris* simultaneously was found for 8.2% of all parasitized birds (0.8% of all birds); this rate is approximately 4.5-times higher than what would be expected by chance ($\chi^2 = 439.1$; $P < 0.001$). All ticks were immature stages with the exception of a single adult female *I. dentatus* collected from a House Wren. The average burden of *I. dentatus* larvae and nymphs on infested birds was 7.0 ± 0.5 (standard error) and 1.7 ± 0.2, respectively. Peak *I. dentatus* larval burden occurred on an Eastern Towhee which harbored 162 larvae that were removed and an estimated 80 ticks that were left on the bird. Peak *I. dentatus* nymphal burden was 11 on a Brown Thrasher that also harbored 14 other ticks. The average burden of *H. leporispalustris* larvae and nymphs on infested birds was 4.2 ± 0.5 and 1.2 ± 0.1, respectively, with a peak larval burden of 56 on a Swainson's Thrush that harbored 2 additional ticks, and a peak nymphal burden of 4 on a White-throated Sparrow that harbored 23 additional ticks.

Across all sampling, only three individual birds harbored *I. scapularis*: a Swainson's Thrush, a Hermit Thrush, and a Connecticut Warbler. All three were hatch year birds upon their first capture during in late August and September of 2004-2005 and were not captured subsequently. The Hermit Thrush was the most heavily infested, with 6 larvae and 5 nymphal *I. scapularis*, in addition to 2 *H. leporispalustris* larvae. The Connecticut Warbler harbored a single *I. scapularis* larva. The Swainson's Thrush harbored a single *I. scapularis* nymph, in addition to 10 and 3 *H. leporispalustris* larvae and nymphs, respectively. Communication with local ornithologists and review of

species-specific breeding and migratory data (Brewer et al. 1991) indicate that these three captures represent fall migrants from further north on a migratory stopover.

Phenology of bird-associated ticks. *I. dentatus* larvae exhibited bimodal peaks of activity, with the earlier peak in June and the second peak in October-November, whereas *I. dentatus* nymphs were mostly active in May-July, with smaller numbers throughout the fall (Figure 5.2a). Conversely, *H. leporispalustris* larvae were most active in August-September, when both stages of *I. dentatus* were least active. *H. leporispalustris* nymphs had a low level of activity throughout the sampling period (Figure 5.2b).

Ticks on eastern cottontails. Nineteen captures of eastern cottontails represented 15 individual rabbits; all recaptures occurred at least 7 days after initial trapping and therefore are considered as independent in the calculations of infestation prevalences. Of these captures, 73.7% were infested by ticks of three species. The two most common tick species on cottontails were *I. dentatus* and *H. leporispalustris*, which parasitized 63.2 and 26.3% of captures, respectively. Nymphs and adults of each species were found, with maximal burdens of 1 and 24 for *I. dentatus* and 1 and 3 for *H. leporispalustris*, respectively. Three cottontails were infested by both species simultaneously, and the maximum overall tick burden was 27. One rabbit was infested by two adult male *Dermacentor variabilis* in addition to nine *I. dentatus* adults.

***Ixodes scapularis* on white-footed mice and chipmunks.** At Pitsfield, no *I. scapularis* were found on 65 mice and 59 chipmunks trapped in May-June of 2005-2009. Fourteen

of these mammals were trapped again the day after initial processing, and none harbored ticks. Conversely, at Van Buren, 69.3% of 179 mice and 100% of 23 chipmunks were infested with *I. scapularis* (Table 5.2); these mammals were all trapped within two weeks of the dates of mammal trapping at Pitsfield so as to control for *I. scapularis* phenology.

Ticks on non-target captures. Non-target captures at Pitsfield included 3 raccoons, 1 fox squirrel (*Sciurus niger*), 3 meadow jumping mice (*Zapus hudsonius*), 1 red squirrel (*Tamiasciurus hudsonicus*), 3 northern short-tailed shrews (*Blarina brevaucada*), 4 Virginia opossum, and 3 woodchuck. Prevalence of ticks on these hosts included 33.3% of raccoons, 25% of opossums, and 33.3% of woodchuck with *D. variabilis*, 66.6% of woodchuck with *I. cookei*, and 66.6% of meadow jumping mice with *I. dentatus.*

***Borrelia* infection in bird-associated ticks.** A total of 2202 ticks/larval pools were assayed for infection with *Borrelia* species. Of these, 146 (6.6%) tested positive for *Borrelia* species, and DNA sequencing of the rDNA IGS and/or 16S gene was used to identify the pathogen in 128 samples: 78 (3.5%) were positive for *B. burgdorferi,* 35 (1.6%) were positive for *B. andersonii*, and 15 (0.7%) were positive for *B. miyamotoi* spirochetes. There were 18 additional samples which produced IGS bands at approximately 980 base pairs in size (indicative of either *B. burgdorferi* or *B. andersonii*) and an additional 22 samples that produced faint IGS bands at approximately 500 base pair size (indicative of *B. miyamotoi*) that were unsuccessfully sequenced. Accordingly, the infection prevalences for all species should be considered as minimum. *B. burgdorferi* and *B. andersonii* infection prevalence in nymphs was significantly greater than that of

larval pools (*B. burgdorferi*: 5.2 and 3.2%, respectively; P = 0.02; *B. andersonii*: 6.2 and 0.5%, respectively; P < 0.0001). Conversely, 14 of the 15 sequence-confirmed *B. miyamotoi*-positive samples were pools of larval *I. dentatus* (0.8% larval MIP), and a single sample was a nymphal *I. dentatus* (0.3% nymphal infection prevalence; Table 5.3).

Infection of *B. burgdorferi* and *B. andersonii* was found in *I. dentatus, H. leporispalustris*, and *I. scapularis*, whereas all *B. miyamotoi*-infected ticks were *I. dentatus* (Table 5.3). Of the small number of *I. scapularis* found in our study, one of the 5 nymphal *I. scapularis* removed from the Hermit Thrush was infected with *B. burgdorferi*, and the single larva removed from the Connecticut Warbler was infected with *B. andersonii*.

All three *Borrelia* species were found in ticks removed from birds of both age classes, and the prevalence of infection with each pathogen in each age class was not different that what would be expected based on the age structure and overall tick infection rates (hatch year, $\chi^2 = 0.73$. $df = 3$, P = 0.87; after hatch year, $\chi^2 = 2.88$, $df = 3$, P = 0.41). Similarly, all three species infected ticks removed from both sexes of bird in proportion to the sex structure and overall tick infection rates (female, $\chi^2 = 3.35$. $df = 3$, P = 0.34; male, $\chi^2 = 1.98$, $df = 3$, P = 0.58). Annual variation in tick infection prevalence was not significant *B. burgdorferi* (0.5, 6.3, 1.0, and 5.0%, in 2004-2007, respectively; $R^2 = 0.16$; P = 1) or *B. andersonii* (0.3, 2.7, 1.0, and 1.8% in 2004-2007, respectively; $R^2 = 0.23$; P = 1). All 15 *B. miyamotoi*-infected ticks were collected in 2007 (additional samples suspect-positive for *B. miyamotoi* were collected in earlier years, but were not sequence-confirmed; see above). Tick infection prevalence varied seasonally within years (Figure 5.3). Aggregating all four years of the study, *B. burgdorferi* was present in

all months of May-November with the highest monthly prevalence of 11-12% in May and July (Figure 5.3). *B. andersonii* was present in the months of May – October with the highest monthly prevalence of 4.9-6% in May-July. *B. miyamotoi* was only found in October and November with monthly prevalence of 1 and 5%, respectively. In the months of June-August only, a time period which largely excludes the spring and fall migrations, we detected 27 *B. burgdorferi*-positive ticks/pools (5.4% of ticks/pools tested in those months) and 21 *B. andersoni*-positive ticks (4.2% of ticks/pools tested in those months), comprising 34.6 and 60% of all positives, respectively. Of these June-August positive samples, 59.3 and 61.9% were from hatch year birds, respectively.

Assuming no significant transovarial or co-feeding transmission, our data on production of infected larval ticks (i.e natural xenodiagnosis; Table 5.1) implicate 19 species of bird as reservoir-competent for *B. burgdorferi*, and 7 species of bird as reservoir-competent for *B. andersonii*. As further evidence of local infectious hosts for both pathogens, 9 birds were associated with multiple infected ticks/tick pools, removed during one or more separate capture events (*B. burgdorferi*: American Robin, Song Sparrow, Swainson's Thrush, and White-throated Sparrow; *B. andersonii*: Brown-headed Cowbird, Hermit Thrush, Northern Cardinal, and Song Sparrow). In contrast, *B. miyamotoi* is a transovarially-transmitted spirochete (Scoles et al. 2004), and our findings of infected ticks on birds do not necessarily implicate host reservoir competence. We removed *B. miyamotoi* -positive ticks/tick pools from three host species, of which a majority (73.3%) were from Northern Cardinal, and a minority (13.3% each) was from American Robin and Hermit Thrush.

***Borrelia* infection in mammal-associated ticks.** A total of 150 ticks removed from white-footed mice, chipmunks, eastern cottontails, and non-target captures at Pitsfield were assayed for infection with *Borrelia* species. The only host species associated with positive ticks was the eastern cottontail, in which 15 of 111 (13.5%) *I. dentatus* adults tested positive, with a minimum of four (3.6%) *B. burgdorferi*-positive ticks, a minimum of 7 (6.3%) *B. andersonii*-positive ticks, and 4 samples of unknown pathogen identity (because sequencing was unsuccessful).

***Borrelia* infection in mammal ear biopsies.** At Pitsfield, one of 61 (1.6%) and two of 55 (3.6%) white-footed mouse and chipmunk ears tested positive for *B. burgdorferi*, whereas the equivalent prevalences at Van Buren were 30.4% of 171 and 34.8% of 23, respectively (Table 5.2). At Pitsfield, seven of 20 (35%) cottontail ear biopsies were positive for *Borrelia* species, which included a minimum of four (20%) *B. burgdorferi*-positive ears, a minimum of 1 (5%) *B. andersonii*-positive ears, and 2 ears of unknown pathogen identity (no sequence). None of 13 ear biopsies from the non-target captures tested positive.

***B. burgdorferi* genotypes.** *B. burgdorferi* IGS PCR products were successfully sequenced from 80 samples, including 71 ticks/larval pools removed from birds (including 39 larval pools and 11 nymphs of *I. dentatus*, 14 larval pools and six nymphs of *H. leporispalustris*, and one nymph of *I. scapularis*) and nine mammal-associated samples (ear tissue from one white-footed mouse, two eastern chipmunks, and one eastern cottontail, and from five adult *I. dentatus* removed from five different rabbits).

Of the 80 sequences, 10% were interpreted as mixed strain infections due to the presence of double-nucleotide peaks at polymorphic sites. These mixed strain infections comprised six tick samples removed from birds, one chipmunk ear, and one tick removed from a rabbit, and are not considered in further analyses. Among all IGS strains, we found no evidence for recombination using Sawyer's test. Within the 500 nucleotide IGS fragment, 58 sites were found to be polymorphic, including one indel block of 7 nucleotides (treated as a single polymorphism).

In total, 25 IGS strains were found among 72 samples, including strains within all three RST groups (GU993279- GU993303). Of all strains, 32% are 'ubiquitous' (i.e., also found in association with classic transmission cycles in the Northeast (Bunikis et al. 2004a) and/or Midwest (S. Hamer, unpublished)) and 68% are 'indigenous'(i.e., novel IGS mutants found only at Pitsfield in cryptic transmission; Figure 5.4). The only strains found more than once were ubiquitous strains; all indigenous strains were singletons, many being single or double nucleotide polymorphisms of ubiquitous strains. A MST of Pitsfield strains shows, for example, that there were many indigenous strains very similar to the ubiquitous strain IGS 2D, including 6 single nucleotide polymorphism mutants and 3 double nucleotide polymorphism mutants (Figure 5.5). A rarefaction curve to assess strain richness based on the frequency and diversity of strains we sampled suggests that true *B. burgdorferi* strain richness at Pitsfield is vast, as the rate at which new strains were found per unit of individuals sequenced showed no sign of approaching an asymptote (Figure 5.6). While we detected a total of 25 IGS strains within 72 samples derived from investigations of over 19,000 birds and a small number of mammals, the Chao-1 non-parametric estimator of true species richness is 245.5 ± 100.7 strains.

The single bird-borne *B. burgdorferi*-positive from a *I. scapularis* nymph was type 'Midwest A' of RST group 2 (Figures 5.4 and 5.5), a ubiquitous strain with a wide Midwestern distribution. There was no difference in the proportion of indigenous strains in bird-associated ticks during the migratory (May; September-November) versus non-migratory (June-August) seasons (23.8 and 26.1%, P = 0.84). The avian species that harbored ticks infected with indigenous strains during the migratory seasons included species known to both breed and winter on the property (e.g., Northern Cardinal) as well as species that do not breed on site that could have picked up these indigenous strains elsewhere, or on site during a migratory stopover (e. g., Hermit Thrush and White-throated Sparrow).

Of the seven *B. burgdorferi* sequences identified in mammal tissues or mammal-associated ticks, all but one were ubiquitous strains, with all three RST groups represented (Figure 5.4). The exception was an ear tissue sample from a rabbit that was infected with strain 'Novel II', an indigenous strain within RST 3. The single infected white-footed mouse ear was infected with 'Midwest A', the chipmunk ear was infected with 'Midwest B' and the *I. dentatus* adults removed from rabbits were infected with IGS 2D (n = 2), IGS 1A (n = 1), and 'Midwest K' (n = 1).

***B. andersonii* genotypes.** *B. andersonii* IGS PCR products were successfully sequenced from 33 samples (including 27 nymphs/larval pools from birds, 5 ticks from rabbits, and 1 rabbit ear). We found a total of 12 unique strains ('Michigan Bird A – L', accession nos. HMO15226-237) of which all but two were represented by more than one sequence in our populations. Within the 500 nucleotide IGS fragment, 57 sites were found to be

polymorphic, including three indels (two single nucleotide indels, and one 10 nucleotides indel which was treated as a single polymorphism). The amount of evolutionary change separating *B. andersonii* IGS strains was greater than that for *B. burgdorferi* IGS strains (Figure 5.7).

***B. miyamotoi* genotypes.** *B. miyamotoi* IGS PCR products were successfully sequenced from 12 samples (including 11 larval pools and 1 nymph of *I. dentatus).* A majority (n = 11) were identical to the published North American strain of *B. miyamotoi* (Bunikis et al. 2004b). A single sample from a larval pool had a single nucleotide polymorphism within the spacer, confirmed with reverse strand sequencing (GU993309).

Discussion

Our four-year dataset provides evidence of a unique cryptic transmission system involving three *Borrelia* species- *B. burgdorferi, B. andersonii,* and *B. miyamotoi* – maintained by bird-associated ticks in the apparent absence of the bridge vector *I. scapularis*. The focal site from which we trapped birds is outside the detected range of established *I. scapularis* populations, based on both historic records and our findings over the 2004-2007 sampling period (Hamer et al. 2010). Whereas birds in general have previously been considered to dilute the force of infection in endemic areas, by diverting tick bites away from white-footed mice which are generally able to infect more ticks than do birds (Giardina et al. 2000; LoGiudice et al. 2003), we present an ecological scenario in which birds may accelerate increasing Lyme disease risk through their maintenance of *B. burgdorferi* in the absence of classic *I. scapularis*/white-footed mouse transmission.

Absence of *I. scapularis*. Given our evidence based on small mammal and avian investigations, we conclude that the distribution of *B. burgdorferi* is not congruent with that of *I. scapularis*. *I. scapularis* was not encountered on any mammals or any resident birds at our study site, and was rarely encountered on fall migrants, with three birds found to carry *I. scapularis* during late August through October. Given the species-specific breeding and migratory distribution and timing of these species(Brewer et al. 1991), combined with the endemicity of *I. scapularis* in the southern Upper Peninsula (Walker et al. 1994) and other zones in the upper Midwest, we posit that these migratory birds picked up *I. scapularis* prior to arrival at Pitsfield. Furthermore, all three infested birds were captured during the first two years of this four-year study, and no additional *I. scapularis* infestations on wildlife were subsequently found, suggesting that the infestation of these birds did not represent the beginning of an appreciable process of successful establishment.

Migratory birds are known to be an important source of adventitious *I. scapularis* in areas beyond the range of established *I. scapularis* (Klich et al. 1996), and may be responsible for extending the geographic range of *I. scapularis* (Smith et al. 1996; Ogden et al. 2008) and *B. burgdorferi* sensu lato (Weisbrod and Johnson 1989; Dubska et al. 2009). During the September – November fall migratory period of four years, we searched a total of 14,667 birds, and found 3 (0.02%) to harbor a total of 13 *I. scapularis* ticks. Given this average of 0.0009 *I. scapularis* per migratory bird, and given that not all *I. scapularis* are likely to drop off while their host is on site, it seems unlikely that south-bound migrants alone would seed a new population of *I. scapularis* at this site. This is

likely influenced by the mismatch between peak juvenile *I. scapularis* activity in northern endemic areas and the timing of fall migration. In contrast, Ogden et al. (2008) found that north-bound migratory birds in the spring are very likely to be important in expanding the northern geographic range of *I. scapularis* across a wide front in northern Canada, with 0.35% of birds infested and an average of 0.007 *I. scapularis* per migratory bird. It is likely only a matter of time before a critical mass of occasional bird-derived engorged *I. scapularis* larvae and nymphs, combined with ticks moving in as a continuation of the invasion process occurring to the west (Hamer et al. 2010), will seed a reproducing population.

Bird-associated ticks. The vast majority of ticks removed from birds at our site were *I. dentatus* (86.4%), followed by *H. leporispalustris* (13.4%) and *I. scapularis* (0.1%). In a review of tick species assemblages on avain hosts across both sites endemic and non-endemic for *I. scapularis*, a similar preponderance of *I. dentatus* has not been found. For example, no *I. dentatus* were identified among 1643 ticks removed from 4317 birds from north-central Wisconsin, eastern and central Minnesota, and Michigan's northern Lower Peninsula, where 92-98% of ticks were *H. leporispalustris*, followed by small numbers of *I. scapularis* and *D. variabilis* (Nicholls and Callister 1996). Similarly, no *I. dentatus* were identified among 883 ticks removed from 1693 birds in the central Maryland Piedmont, where 97.4% of ticks were *I. scapularis* (Scharf 2004). In two Lyme disease-endemic foci, *I. dentatus* comprised a small proportion of ticks removed from birds relative to *I. scapularis* (Westchester Co., NY: 1067 ticks; 3.2% *I. dentatus*, 96.5% *I. scapularis* (Battaly and Fish 1993); Lyme, CT: 4,065 ticks; 1.6% *I. dentatus*, 3.7% *H.*

leporispalustris; 94.4% *I. scapularis*; (Stafford et al. 1995). In a recent study of tick parasites of migratory birds in Canada, *I. dentatus* and *I. scapularis* were nearly equal in abundance (34 and 29.3% of ticks, respectively), and *H. leporispalustris* comprised 22.5% of the sample (Ogden et al. 2008). The preponderance of *I. dentatus* among the birds we sampled may reflect a unique area of *I. dentatus* abundance, reflecting the tick community composition prior to *I. scapularis* invasion, and the extent to which *I. dentatus* and *I. scapularis* compete is unknown.

I. dentatus has a widespread distribution throughout the eastern United States, with recoveries from at least 26 states and Ontario (Durden and Keirans 1996); immatures parasitize several species of Passerine birds and all stages parasitize cottontail rabbits. *I. dentatus* may itself be undergoing range expansion, having been detected in Michigan only since 1992, despite an established passive surveillance program since 1968 (Walker et al. 1998). Alternatively, it may be an endemic species that is not readily detected due to its degree of wildlife host specificity. *I. dentatus* has been associated with *Rickettsia rickettsia*, agent of Rocky Mountain spotted fever (Clifford et al. 1969), Connecticut virus (Main and Carey 1980), *B. andersonii* (Anderson et al. 1989; Marconi et al. 1995), and *B. burgdorferi* (Telford and Spielman 1989b) including antigenically variable strains of *B. burgdorferi* (Oliver et al. 1996; Oliver et al. 1998). This tick species has been posited as a 'transition vector' of *B. burgdorferi*, facilitating the movement of the spirochete into new areas beyond the distributional limit of *I. scapularis* (Levine et al. 1991).

Comparatively less is known about *H. leporispalustris*. The cottontail rabbit is the dominant host for all stages of *H. leporispalustris*, and immatures can also be found

on wild birds (Sonenshine and Stout 1970). *H. leporispalustris* is recognized as a vector of the agents of Rocky Mountain spotted fever (Sonenshine and Stout 1970), tularemia (Cooney and Burgdorfer 1974), and Sawgrass vius (Sather et al. 1970). In Lyme disease-endemic areas, few infected *H. leporispalustris* ticks are generally found relative to other species (Stafford et al. 1995), yet infected *H. leporispalustris* have been documented (Anderson and Magnarelli 1984; Levine et al. 1991), and vector competency is unknown. In the current study, *H. leporispalustris* infection rates were slightly higher than that of *I. dentatus*, though *H. leporispalustris* was relatively less abundant. In our study, *H. leporispalustris* generally parasitized the same species as did *I. dentatus*, and a significant rate of simultaneous co-infestation with both species was found, despite the differences in peak activity periods of the ticks. Therefore, infection in *H. leporispalustris* may reflect co-feeding transmission from *I. dentatus*, spirochetemic bloodmeals from infectious hosts, transstadial transmission, or perhaps vector competency.

Ecology of tick-borne pathogens at Pitsfield. Our documentation of infection of bird-associated ticks with at least three species of *Borrelia* underscores the potential importance of birds in *Borrelia* maintenance and dispersal. Local transmission of all three pathogens is evident (see below) in that hatch year birds, known to have been born on-site, harbored infected ticks at times that exclude the migratory period. Furthermore, eastern cottontails – important hosts for feeding adults of both species of bird-associated ticks – were present on site, infected with both *B. burgdorferi* and *B. andersonii*.

B. burgdorferi. We detected a low prevalence of *B. burgdorferi* infection in bird-derived (minimum of 3.5%) and rabbit-derived (minimum of 3.6%) *I. dentatus*, and suggest that this pathogen is being actively transmitted at Pitsfield. Our findings are similar to the scenario reported by Telford and Spielman (1989b), except that this system is operating in the apparent absence of *I. scapularis*. Ogden et al. (2008) reported that no larval and 15.4% of nymphal *I. scapularis* removed from birds were infected with *B. burgdorferi*, whereas 0.3% of *I. dentatus* larvae and no *I. dentatus* nymphs were infected. Stafford et al. (1995) found 4.3% of all *I. dentatus* in a Lyme endemic area to be infected with *B. burgdorferi*, but 0% of *H. leporispalustris*. Because of the narrow host range of *I. dentatus* on birds and rabbits, its involvement in *B. burgdorferi* maintenance is cryptic with respect to disease (Telford and Spielman 1989b).

Vector competency trials have not been conducted for *H. leporispalustris*. As a non-*Ixodes* genus tick, *H. leporispalustris* would not be predicted to be a competent vector for *B. burgdorferi*. Nicholls and Callister (1996), however, report that 5% of 1,184 ticks removed from birds in a Lyme disease-endemic area were *B. burgdorferi*-infected *H. leporispalustris* immatures. Herein we report a 4.2% overall infection prevalence in *H. leporispalustris*, resulting largely from infected larval pools, indicating acquisition of spirochetes from infected avian hosts, or transovarial transmission.

Transovarial transmission is considered negligible for *B. burgdorferi* (Piesman et al. 1986; Magnarelli et al. 1987; Patrican 1997) but cannot be ruled out as a mechanism giving rise to infected larvae in our study. In the absence of local maintenance and transmission at our site, however, the rate of transovarial transmission from adult ticks (infected elsewhere) to larval ticks picked up by local birds would have to be

extraordinarily common to explain the frequent detection of infected larvae in our study (58 larval pools removed from 58 different birds). The diversity of avian hosts able to maintain *B. burgdorferi* infection and infect ticks underscores the complex transmission dynamics of these pathogens. Many species of birds have been proposed as competent *B. burgdorferi* reservoir hosts using laboratory studies and natural xenodiagnostic investigations (Anderson et al. 1990; Rand et al. 1998; Richter et al. 2000; Ginsberg et al. 2005). Using natural xenodiagnosis, we implicate 18 avian species as competent reservoirs for *B. burgdorferi*, including 10 species which have not before been reported: Brown-headed Cowbird, Blue Jay, Eastern Towhee, Fox Sparrow, Magnolia Warbler, Slate-colored Junco, Swainson's Thrush, Tufted Titmouse, White-throated Sparrow, and Yellow-breasted Chat. Given that infective birds were found both during the summer breeding months and fall migratory months, we posit that migration of hosts from sites of cryptic transmission may provide a mechanism for dispersal of cryptic *B. burgdorferi* to new sites. Conversely, Ogden et al. (2008) detected little evidence of infectious reservoir hosts among the Spring migratory birds arriving in Canada, as no larval *I. scapularis* ticks in their study were found to be infected with *B. burgdorferi*. This may result from a waned infection that was contracted during the previous year's nymphal season, or, lack of exposure of these birds to infectious ticks.

In this cryptic cycle, the prevalence of *B. burgdorferi* in bird-associated ticks is quite low, with an overall infection prevalence of 3.5% of ticks/larval pools. This raises questions as to how the cycle can be maintained given this low prevalence, compared to the relatively higher rates of infection in classic transmission cycles involving *I. scapularis*. Adjusting the data to reflect infection in only *I. dentatus* nymphs removed

from only those avian hosts defined by our study as being reservoir competent (see above), the minimum infection prevalence is 5.1%. Infection of adult *I. dentatus* feeding on rabbits makes maintenance of this pathogen more plausible; in our study, a minimum of 20% of rabbits were infected based on the conservative index of infected ear tissue, and a minimum of 28.6% of rabbits were associated with *B. burgdorferi*-infected ticks.

Presence of other *Borrelia spp.* spirochetes. The presence of *B. andersonii* and *B. miyamotoi* at the cryptic site enriches our understanding of the reservoir hosts and vector species that may be important for maintenance and transmission of these spirochetes. Neither *B. andersonii* nor *B. miyamotoi* have been associated with human disease (though *B. andersonii* has been detected in an *I. dentatus* tick removed from a human in Connecticut (Anderson et al. 1996). The presence of these pathogens at our study site is of uncertain epidemiological importance. Twelve unique IGS strains of *B. andersonii* were present among bird and rabbit-associated ticks and rabbit ears at the cryptic site. The amount of evolutionary distance that separates *B. andersonii* strains is much greater than that which separates most cryptic *B. burgdorferi* strains, suggesting that the duration of establishment of the *B. andersonii* maintenance system is longer than that of *B. burgdorferi*, and that *I. dentatus* (if required for pathogen maintenance) may be an endemic tick species in this area.

We report on the first detections of *B. miyamotoi* in ticks removed from passerine birds. *B. miyamotoi* was first detected in North America in *I. scapularis* ticks (Scoles et al. 2004) and was originally described in *I. persulcatus* ticks in Japan (Fukunaga et al. 1995). A *B. miyamotoi* spirochete has also recently been detected in tissues and blood

from Wild Turkey in Tennessee (Scott et al., in review)). A definitive wildlife reservoir, however, has not yet been identified. We suggest the Northern Cardinal may be important in the life history of this species due to the significant association of infected ticks with this species (11 of 15 infected tick pools were derived from 11 separate Northern Cardinals). Northern Cardinals are a permanent resident in Michigan, and most cardinals stay within 8 km of where they were raised (Brewer et al. 1991). While all the birds carrying *B. miyamotoi* -infected ticks were captured during the fall migratory period, it is likely that all hatch year cardinals with infected ticks were born on site (analysis of capture records indicates that 7 hatch year cardinals associated were each captured two to six times (average 3.8) between 2 August and 13 November 2007 - the last day of banding for the season). Conversely, two Hermit Thrushes were carrying infected ticks. As Hermit Thurshes are not known to breed in the area, the timing of our findings suggests that migratory birds from the north may have brought *B. miyamotoi* to the study site. Similarly, we found one American Robin that carried infected ticks in the fall. This species in Michigan is typically a migrant, though a small proportion of birds will overwinter in the southern counties. We thus have evidence to support both local and migratory contributions to *B. miyamotoi* maintenance at Pitsfield. The pattern in our data in which a majority of infected ticks were larvae may parallel the finding of Barbour et al. (2009), in which *B. miyamotoi* infection in mouse hosts rose toward the end of the summer, coincident with the larval phenology (and in this vertically-transmitted system, larvae may infect hosts). Similarly, Scott et al. (in review) found that *B. miyamotoi*-positive turkeys were more likely to be infested with larval as opposed to nymphal ticks. In comparison to the high strain diversity observed within *B. burgdorferi* (see below), the

intraspecific diversity of the *B. miyamotoi* spirochetes at the IGS locus was much less, as we found only two strains. Only a single strain – the same strain that infected 91.2% of our *B. miyamotoi* samples - was present within ticks in Connecticut (Bunikis et al. 2004b).

Bird tick phenology and implications for *Borrelia* species maintenance. Larval *I. dentatus* exhibited bimodal activity peaks in Spring/early summer and again in the fall, with nymphs active in the spring thought mid-summer, similar to the findings of Sonenshine and Stout (1970), Battaly et al. (1987), and Kollars and Oliver (2003). From the conventional perspective of *B. burgdorferi* maintenance by *I. scapularis* in the northeastern US, we expect that nymphal activity should precede larval activity, such that nymphs can infect the hosts prior to their being fed upon by the larvae. At our study site, *I. dentatus* nymphal activity clearly precedes the fall peak in larval activity, but nymphs and the early larvae are simultaneously active. This non-optimal phenology, from the perspective of the pathogen, may partially explain its low prevalence. However, more synchronous feeding nymphal and larval *I. scapularis* has been observed in the Midwest (Gatewood et al. 2009)(Hamer et al. 2010), with similar infection prevalences as found in the Northeast. The longevity of birds, in comparison to mice, may be advantageous to the pathogen in a cryptic scenario (though the duration of infectiousness of *B. burgdorferi* in infected birds is unknown), as a bird infected by a nymph in the previous year may infect a larva in the spring in the absence of infectious nymphs. Given that peak infection prevalence in ticks for both *B. burgdorferi* and *B. andersonii* was toward the beginning of the season in May-July, coincident with the synchronous activity of larvae and nymphs,

we suggest that co-feeding of larval and nymphal *I. dentatus* on the same avian host may be important in pathogen transmission. Co-feeding was observed in 116 cases, 73 of which occurred during weeks 20-29 of the spring/early summer tick activity peak, thus offering a mechanism for *Borrelia spp.* maintenance. There are three birds in our dataset from which we simultaneously removed both *B. burgdorferi*-positive larvae and nymphs. Furthermore, birds have been shown to reactivate latent *B. burgdorferi* infection in the face of migratory stress (Gylfe et al. 2000), and this may contribute to the infection in spring and early summer larvae that we observed.

Strain diversity of *B. burgdorferi.* We detected an unprecedented level of diversity at the 16S-23S ribosomal intergenic spacer of cryptic *B. burgforferi* within host and tick populations not classically expected to be infected. Rarefaction analysis suggests that our sampled richness reflects only a small proportion of true strain richness. Intergenic spacer regions generally accumulate higher degrees of sequence variation between related species than do coding regions, because spacers do not produce a functional gene product and are free of selective constraint (with the exception of a tRNA gene that occurs within the IGS of *Borrelia* species). Furthermore, because no detectable recombination is found within the IGS (which is chromosomally-located), in contrast to other *B. burgdorferi* typing schemes (such as *ospC* , which is located on a plasmid), IGS is a sensitive marker of evolutionary change (Liveris et al. 1995), and alone is an efficacious genetic marker to differentiate among strains of *B. burgdorferi* (Bunikis et al. 2004a).

At least three evolutionary mechanisms may contribute to the pattern of genetic diversity we observed within the cryptic *Borrelia*, which is characterized by a small

number of ubiquitous strains that are also found in classic transmission at Lyme disease-endemic sites (present within more than one individual in our sampled population), and a larger number of indigenous strains that are novel mutants (present as singletons in our sampled population). (1) Ecological opportunity is present by the invasion of a new vector (*I. dentatus*), new host species (birds/rabbits) or new geographical regions (Pitsfield) by *B. burgdorferi,* after which the one or few invading genotypes become the most common. Among others, strain IGS 2D was likely a founder at Pitsfield, as it is the most common strain, comprising 40.3% of all sequences, and is present in ticks removed from both birds and rabbits. This bottleneck in the *B. burgdorferi* population, represented by the presence of only a few founders, is followed by clonal expansion and diversification of founders. This diversification is evident as a star phylogeny in the MST, in which each novel strain is derived independently from its common ancestor. Because of the nature of cryptic transmission, these novel variants are permitted within birds, rabbits, and I. dentatus, but are not necessarily exposed to the mammalian immune system to the same extent as would be newly-evolved strains in classic transmission scenarios. Thus, strains that may otherwise have been 'purged' by a host immune system in classic transmission are instead present in cryptic transmission. (2) Multiple-niche polymorphism, also known as diversifying selection, can maintain diversity within the population when the environment is heterogenous and no single genotype has the highest fitness in all environments (Brisson and Dykhuizen 2004). This selection does not occur on the IGS region itself, but on other areas of the genome which are linked to the IGS region such as *ospC*. The environment experienced by cryptic *B. burgdorferi*, including *I. dentatus*, rabbits, and at least 12 species of birds in our study, may therefore influence

the frequency with which we observed genotypes. For example, that strain IGS 2D was so common at Pitsfield may reflect selection for this strain at this site in avian hosts and the *I. dentatus* vector, or, its selection within classic transmission cycles (it is among the most common strains across the Midwest) and spillover into this cryptic cycle. Host associations, facilitated by selective killing of strains by host-specific innate immune factors (Kurtenbach et al. 2002), have been documented as a factor leading to the diversifying selection of Lyme disease spirochetes, at both the inter-species level (e.g. in Europe, *B. burgdorferi* and *B. afzelii* are mammal-associated, whereas *B. garinii* and *B. valaisiana* are bird-associated (Dubska et al. 2009)) and at the intraspecies level of *B. burgdorferi* (Brisson and Dykhuizen 2004; Hanincova et al. 2006). (3) Negative frequency-dependant balancing selection has been described for *B. burgdorferi*, in which antigenic types that have already infected a host cannot establish a subsequent infection in the same host because of the immune response, and therefore rare antigenic types are more likely to establish infection in hosts infected with multiple strains, leading to high levels of variation (Wang et al. 1999). Balancing selection acts not only on the *ospC* locus, but on closely linked loci (Brisson and Dykhuizen 2004).

Given the low overall prevalence of *B. burgdorferi* infection in this cryptic system, the degree to which we identified mixed strain infections (10% of all infected samples) was surprisingly high. We recently identified 4% of adult *I. scapularis* from Van Buren State Park within the recently-invaded area in Michigan's Lower Peninsula as harboring mixed-strain infections (unpublished data). (Furthermore, adults are likely to have higher levels of mixing as compared to nymphs, given the additional bloodmeal they have consumed). Thus, our encountering of mixed infections at 2.5 the prevalence

in the cryptic zone suggests that the degree of interaction among infected ticks and hosts is high, and that hosts are being exposed to multiple strains. Mixed strain infections are generally expected to occur in highest frequency in sites with well-established transmission as opposed to recently-colonized foci with many naïve hosts and a reduced diversity of strains due to invasion by one or a few founding organisms strains.

What is the epidemiological significance of a cryptic cycle? The epidemiological significance of a cryptic cycle is dictated by a combination of the following five parameters: (1) the infectivity of cryptic strains to humans; (2) the frequency at which infected cryptic vectors directly feed on humans; (3) the presence or imminent invasion of a bridging vector; (4) the frequencies at which the cryptic vector and bridging vector share hosts; and/or (5) the extent to which the cryptic *B. burgdorferi* introduced into the bridging vector serves as additive infection, above the level of infection that invading *I. scapularis* may bring to the site, or the extent to which the cryptic B. burgdorferi strains replace strains that the bridging vector circulates

(1) Two main genetic typing schemes are useful in assessing virulence of different *B. burgdorferi* strains in humans: RST (a tripartite classification of the 25+ IGS strains) and outer surface protein C (*ospC*; an antigenic plasmid-borne gene); *ospC* is in positive linkage disequilibrium with IGS (Bunikis et al. 2004a). Some RST 1 strains are associated with a higher frequency of disseminated infection in humans and more invasive disease in experimental animals (Seinost et al. 1999; Wang et al. 2002; Derdakova et al. 2004; Hanincova et al. 2008; Wormser et al. 2008), and a bias toward the relatively less invasive RST 2 and 3 strains has been found among infected *I.*

scapularis in the Midwest (Gatewood et al. 2009). At our cryptic site, we document the presence of two RST 1 strains (IGS 1A and 'Novel PP') which alone underscores the potential epidemiological significance of this cryptic cycle.

The pathogenicity in humans of the large number of novel IGS mutant strains is unknown, though we have no *a priori* reason to suggest that single or double nucleotide polymorphisms within the non-coding intergenic spacer region would necessarily correspond to a change in virulence. To begin to assess the epidemiological risk associated with novel strains found within this cryptic cycle, we sent total DNA from 11 of the samples with novel IGS types (Novels NN, O, OO, P, PP, R, S, T, V, W and X; all were derived from ticks removed from birds) to a laboratory at the University of California-Irvine for direct *ospC* typing (B. Travinsky and A. Barbour, unpublished data). Of these, *ospC* was successfully amplified and sequenced from 10 samples, resulting in detection of *ospC* A, I3, and K, which represent three of the four ospC major groups associated with disseminated human Lyme disease (Seinost et al. 1999). Furthermore, many mixed strain infections were found at the *ospC* locus. Given that no evidence of mixing was apparent in the portion of IGS that we scrutinized from these samples, this suggests that *ospC* may more readily be mixed, and further underscores the complexity of the pathogen diversity maintained at this site.

(2) Documented human-biting in *I. dentatus* is rare, yet has been reported a total of 13 people across Michigan (Walker et al. 1992), North Carolina (Harrison et al. 1997) West Virginia (Hall et al. 1991), Washington D. C. (Sollers 1955), Connecticut (Anderson et al. 1996) and Maine (Keirans and Lacombe 1998).

(3) *I. scapularis* is invading from the west (Hamer et al. 2010). Pitsfield Banding Station is on the trajectory for continued invasion, and has habitat features predicted to support *I. scapularis* populations should they become introduced (Guerra et al. 2002).

(4) We document *I. dentatus* feeding on two meadow jumping mice of a total of three captured at Pitsfield. This represents an expansion of the documented host range for this tick species, and may afford the opportunity for small mammals to become infected with cryptic *Borrelia spp.* (though the two mouse-infesting *I. dentatus* were both uninfected larvae in our study). Similarly, an *I. dentatus* larva was found on a white-footed mouse in Tennessee (Kollars 1992). As mice are key hosts for the bridging vector *I. scapularis*, such adventitious feeding events by the cryptic vector on atypical hosts may offer a mechanism for transmission of cryptic strains to bridging vectors and subsequently, humans. In addition to atypical host utilization by the cryptic vector as a mechanism for merging cryptic and classic transmission cycles, the bridge vector may feed directly on cryptically-infected hosts. The frequency at which *I. scapularis* feeds on rabbits in endemic areas is not widely studied. Telford and Spielman (1989b) found that 41, 38, 3% of rabbits were infested with *I. scapularis* larvae, nymphs, and adults, respectively, and that 85, 62, and 85% were infested with the equivalent life stages of *I. dentatus*. Thus, there exists potential for introduction of cryptic *Borrelia* into *I. scapularis* populations through direct feeding by the bridge vector on cryptically-infected wildlife.

(5) It is currently unknown whether the infection prevalence in invading *I. scapularis* will be elevated due to the existence of cryptic *Borrelia* in the region undergoing invasion. In southwest Michigan, *B. burgdorferi* was found within recently

invaded *I. scapularis* populations, and it is plausible that both *I. scapularis* and *B. burgdorferi* may invade simultaneously (Hamer et al. 2010). Upon invasion into a zone of cryptic transmission, the frequency with which *I. scapularis* feeds on infectious hosts will likely dictate the additive nature of cryptic infection. The degree to which invading strains may replace cryptic strains, and how host associations of strains may affect their persistence and the blending of the invading and cryptic cycles is unknown.

We hypothesize that the presence of *B. burgdorferi* in birds and rabbits, which precedes the arrival of *I. scapularis*, may reduce the time lag between *I. scapularis* invasion and build-up of infection prevalence in *I. scapularis*. Thus, mobile infected birds and cryptic cycles may have the potential to accelerate the increase in human disease risk within an invasion zone, or, facilitate the establishment phase of invasion. Infection of birds from cryptic cycles may then allow bird dispersal of *B. burgdorferi* strains such that the movement and maintenance of *B. burgdorferi* is un-linked from that of the main bridging vector. Further studies are needed to understand the role of birds in generating or maintaining genetic diversity of *B. burgdorferi*, as well as *B. andersonii* and *B. miyamotoi*, and the consequences for infection of humans or canines with these bird-associated strains.

Table 5.1a. Infestation prevalences of all parasitized bird species with three species of tick, Pitsfield Banding Station, 2004-2007. Some ticks were observed on birds yet not removed and are categorized as unknown identity. Tick species-specific infestation prevalences therefore underestimate true infestation. Additionally, a single Song Sparrow harbored 6 *Dermacentor variabilis* larvae.

Common name	Total	Overall	*I. dentatus*	*H. lep.*	*I. scapularis*	*Co-infestation I. dent, H. lep*	Un-identified
American Goldfinch	1398	0.9	0.6	0.7			0.1
American Redstart	222	1.0		1.0			
American Robin*	566	34.9	26.5	1.9		1.6	8.0
Black and White Warbler	60	1.7		1.7			
Black-capped Chickadee	716	3.9	3.6	0.1		0.1	0.3
Blue Jay*	109	28.4	2.2				8.3
Brown Thrasher*†	104	46.2	44.2	11.5		1.6	1.0
Brown-headed Cowbird*	29	13.8	13.8				
Canada Warbler	42	7.1		7.1			
Carolina Wren*	21	42.9	28.6	19.5		4.8	
Chestnut-sided Warbler†	114						
Common Grackle	159	27.4	2.8	1.3		0.6	5.7
Common Yellowthroat*	326	7.4	3.7	5.0		0.6	
Connecticut Warbler	9	11.1			11.1		
Downy Woodpecker	212	1.4	1.4				
Eastern Bluebird	31	3.2		3.2			
Eastern Towhee*	75	42.7	29.3	17.3		6.7	2.7
E. White-crowned Sparrow	53	22.6	17.0	13.3		7.5	
Field Sparrow	196	9.7	7.7	1.5		0.5	1.2
Fox Sparrow*	94	27.7	25.5	3.2		2.1	1.6
Golden-crowned Kinglet	249	0.4		0.4			
Gray Catbird*†	2775	4.9	3.3	2.1		0.3	0.2
Gray-cheeked Thrush	58	19.0	13.8	1.3		5.2	
Hairy Woodpecker	22	4.5	4.5				
Hermit Thrush*†	563	35.2	26.6	2.8	0.2	1.8	7.5
House Finch	252	1.6	1.6				
House Wren*‡	135	31.1	14.8	23.0		6.7	
Indigo Bunting	64	3.1	1.6	1.6		1.6	1.6
Lincoln's Sparrow	24	37.5	37.5	12.5		12.5	
Magnolia Warbler*	777	0.8	0.5	0.3			
Mourning Warbler	54	5.6		5.6			
Myrtle Warbler	1665	3.5	2.9	0.2		0.7	0.5
Nashville Warbler	428	1.6	0.9	0.8			
Northern Cardinal*	768	27.9	22.9	4.0		1.8	2.9
Northern Waterthrush	27	3.7	3.7				
Orange-crowned Warbler	57	1.8	1.8				
Ovenbird*	384	3.1	0.8	2.3			
Purple Finch	64	7.8	6.3	1.6			
Red-breasted Nuthatch	25	4.0	4.0				
Ruby-crowned Kinglet	660	1.7	1.4	0.3			
Slate-colored Junco*	466	7.8	5.2	2.1		0.9	0.6
Song Sparrow*†‡	500	39.0	28.4	13.0		5.6	3.2
Swainson's Thrush*	364	13.2	3.6	1.2	0.3	0.5	
Swamp Sparrow†	37	18.9	1.8	2.7			5.5
Tennessee Warbler	790	0.4	0.1	0.3			
Tufted Titmouse*†	302	19.9	16.6				3.3
Veery	22	22.7	13.6	10.0			
White-breasted Nuthatch	86	1.2	1.2				
White-throated Sparrow*	1159	4.8	29.9	4.6		2.2	9.6
Wilson's Warbler	165	0.7		0.7			
Winter Wren	29	24.1	24.1				
Wood Thrush	209	8.6	6.7	2.4		0.5	
Yellow Warbler	187	0.5	0.5				
Yellow-breasted Chat*	6	33.3	33.3				

* designated as competent reservoir for *B. burgdorferi*
† designated as competent reservoir for *B. andersoni*
‡ a single Song Sparrow harbored 6 *D. variabilis* larvae and a single House Wren harbored one *D. variabilis* adult

Table 5.1b. Bird species investigated for ticks and found to be uninfested, Pistfield Banding Station, 2004-2007.

Common name	Total captured
Acadian Flycatcher	5
American Woodcock	5
American Tree Sparrow	45
Baltimore Oriole	28
Black-billed Cuckoo	2
Bay-breasted Warbler	44
Blue-gray Gnatcatcher	4
Blue-headed Vireo	37
Blackburnian Warbler	9
Blackpoll Warbler	56
Brown Creeper	42
Brewster's Warbler	1
Black-throated Blue Warbler	46
Black-throated Green Warbler	54
Blue-winged Warbler	93
Cedar Waxwing	157
Cerulean Warbler	1
Chipping Sparrow	20
Cape May Warbler	9
Cooper's Hawk	2
Eastern Kingbird	1
Eastern Phoebe	48
Eastern Wood-Pewee	70
Great Crested Flycatcher	2
Golden-winged Warbler	9
House Sparrow	2
Hooded Warbler	5
Least Flycatcher	40
Mourning Dove	19
Northern Parula	1
Orchard Oriole	1
Philadelphia Vireo	3
Prairie Warbler	2
Prothonotary Warbler	1
Rose-breasted Grosbeak	141
Red-bellied Woodpecker	34
Red-eyed Vireo	73
Ruby-throated Hummingbird	319
Red-winged Blackbird	4
Scarlet Tanager	36
Sharp-shinned Hawk	15
Tree Swallow	2
Traill's Flycatcher	63
Virginia's Warbler	1
Warbling Vireo	38
White-eyed Vireo	12
Western Palm Warbler	13
Yellow-billed Cuckoo	9
Yellow-bellied Flycatcher	57
Yellow-shafted Flicker	36
Yellow-throated Vireo	5

Table 5.2. Small mammals as sentinels for *I. scapularis* and *B. burgdorferi* presence at Pitsfield Bird Banding Station and Van Buren State Park, a hotspot for recently-invaded *I. scapularis* 90 km west of Pitsfield. Sample size of mammals captured and infestation prevalence with two species of ticks is followed by sample size of ear biopsies tested and infection prevalence with *B. burgdorferi*.

	White-footed mouse					Eastern chipmunk				
		Infestation (%)		Ear biospy			Infestation (%)		Ear biospy	
Site	No. trapped	*I. scapularis*	*D. variabilis*	No. tested	Prevalence (%)	No. trapped	*I. scapularis*	*D. variabilis*	No. tested	Prevalence (%)
Pitsfield	65	0	9.2	61	1.6	59	0	8.5	55	3.6
Van Buren	179	69.3	39.1	171	30.4	23	100	8.7	23	34.8

Table 5.3. Tick species-specific infection prevalence with 3 different *Borrelia* pathogens, 2004-2007, Pitsfield Banding Station. In addition to samples listed, a single adult female *I. dentatus* and a pool of 6 *D. variabilis* larvae were negative for all pathogens.

	Larval pools				**Nymphs**			
		Percent Infected				Percent Infected		
	No. tested	*B. burgdorferi*	*B. andersoni*	*B. miyamotoi*	No. tested	*B. burgdorferi*	*B. andersoni*	*B. miyamotoi*
I. dentatus	1452	3.03	0.41	0.96	262	4.96	9.16	0.38
H. leporispalustris	364	3.85	0.55		114	5.26	1.75	
I. scapularis	2		50		6	16.67		
All species	1818	3.19	0.50	0.77	382	5.23	6.81	0.26

Figure 5.1. Pitsfield Bird Banding Station in Vicksburg, MI (blue triangle) and Van Buren State Park, the site of comparative small mammal sampling (red circle). Shading indicates counties with documented established populations of blacklegged ticks at the time the study started in 2004 (dark gray) and at the time the study ended in 2007 (light gray), and the endemic population (diagonal lines; Hamer et al. 2010). *H. lep.* = *H. leporispalustris*; *I. dent.* = *I. dentatus.*

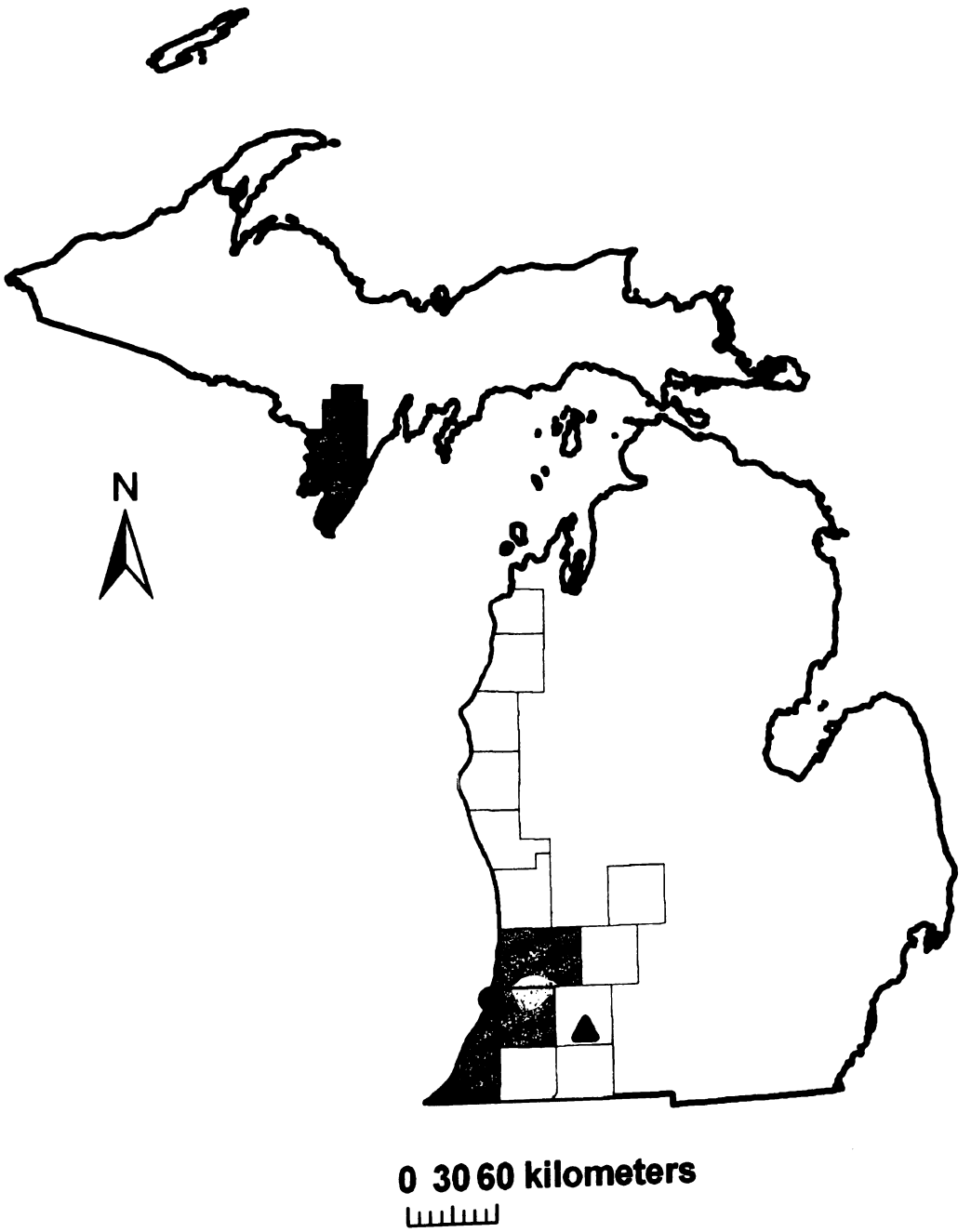


Figure 5.2. Phenology of larval and nymphal bird-associated ticks (a, *I. dentatus*; b, *H. leporispalustris*) depicted as weekly mean proportions of infested birds (error bars are standard error of the mean using year as replicate).

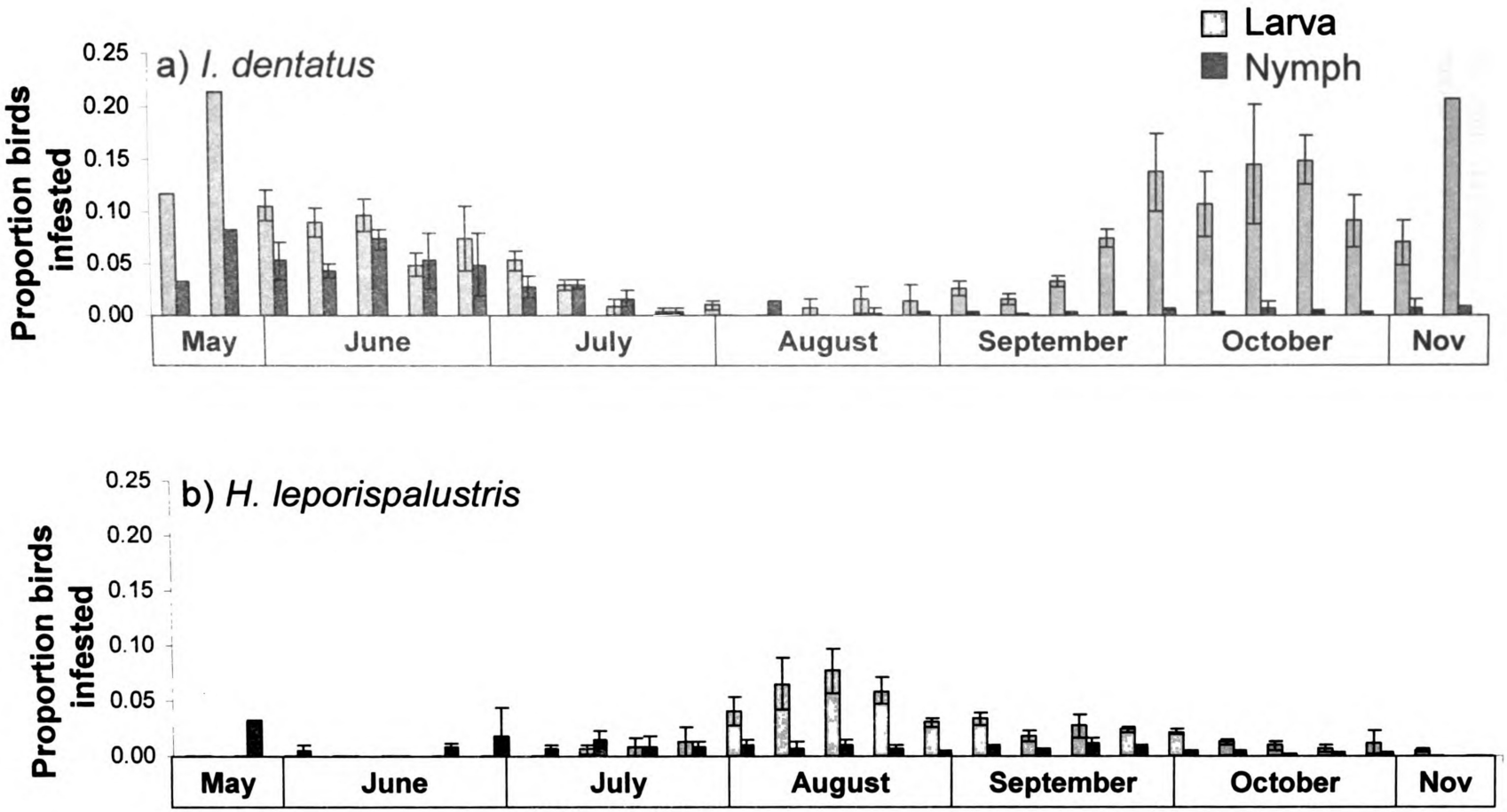


Figure 5.3. Temporal variation in infection prevalence of *B. burgdorferi*, *B. andersonii*, and *B. miyamotoi* from May-November, 2004-2007. Monthly mean infection prevalences across all years (± SE of mean) is plotted. All tick species are aggregated.

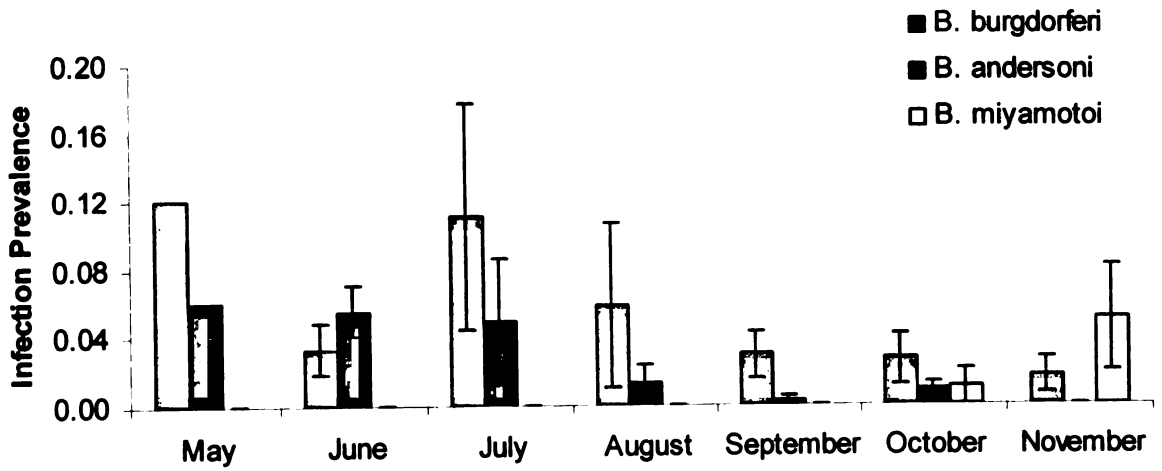


Figure 5.4. Neighbor-joining phylogenetic tree and frequency distribution of *B. burgdorferi* IGS haplotypes collected from Pitsfield Banding Station, 2004-2007. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Indigenous strains (novel IGS mutants not previously reported) are indicated with a blue triangle; ubiquitous strains (haplotypes previously reported in Bunikis et al. (2004a) or detected in *I. scapularis* across the Midwest) are indicated with a red circle. Sequences conforming to the 16S – 23S RST group 1, 2, and 3 designations by the criteria of Liveris et al. (1995) are demarcated by the labels at the top left of shaded or unshaded groups. Total samples size of each strain is indicated at the end of each branch label, of which a majority is from bird-derived ticks; the 7 mammal-associated samples (from rabbit ticks and ear and small mammal ears) are denoted in parentheses.

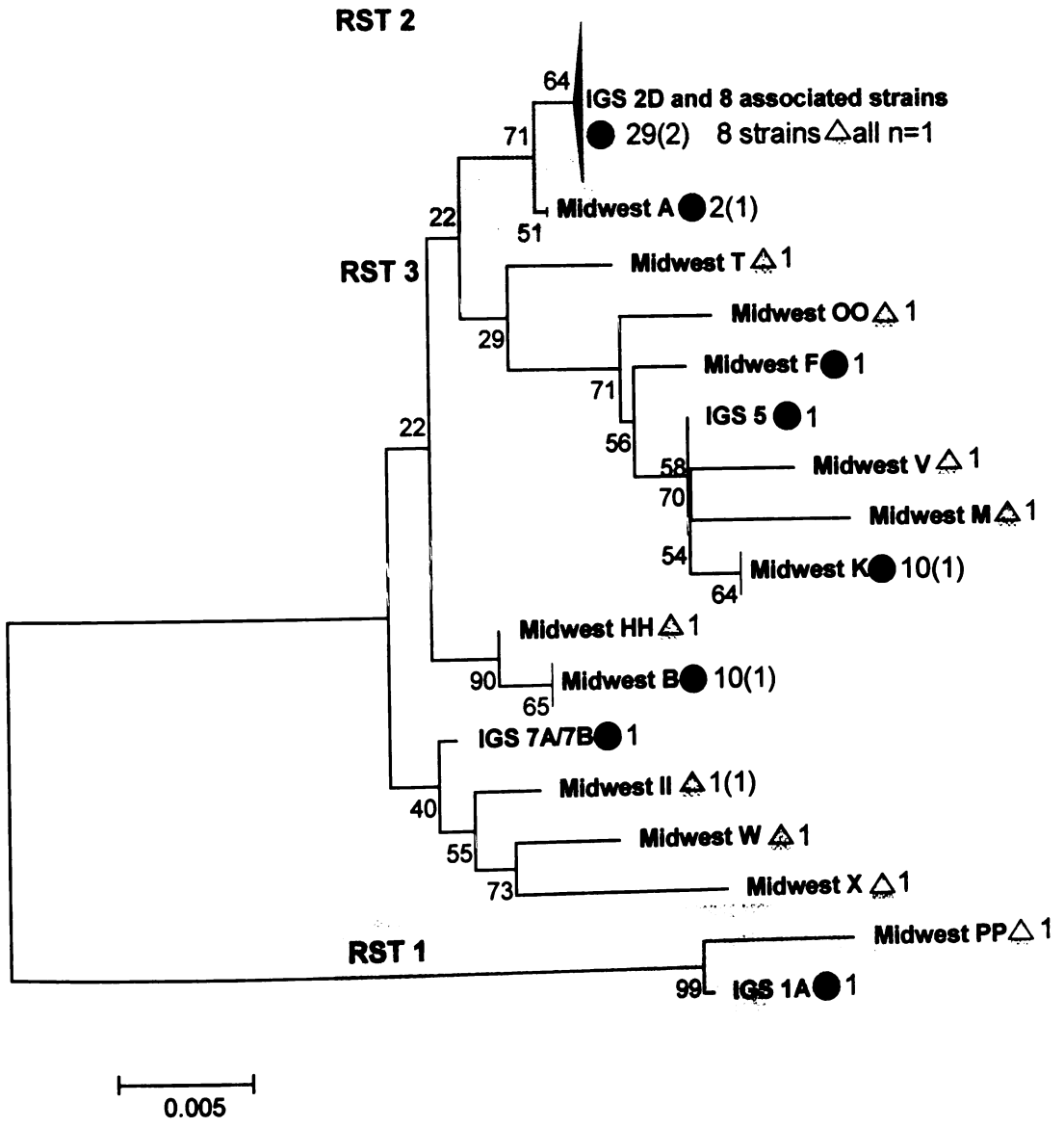


Figure 5.5. Minimum spanning tree (MST) of *B. burgdorferi* IGS haplotypes collected from Pitsfield Banding Station, 2004-2007. Each black circular node connecting haplotypes represents one mutational change; a 7 base pair indel that occurs within the IGS is considered as one change. The size of each haplotype is proportional to its frequency within the sampled population. The rectangles represent the haplotypes with the highest outgroup weight, which correlates with haplotype age. The two haplotpyes within RST 1 (IGS 1A and Novel PP) are linked to each other by two mutational changes, yet unlinked to the rest of the network. Indigenous strains (novel IGS mutants not previously reported) are indicated with a blue triangle; ubiquitous strains (haplotypes previously reported by Bunikis et al. (2004a) or detected in *I. scapularis* across the Midwest) are indicated with a red circle.

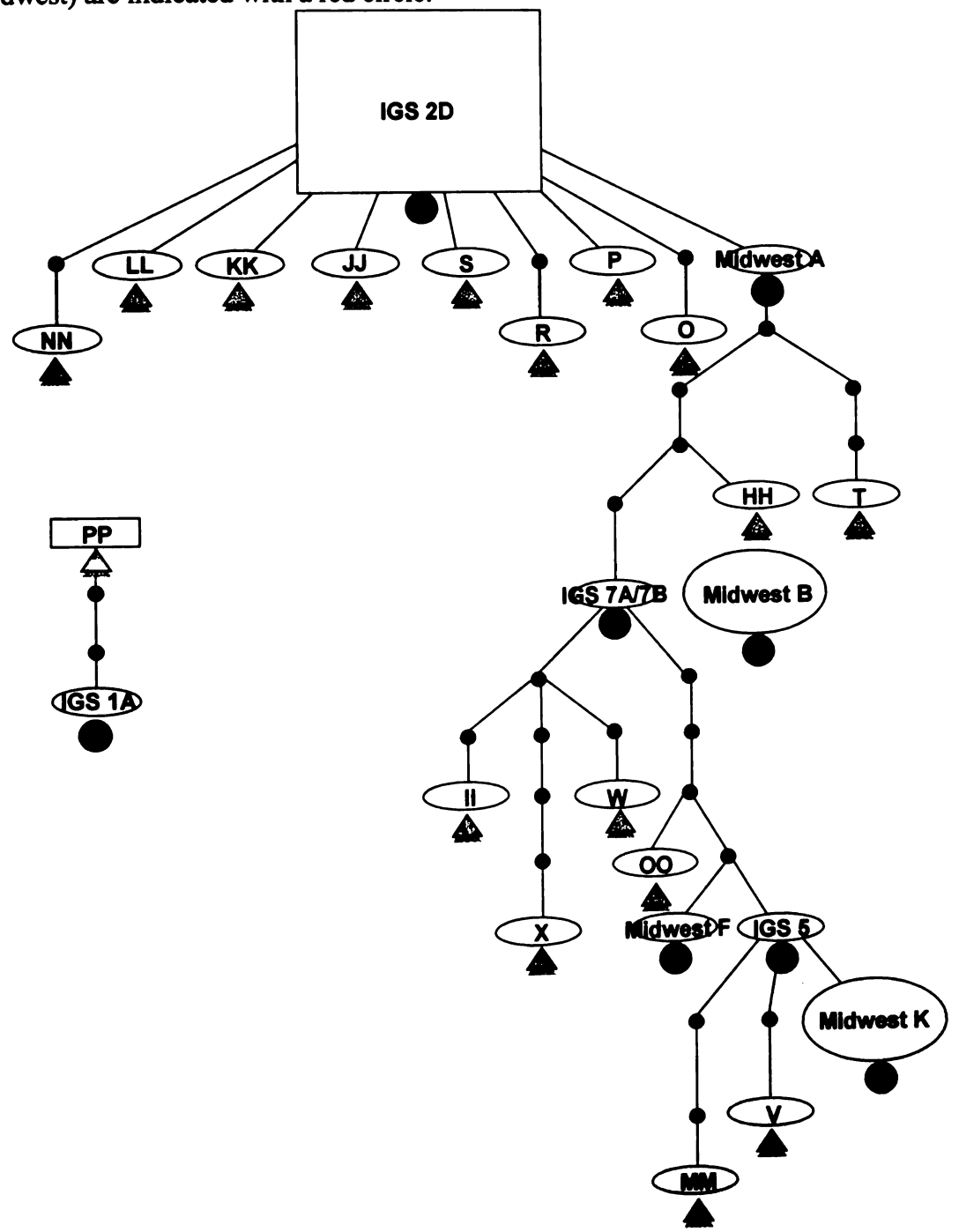


Figure 5.6. Rarefaction curve for *B. burgdorferi* strains derived from Pitsfield Banding Station, 2004-2007. Mean and standard deviations of the number of strains found in subsamples are shown.

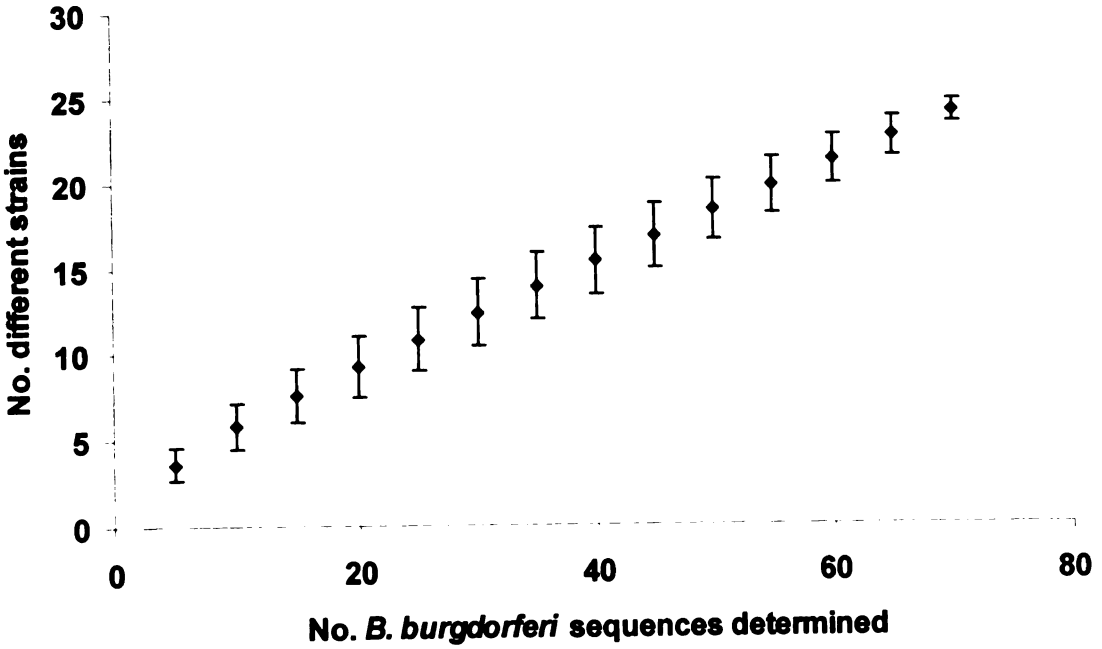
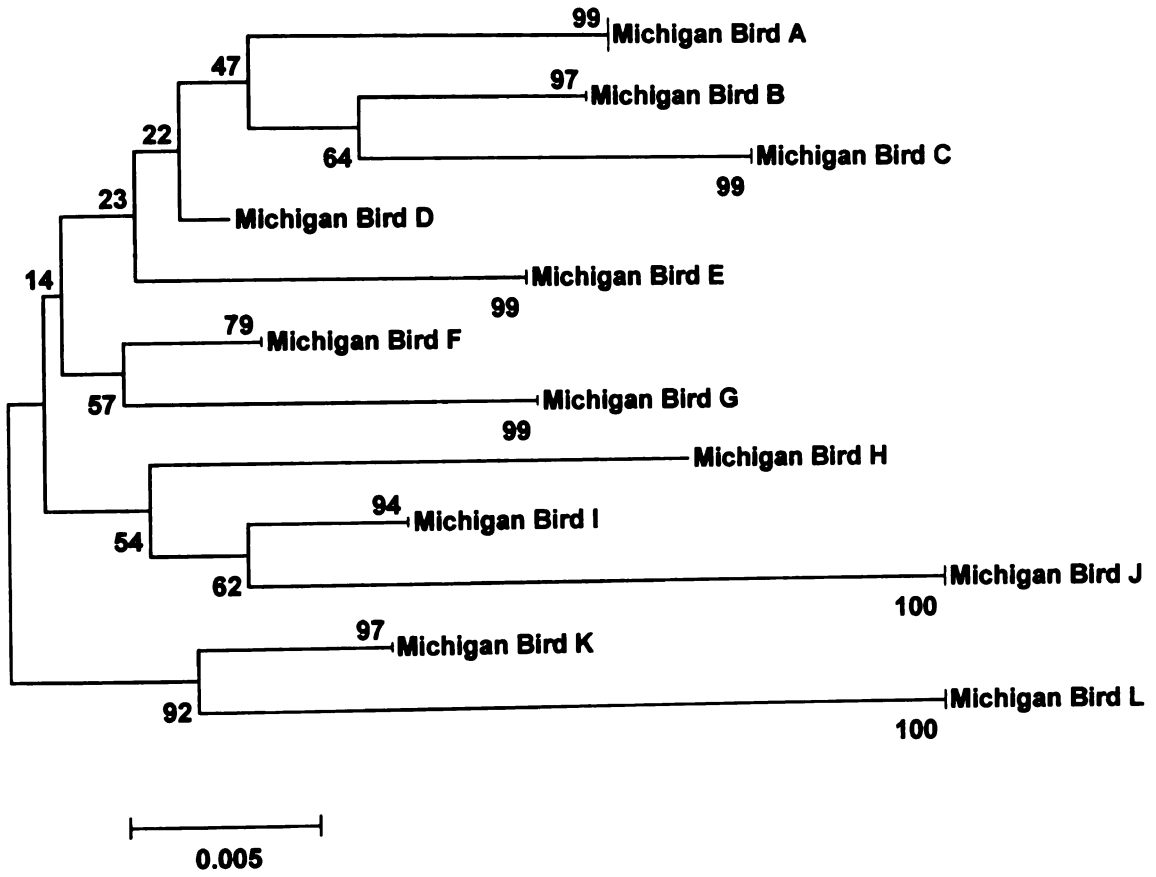


Figure 5.7. Neighbor-joining phylogenetic tree and frequency distribution of *B. andersoniii* IGS haplotypes collected from Pitsfield Banding Station, 2004-2007. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Total samples size of each strain is indicated at the end of each branch label, of which a majority is from bird-derived ticks; the 7 rabbit-associated samples (from rabbit ticks and a rabbit ear) are denoted in parentheses.



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CHAPTER 6

Diversity of zoonotic pathogens in *Ixodes scapularis* as a marker of its invasion of across the Midwestern United States

Abstract

The center of origin theory predicts that genetic diversity will be greatest at the site of origin because of a long period of time available for evolution. Conversely, diversity is predicted to decrease with distance from the origin, because invasion and colonization are associated with founder effects that reduce genetic variation in newly-arrived populations. Given the close association of *Ixodes scapularis* - the blacklegged tick - and the suite of zoonotic pathogens it transmits, we posited that patterns of diversity of *I. scapularis*-borne pathogens across its Midwestern range may be useful in better understanding the broad-scale invasion of this tick and subsequent disease emergence. Analysis of 1565 adult *I. scapularis* ticks from 13 sites across five Midwestern states reveals that tick infection prevalence with multiple microbial agents, co-infections, and strain diversity of *Borrelia burgdorferi* - agent of Lyme disease - were positively correlated with the duration of establishment of tick populations. The observed differences in these parameters across gradients of establishment were, however, subtle, as recently-invaded ticks harbored diverse infections. Our data suggest that the invasion of ticks and emergence of various tick-borne diseases may be more complex than the traditional scenario whereby infected, invading ticks are the only means of introduction of pathogens to naïve communities.

Introduction

The distribution and incidence of vector-borne pathogens and the diseases they cause are inextricably linked to that of their invertebrate vectors (Gubler 1998; Otranto et al. 2009). As such, patterns of invasion and establishment of vectors can be used to predict disease, and similarly, pathogen diversity and prevalence may provide an indirect understanding of the endemicity of the disease system. In disease ecology, a disease system is considered endemic in an area when a pathogen persists in a given vector and/or host population without the need for external inputs. *Ixodes scapularis*, the blacklegged tick, is an epidemiologically important vector of multiple zoonotic pathogens with an endemic distribution in the upper Midwestern and Northeastern United States. Blacklegged ticks were widespread prior to the Pleistocene glaciation, which receded 10,000 years ago, throughout which time relict populations remained in refugia in the Northeastern United States and in Northwestern Wisconsin (Spielman 1988). In the mid-twentieth century, the reversion of agricultural lands to forest and implementation of deer hunting regulations that allowed deer populations to increase in number supported expansion of *I. scapularis* and its vertebrate hosts from refugia (Steere et al. 2004). *I. scapularis* continues to spread from endemic areas both in the Northeastern and Midwestern United States, but the mechanisms for this spread are not established.

The establishment of a new population of ticks requires the invasion of a new site by a sufficient propagule, and the new site must be receptive with appropriate biotic and abiotic features to allow for successful tick survival and reproduction. With regard to I. *scapularis*, the Centers for Disease Control and Prevention (CDC) defines an established population as one in which at least six individual ticks of a single life stage, or at least

two life stages, are found (Dennis et al. 1998). With regard to pathogens, establishment is a description of the stability of the infection. Mathematically, establishment can be demonstrated when the basic reproductive rate (R_0) is greater than one; that is, the more than one secondary infections result from a single infectious unit (Anderson and May 1978), and the extent to which R_0 exceeds one may dictate the magnitude of zoonotic risk (Telford et al. 1991). Across the Midwest, a non-monotonic continuum of establishment of *I. scapularis* is apparent (Table 6.1), with tick populations first detected in Wisconsin, Minnesota, Michigan's Upper Peninsula, and Northwestern Illinois (Jackson and Defoliart 1970; Drew et al. 1988; Bouseman et al. 1990; Strand et al. 1992), later detected in northern Indiana (Pinger and Glancy 1989; Bouseman et al. 1990; Pinger et al. 1991; Pinger et al. 1996; Cortinas and Kitron 2006), and most recently detected in the Chicago-area and Michigan's Lower Peninsula (Foster 2004; Jobe et al. 2006; Hamer et al. 2007; Jobe et al. 2007). The current tick invasion in lower Michigan is particularly well-documented, with detectable new wildlife infestations moving northward annually (Hamer et al. 2010).

The three most significant *I. scapularis*-borne zoonotic pathogens are *Borrelia burgdorferi, Anaplasma phagocytophilum,* and *Babesia microti.* All three pathogens have similar life histories, in which wild rodents serve as important (although not the only) natural reservoirs (Donahue et al. 1987; Telford and Spielman 1993; Levin et al. 2002)and disease results when *I. scapularis* serves as a bridging vector through becoming infected by infectious wildlife and subsequently delivering the pathogens to humans. The epidemiology of each disease reflects the geographic distribution and phenology of *I. scapularis* as well as the human behaviors that influence exposure to ticks. *B.*

burgdorferi is a spirochetal bacterium that causes Lyme disease, the most frequent human vector-borne disease in the northern hemisphere (Bacon et al. 2007). First reported in 1975 in Old Lyme, Connecticut (Steere et al. 1977), over 20,000 human cases are now reported annually in the United States, with increasing annual incidence (Bacon et al. 2007). *Anaplasma phagocytophilum*, a gram-negative, obligate intracellular rickettsial bacterium of neutrophils, causes human anaplasmosis, formerly known as human granulocytic ehrlichiosis. This disease was first reported among patients in Minnesota and Wisconsin in 1994 (Bakken et al. 1994; Chen et al. 1994). In the United States, a 130% increase in incidence of human anaplasmosis has been reported in 2007 over 2003 levels with a majority of cases reported from the I. scapularis-endemic foci in the Northeast and upper Midwest (Hall-Baker et al. 2009). *Babesia microti* is one of four apicomplexan parasites of the genus *Babesia* that infect erythrocytes and causes a malaria-like disease called babesiosis in humans. The first significant cluster of disease was reported among residents of Nantucket Island, Massachusetts, in 1977 (Ruebush et al. 1977). Babesiosis is usually self-limiting, but can cause severe morbidity and mortality in asplenic, elderly, and immunecompromised patients. Whereas both Lyme disease and anaplasmosis are reportable diseases, babesiosis is not, and so incidence estimates are not reliable – nevertheless this disease is considered to be emerging (Vannier et al. 2008). In a review of co-infections of the above pathogens within *Ixodes* ticks from California, Wisconsin, and the Northeast, the prevalence of co-infections was found to be highest among ticks collected from regions of Lyme disease endemicity in the Northeastern states, where infection with each individual pathogen is also highest (Swanson et al. 2006).

In addition to variation in the species of pathogens infecting ticks at different locations, within-pathogen variation occurs. This latter variation has implications both for altering the risk of pathogen transmission and for efficient diagnosis and treatment (Swanson et al. 2006). Strain-level variation has been best characterized for *B. burgdorferi* and is maintained through diversifying selection, as the environment is heterogenous and no single genotype has the highest fitness in all environments (Brisson and Dykhuizen 2004).

A center of origin is a geographical area where a group of organisms, either domesticated or wild, first developed its distinctive properties (Cain 1944). This concept is typically applied to studies of crop domestication, and is a core foundation of dispersal biogeography (Bremer 1992). The center of origin of a pathogen is generally also its center of genetic diversity, and the former is hypothesized to correspond with the center of origin of its host if host/pathogen coevolution has occurred. For example, the leaf spotting cereal pathogen, *Mycosphaerella graminicola*, was found to have highest levels of gene diversity and allele richness in Israel – the center of origin of wheat (Zhan et al. 2003; Banke et al. 2004). The diversity of a pathogen, however, is not always highest at the center of origin of its host. For example, while high genetic diversity of the scale pathogen of barley, *Rhynchosporium secalis*, was expected in the Fertile Crescent of the Middle East where barley has been cultivated for thousands of years, diversity was instead greatest in Scandanavia and Switzerland, leading the authors to suggest that cultivated barley was not the original host of the pathogen (Zaffarano et al. 2006). Diversity is predicted to decrease with distance from the origin, because invasion and

colonization events are associated with founder effects that reduce genetic variation in newly-arrived populations (Carlson and Templeton 1984).

We tested the center of origin theory using *I. scapularis* and associated pathogens not only to apply this theory to a vector-borne disease system for the first time, but also to learn more about the biology of these organisms. Given the close association of *I. scapularis* and the pathogens it transmits, we posited that patterns of prevalence and diversity of *I. scapularis*-borne pathogens across its Midwestern range may be useful in better understanding the mechanisms underlying the broad-scale invasion of this tick and subsequent disease emergence. Assuming a clonal evolution of *B. burgdorferi* (Dykhuizen et al. 1993), a population with greater genetic diversity is likely to have had a longer period of time available for evolution (Wang et al. 1999a). Herein we hypothesize that infection prevalence, interspecific, and intraspecific diversity of pathogens within ticks will positively correlate with the duration of establishment of tick populations, due to the longer period of time for evolution in areas with established population. The objectives of this study were to test the predictions for a center of origin hypothesis through assessment of the following traits of the disease system across a continuum of *I. scapularis* establishment in the Midwest: (1) the population abundance of *I. scapularis*; (2) prevalence of *Borrelia* spp., *Anaplasma phagocytophilum*, and *Babesia* spp., and mixed infections in *I. scapularis;* and (3) strain-level diversity of *B. burgdorferi*.

Materials and Methods

Tick collections. Ticks were collected by drag cloth during the spring adult questing season (15 April – 15 May) of 2006-2007 at a total of 13 sites across the Midwest (Figure

6.1a): Castle Rock State Park, Ogle Co., IL; Indiana Dunes National Lakeshore, Porter Co., IN; Tippecanoe River State Park, Pulaski Co., IN; Van Buren State Park, Van Buren Co., MI; Duck Lake State Park, Muskegon Co., MI; Fort McCoy Military Instillation, Monroe Co., WI; Governor Dodge State Park, Iowa Co., WI; Saugatuck Dunes State Park, Allegan Co., MI; Menominee North and South, Menominee Co., MI; Churchill Woods Forest Preserve, DuPage Co., IL; Fort Sheridan, Lake Co., IL; and St. Croix State Forest, Pine Co., MN. We targeted our sampling time for the peak period of activity of the adult life stage of *I. scapularis* because adults have had two prior bloodmeals and therefore have had a greater chance of being infected in comparison to nymphs. Due to close geographic proximity (between 30-70 km between sites) and tick establishment status, similar drag densities, and inadequate individual sample sizes, we grouped the two Chicago area sites (Chicago North and South), the two Menominee Co. sites (Menominee North and South), and two sites in Michigan's Lower Peninsula (Saugatuck and Duck Lake) for our analyses. Based on the time of first documented *I. scapularis* within these regions, we assigned sites to establishment groups as follows: Minnesota, Wisconsin, western Illinois, and Michigan Upper Peninsula sites are 'long-established' tick populations; northern Indiana sites are 'intermediate-established'; and Chicago-area and lower Michigan sites are 'recently-invaded'.

Each site was sampled for questing ticks (Figure 6.1b) by dragging a 1-m^2 white corduroy cloth (Falco and Fish 1992) on rain-free days in the late morning or late afternoon to avoid the hottest and least-humid times of day (Schulze et al. 2001; Diuk-Wasser et al. 2006). The cloth was inspected every 20 m, and attached ticks were stored in 70% ethanol. Drag-sampling was not performed on excessively hot or wet days. In

2006, ticks were removed from the cloth and placed into chambers with a piece of vegetation for moisture and were kept alive for transport to the laboratory at Michigan State University. In 2007, ticks were removed from the cloth and placed directly into 70% ethanol. At each site, we attempted to collect a quota of 100 adults to allow for an adequate sample size of infected ticks for analysis, but this was not always possible in low-density sties. In some cases, the distance that was sampled was not directly recorded, and was estimated based on the duration of time spent sampling and number of ticks collected. An index of tick abundance was calculated as the number of adult *I. scapularis* collected per $1000m^2$ of drag sampling.

Pathogen detection. Ticks were identified to species and stage (Keirans and Clifford 1978; Sonenshine 1979; Durden and Keirans 1996). In 2006, live ticks were aseptically bisected with one half used to culture *B. burgdorferi* (data not presented). Total DNA from the remaining half tick in 2006 and the whole tick in 2007 was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) following the manufacturer's animal tissue protocol, with modifications as described in Hamer et al. (2010). *B. burgdorferi* strain B31-infected nymphal *I. scapularis* kindly provided by the Centers for Disease Control and Prevention (CDC) served as the positive extraction control, and water as a negative extraction control.

All ticks were tested for the presence of three classes of pathogens in three separate PCR reactions -- for *Borrelia* species, *Babesia* species, and *Anaplasma phagocytophilum*,--with subsequent sequencing of *Borrelia*-positive and *Babesia*-positive samples to identify the species and/or strain. A PCR enzyme kit was used in all assays

(PCR Supermix, Invitrogen, Carlsbad, CA), and water was used as a negative control in all assays. All assays were run in a 50 µl reaction volume. *Borrelia* species were detected using a nested polymerase chain reaction (PCR) for the 16S – 23S rRNA intergenic spacer region (IGS) as described by Bunikis et al. (2004a), resulting in a product size of approximately 980bp for *B. burgdorferi* and 500bp for *B. miyamotoi*. DNA from *B. burgdorferi* strain B31-infected ticks kindly provided by the CDC served as a positive PCR control. Additionally, we screened 2007 samples for *B. burgdorferi* using a quantitative PCR (qPCR) targeting the 16S gene following the protocol of Tsao et al. (2004), and the reported infection prevalence includes samples positive on either assay. *Babesia* genus-specific PCR was performed using primers for the 18S rRNA gene to produce a fragment of variable size, including a 408bp fragment for *B. microti* or a 437-bp fragment for *B. odocoilei* (Armstrong et al. 1998). Commercially-available *B. microti* organism (ATCC, Manassas, VA) was extracted as above and used as a positive control. The *p44* gene of *A. phagocytophilum* (Zeidner et al. 2000) was amplified using a touchdown PCR program described in Steiner et al. (2006) to produce a 334-bp fragment. Infected laboratory colony *I. scapularis* nymphs provided by D. Fish at Yale University were extracted as above and used as a positive control.

Nucleotide sequencing. Species identification and strain typing of *Borrelia*-positive and *Babesia*-positive ticks was attained through DNA sequencing. Additionally, a subset of randomly selected samples that were PCR-positive for *A. phagocytophilum* was sequenced to confirm the species identity. All products were purified (Qiagen PCR Purification Kit; Qiagen, Valencia, CA) and sequences were determined on an ABI Prism

3100 Genetic Analyzer (Applied Biosystems, Foster City, CA) using the primers that were also used in PCR. *Borrelia* sequences were identified as either *B. burgdorferi* or *B. miyamotoi* and *Babesia* sequences were identified as either *Ba. microti or Ba. odocoilei*, based on comparisons to published sequences using the basic local alignment search tool in GenBank (Altschul et al. 1990).

Strain-level analyses were further conducted for approximately half of all *B. burgdorferi* samples, and all *B. miyamotoi* samples For *B. burgdorferi* sequences, a 500 nucleotide segment of the IGS was aligned with the prototypical strains published in Bunikis et al. (2004a) using the ClustalW algorithims within the program Mega4 (Tamura et al. 2007). Analysis of this fragment size allowed identification of the 10 main IGS groups (groups 1-10; the minimal matrix for differentiation of these 10 groups includes mutations which all occur within the first 309 nucleotides of the fragment), and of 20 IGS subtypes within the main groups (1A, 1B, 2A/C, 2B, 2D, 3A, 3B/C, 3D, 4A, 4B, 5, 6A, 6B, 6C, 7A/B, 8A/C, 8B, 8D, 9, 10), as presented in Bunikis et al. (2004a). Additionally, sequences were identified to broad ribosomal spacer type (RST 1, 2, or 3; (Liveris et al. 1995) based on clustering topology of the IGS phylogenetic trees. In the case that a sequence we derived did not completely match any published strain, we classified it as novel IGS mutant and assigned an alphabetical nomenclature beginning with 'Midwest'. Sequence chromatographs were manually scrutinized for confidence in nucleotide assignments and evidence of mixed strain infections, which were excluded from analyses. For at least one representative of each detected strain, we also determined the sequence in the reverse direction and/or determined the sequence in the forward direction twice to validate the occurrence of unique mutations. A similar protocol was

followed for *B. miyamotoi* sequences, for which prototypical IGS strains for use in alignments were obtained from Bunikis et al. (2004b).

Statistics. Kruskal-Wallis non-parametric one-way analysis of variance was used to assess differences in the index of population abundance of ticks and tick and infection prevalence among establishment groups. Comparisons in infection between male and female ticks were made by calculating the z-ratio and associated probability for the difference between two independent proportions. Chi-squared test for independence was used to assess co-infections. Statistics were performed using Statistix 8 (Analytical Software, Tallahassee, FL). The effect of sample size on strain richness was assessed using a web-based program called the rarefaction calculator (University of Alberta, Edmonton, Canada; http://www.biology.ualberta.ca/jbrzusto/rarefact.php). Strain richness was estimated by using the nonparametric model of Chao, which considers the number of operational taxonomic units observed and the frequency with which each was observed to estimate the total population strain richness including unsampled strains (Chao and Tsung-Jen 2003).

A series of standard ecological diversity indices were computed to assess the *B. burgdorferi* strain-level diversity within and among the three establishment groups. Strain richness (alpha diversity) was tabulated as a simple count of the number of strain types in each group. Evenness is a measure of the relative abundance of the different species making up the richness of an area (Krebs 1978). This measure is constrained between zero and one in which the most even (least variable) community has a value close to one. Shannon's Diversity Index considers both richness and evenness (Shannon

and Weaver 1949). Sørensen's Similarity Index (a measure of Beta diversity) was computed for each pairwise combination of establishment groups (Sørensen (1948); this measure is constrained between zero and one in which a value of zero indicates no overlap between communities, whereas a value of one indicates that exactly the same strains are found in both communities.

Genetic differentiation within a pathogen among geographic populations was tested using an analysis of molecular variance (AMOVA) (Excoffier et al. 1992) using Arlequin 3.1 (Excoffier et al. 2005) to partition genetic variation into within- versus among-population components. The fixation index (F_{ST}) is a measure of the level of population genetic differentiation that reflects the proportion of total genetic variability that is due to the net differences between populations(Weir and Cockerham 1984). F_{ST} ranges from zero to one, where a value of one indicates that sub-populations are completely different and values <0.1 indicate that subpopulations are not different. The significance of the fixation index is tested using a nonparametric permutation approach (Excoffier et al. 1992). Population pairwise F_{ST} values were computed to test the null hypothesis of no difference between populations by permutating haplotypes between populations, in which the P-value of the test is the proportion of permutations leading to a F_{ST} value larger or equal to the observed one (Reynolds et al. 1983). The molecular diversity index Theta_k (an estimate of Theta obtained from the observed number of alleles (Ewens 1972)) and the mismatch distribution (Rogers and Harpending 1992) were computed for each establishment group. Mismatch distribution assesses the distribution of the number of differences (mismatches) between pairs of DNA sequences in a sample. The shape of this distribution is affected by the past demography of a population, such

that the distribution is usually multimodal in samples from populations at demographic equilibrium, reflecting the highly stochastic shape of gene trees. Conversely, a unimodal mismatch distribution is characteristic of a population that has passed through a recent demographic expansion. Goodness of fit of the observed mismatch distribution to the sudden expansion model (unimodal distribution) allowed for assessment of significance through the calculation of Harpending's Raggedness Index (Harpending 1994).

To evaluate phylogenetic relationships among *B. burgdorferi* haplotypes, we constructed an unrooted neighbor-joining phylogeny and minimum spanning network (MSN) using Mega4 and TCS 1.21, (Clement et al. 2000; Tamura et al. 2007) respectively. Evolutionary distances were computed using the Kimura 2-parameter method and are in units of number of base substitutions per site. Percentage support values for clades within the neighbor-joining tree were obtained from 1000 bootstrap iterations. The MSN method determines the gene network in which the total length of the branches that connect haplotypes is minimized; discrimination among equal-length MSNs was achieved by assuming older alleles are more common than recently derived alleles, and that new mutations are likely to be found in the same population as their ancestor. Evidence for gene conversion was examined using Sawyer's Test in GENECONV version 1.81 (http://www.math.wustl.edu/~sawyer/geneconv/), which tests the null hypothesis that nucleotide substitutions are randomly distributed (Sawyer 1989).

Results

Index of abundance of *I. scapularis*. Across all sites, a total of 1655 questing ticks were collected, the majority (94.6%) of which were *I. scapularis* adults (816 females and 749

males). Also collected were 29 nymphal and one larval *I. scapularis*, 59 adult *Dermacentor variabilis*, and one adult *Ambloymma americanum*. The indices of abundance of *I. scapularis* adults were highly variable within each establishment group, and were not significantly different among groups (P = 0.95), although the trend was for abundance to correlate positively with tick establishment status (26.6, 23.7, and 17.6 adults/1000m^2, at long-established, intermediate-established, and recently-invaded sites, respectively; Table 6.2).

Infection of ticks with multiple microorganisms. Five different microorganisms - *B. burgdorferi, B. miyamotoi, A. phagocytophilum, Ba. microti,* and *Ba. odocoilei* - were found among 1565 adult *I. scapularis* from across the Midwest, with overall percent prevalences of 51.3, 2.2, 8.9, 0.3, and 4.5, respectively (Figure 6.2; Table 6.2). There was no difference in infection between females and males for any microbe except *B. miyamotoi* (females, 1.3%; males, 2.9%; P = 0.03) and *A. phagocytophilum* (females, 7.1%; males, 10.8%; P = 0.01). All microbes except *Ba. microti* were found in ticks of all three establishment groups; *Ba. microti* was found in only two long-established populations. Infection prevalence for all microbes and co-infections except *Ba. odocoilei* trended highest in the long-established ticks, though prevalence was only significantly higher in long-established ticks for *A. phagocytophilum* (11.4% versus 6.4 and 3.7% for intermediate-established and recently-invaded ticks, respectively; P = 0.004; Table 6.2). *Ba. odocoilei* infection prevalences trended highest in the recently-invaded ticks (7.0%; versus 2.9 and 3.7% for long-established and intermediate-established ticks; P = 0.35).

The IGS of all 34 *B. miyamotoi*-positive samples was sequenced, and all samples were 100% homologous to 'Type 4' North American *B. miyamotoi*.

Overall, 8.9% of all ticks collected in the Midwest were co-infected with two or more microbes (Table 6.2; Figure 6.2). Male ticks had a higher prevalence of co-infections (11.2%) than females (6.9%; P = 0.003). Co-infections were found in all three establishment groups and were most prevalent among long-established ticks (10.4%; P = 0.03) as compared to intermediate-established (6.7%) and recently-invaded ticks (6.4%; Table 6.3). Across all data, the most common co-infection resulted from *B. burgdorferi* and *A. phagocytophilum* which occurred in 90 ticks (5.8% of all ticks); this rate of co-infection is 1.3 times higher than the expectation given the prevalence of each microbe within the population (χ^2 = 11.03; P = 0.0009). Other co-infections that were observed in a frequency that is significantly different than expected included *B. burgdorferi* and *B. miyamotoi* (0.05% of all ticks; 2.1 times lower than expected; χ^2 = 9.89; P = 0.001) and *B. miyamotoi* and *Ba. odocoilei* (0.3% of all ticks; 2.7 times higher than expected; χ^2 = 4.62; P = 0.03). The co-infections that were observed at an expected frequency included *B. burgdorferi* and *Ba. microti* (0.1% of all ticks; χ^2 = 0.26; P = 0.61); *B. burgdorferi* and *Ba. odocoilei* (2.4% of all ticks; χ^2 = 0.26; P = 0.61); *B. miyamotoi* and *A. phagocytophilum* (0.3% of all ticks; χ^2 = 0.44; P = 0.51); *A. phagocytophilum* and *Ba. microti* (0.06% of all ticks; χ^2 = 0.77; P = 0.38); and *A. phagocytophilum* and *Ba. odocoilei* (0.3% of all ticks; χ^2 = 0.91; P = 0.34). Additionally, infection of ticks with three microbes was observed 3 times, and a single tick was infected with four microbes. All of these co-infections of 3 or more microbes occurred in ticks collected at long-established sites.

***B. burgdorferi* genotypes.** The IGS of 458 *B. burgdorferi*-positive samples (57.0% of all positives) was sequenced, which included 62.0, 52.7, and 44.7% of PCR-positive samples from the long-established, intermediate-established, and recently-invaded ticks, respectively. Across all sites, 21 (4.6%) had evidence of mixed-strain infections, based on the presence of double-nucleotide peaks in the chromatograph at polymorphic sites as defined by Bunikis et al. (2004a). The highest proportion of mixed strain infections was found among long-established ticks (5.1%), followed by intermediate-established (3.9%) and recently-invaded (2.9%; $P = 0.35$); mixed strain infections were present at all sites harboring long-established ticks and intermediate-established ticks, but were only present in one of the three sites harboring recently-invaded ticks (Table 6.4).

***B. burgdorferi* RST groups.** Among the 437 single strain infections, all three RST groups were represented, with RST 3 in highest abundance (58.3%) followed by RST 2 (35.5%) and RST 1 (6.2%). Strains of RST 1 were least abundant at all sites (Figure 6.3), and were found at 3 of 5 long-established sites, both intermediate-established sites, and two of three recently-invaded sites.

***B. burgdorferi* strain richness and diversity.** We detected a total of 22 IGS strains within 437 *B. burgdorferi* sequences across the Midwest. These 22 strain types included 8 prototypical IGS strains previously described by Bunikis et al. (2004a) from the Northeastern United States (IGS 5, 1A, 2A/C, 2D, 4A, 6A, 7A/B, and 8A/C), 4 strains that we have recently detected in bird-associated ticks (Midwest A, B, F, and K; Hamer et al. unpublished) and 10 novel IGS variants that differed from published strains by at least

one nucleotide (Midwest C, D, E, G, H, I, J, L, M, and N; accession numbers HM015238-HM015247). Observed *B. burgdorferi* strain richness was highest within ticks of the long-established group (19 strains), followed by intermediate-established (12 strains) and recently-invaded (10 strains), though this relationship is likely influenced by sample size (297, 74, and 66 sequences determined in the three groups, respectively; Table 6.4). Using a rarefaction analysis, we determined the rate at which new strains are found per unit of individuals sequenced (Figure 6.4). At a common sample size of at least 50, the long-established group is more rich than the intermediate-established group, which is more rich than the recently-invaded group. In a separate analysis, the Chao-1 non-parametric estimator of true species richness using data across all sites in the Midwest is 46.5 ± 19.1. Richness is predicted to be greatest at long-established sites (27 +/- 8.3) followed by intermediate-established sites (14.3 +/- 2.8), and lowest at recently-invaded sites (11 +/- 1.6). The percent of samples that comprised indigenous strains (found in a single establishment group only) was greatest among long-established ticks (11.8%) as compared to intermediate-established (2.7%) and recently-invaded (1.5%; $\chi^2 = 11.9$, $df = 2$, $P = 0.003$; Figure 6.5).

Using a standard ecological index, we found that strain evenness among all three establishment groups was comparable (0.83-0.85). Shannon diversity was greatest among the long-established ticks (2.45), followed by intermediate-established (2.06) and recently-invaded (1.95). The Sorenson's Similarity Indices computed for pair wise comparisons of establishment groups indicated that the intermediate-established versus recently-invaded strain diversities were most similar (0.73), the long-established versus

recently-invaded was least similar (0.62), and long-established versus intermediate-established was in-between (0.65).

Of the 500 nucleotides of the IGS that were assessed, the total number of polymorphic sites across all sites was 42, including one indel block of 7 nucleotides that was treated as a single polymorphism. Polymorphic sites were most common among long-established samples (n = 40 polymorphic sites), followed by intermediate-established (n = 36) and recently-invaded (n = 34). No significant fragments were found using Sawyer's test, which indicates that there is no evidence for recombination at this locus. The molecular diversity index Theta_k was greatest in long-established sites and smallest in recently-invaded sites, yet differences were not significant (Table 6.4). The mismatch distribution was a significant fit to the model of sudden expansion for the recently-invaded population (Harpending's raggedness index = 0.063; P = 0.02), marginally significant for the intermediate-established population (Harpending's raggedness index = 0.059; P = 0.05), and non-significant for the long-established population (Harpending's raggedness index = 0.016; P = 0.41).

***B. burgdorferi* population structure.** A total of 99.11% of IGS molecular variation occurred within establishment groups, and 0.89% occurred among establishment groups. Marginally significant differences were found in the identity and frequency of IGS haplotypes among the three establishment groups (F_{ST} 0.0089; P = 0.06), indicating that the majority of haplotypes are present in all three groups and that high genetic diversity is maintained across the Midwest. Population pairwise F_{ST} analysis indicates that IGS haplotype frequencies of long-established and intermediate-established populations were

not different (P = 0.32), whereas differences were significant between long-established and recently-invaded populations (P = 0.07) and intermediate-established and recently-invaded populations (P = 0.03). These differences in population structure are driven by the differences in relative abundance of certain strains: most notably, IGS 8A/C comprised 14.1 and 29.7% of long-established and intermediate-established strains, respectively, yet was not present in the recently-invaded population. IGS 6A comprised 18.2% of the recently-invaded population, but only 8.1 and 2.7% of long-established and intermediate-established populations (Figure 6.5).

***B. burgdorferi* phylogeny and network analysis.** The topology of the IGS phylogenetic tree (Figure 6.5) – with broad grouping into three RST groups of which RST 3 is paraphyletic, and further delineation of *B. burgdorferi* into about a dozen intraspecific lineages – is well described (Bunikis et al. 2004a; Attie et al. 2007). Some of the tree topology is star-shaped with polytomies that occurred only at sequences that differ by one or two nucleotides. Midwest A was the most common strain across the Midwest and was the only strain to be found at all sites; it constituted 19.5, 13.5, and 21.2% of samples from long-established, intermediate-established, and recently-invaded ticks, respectively. Seven strains were singletons within our sampled population (IGS 2A/C, Midwest E, I, J, L, M, and N), and the percent of all strains that were singletons in each establishment group did not differ significantly (21.1, 25.0, and 20% in long-established, intermediate-established, and recently-invaded ticks; P = 0.95). Strains shared by more than one establishment group were the most abundant within the sample, with eight ubiquitous strains found in all groups and three strains found in two groups. Of the 11 strains that

were only found within one establishment group, the majority (72.7%) occurred in long-established ticks, whereas two such strains occurred in intermediate-established ticks, and a single such strain occurred in recently-invaded ticks. The unique IGS strains from each establishment group are inter-digitated with those of the other establishment groups as well as with ubiquitous strains, suggesting a recent shared history. Within our sampled population, the maximal number of mutational steps separating a strain from the next most homologous strain was three (Figure 6.6). There was no apparent geographical structure in the relationships among the haplotypes; with the exception of the unlinked RST 1 strains, the network exhibited some star-like topology with low levels of sequence divergence and a high frequency of unique mutations.

Discussion

There have been many criteria proposed for establishing the center of origin of a taxon, none of which independently is useful, but together may provide a framework for assessing diversity within and among species in relation to establishment and invasion (Cain 1944). These criteria were originally developed in the study of plant geography, but some of the more frequently used criteria (as listed in (Crisci et al. 2003) and detailed below) have direct application to better understanding the establishment of the *I. scapularis*-borne disease systems. Below we recapitulate our data on relative tick abundance, interspecific microbe diversity, and intraspecific microbe diversity across a continuum of reported *I. scapularis* establishment in the Midwest and conclude that the long-established populations of *I. scapularis* serve as a center of origin for the Midwestern *I. scapularis*-borne disease system. The observed gradients of relative

diversity are, however, quite shallow, and mechanisms accounting for these patterns are speculated below.

1. The location of the area of greatest dominance and density of distribution. At the tick-level, a gradient of was apparent in the indices of abundance of *I. scapularis* in which the highest tick abundances occurred within the most long-established populations, similar to the findings of Walk et al. (2009) in New Hampshire. Similarly, in a standardized survey for *I. scapularis* nymphs across their distributional range, Duik-Wasser et al. (2006) found that within the Midwestern sampling sites, highest densities were in Minnesota and Wisconsin, at densities comparable to those in Northeastern endemic areas. While the overall trend in our dataset was for highest tick abundance in longer-established populations, variation was apparent; most notably, the highest index of abundance of adults found in the study was at Van Buren State Park, which was invaded in the early 2000s (Foster 2004). We conducted a longitudinal study of questing times at Van Buren, and found that adult activity is greater in the Spring than it is in the Fall (unpublished data), which is consistent with other findings in the Midwest (Strand et al. 1992; Stancil 1999; Jones and Kitron 2000), but different than the characteristic phenology in the Northeast in which adults are more active in the Fall (Fish 1993).

At the microbe-level, the prevalence of four of the five microbes we detected was greatest in highly-established ticks compared to intermediate-established and recently-invaded ticks (though statistical difference was only detected for *A. phagocytophilum*). At highly-established sites, the infection prevalences we report for *B. burgdorferi* (52.1%), *A. phagocytophilum* (11.2%), and *B. microti* (0.5%) are similar to those reported by Steiner et al. (2008) from three sites across the Midwest, except the latter report no *B.*

microti. In two different Lyme disease-endemic areas in New Jersey in the Northeast, Varde et al. (1998) report 43, 17, and 5% infection with these three agents, and Adelson et al. (2004) report 33.6, 1.9, and 8.4% infection with these agents, indicating variability in infection within endemic foci. While all three of these pathogens share wildlife reservoirs (white-footed mouse) and a tick vector (*Ixodes scapularis*), the degree to which other reservoirs and vectors are involved in transmission, and the efficiency of the *I. scapularis* transmission cycles, may influence the infection prevalence we observed.

Walk et al. (2009) and Hamer et al. (2007) found evidence that *B. burgdorferi* infection prevalence is notably higher in Lyme disease endemic areas. Using a larger dataset over a broader spatial and temporal scale, we found that newly-invaded populations had nearly equivalent *B. burgdorferi* prevalence prevalences to long-established populations, which suggests that there is not a long time lag between tick invasion and build-up of high density pathogen prevalence within these populations. This observation may result from a simultaneous dual-invasion process of both the tick and the pathogen (Hamer et al. 2010), or from the existence of cryptic pathogen maintenance cycles, in which certain hosts across the landscape are infected prior to the arrival of the bridging vector *I. scapularis* (Chapter 5). Also, it may be that very soon after ticks invade an area, infection prevalence may be low, but once tick densities build and more time has passed, prevalence builds quickly.

In addition to detecting the three zoonotic pathogens of main interest, our sampling protocols also allowed for simultaneous detection of two tick-borne microbes not known to be of epidemiological importance: *B. miyamotoi* and *Ba. odocoilei*. *B. miyamotoi* was originally described in *I. persulcatus* ticks in Japan (Fukunaga et al. 1995)

and was first detected in North America in *I. scapularis* ticks (Scoles et al. 2004). These spirochetes group genetically with the spirochetes responsible for relapsing fever diseases in humans, are transovarially-transmitted, and rodents serve as a reservoir (Barbour et al. 2009). Across our samples from the Midwest, only one strain of *B. miyamotoi* was present, which was the only strain reported among 22 samples from Connecticut (Bunikis et al. 2004b)and this was the same strain that was identified among 22 field samples in Connecticut (*Ba. odocoilei* is an intraerythrocytic protozoan parasite associated with white-tailed deer and other cervids, and is genetically similar to *Ba. divergans*, the causative agent of babesiosis in cattle, and a zoonotic *Babesia* species (Herwaldt et al. 2003). This parasite has a northcentral and northeastern distribution in the United States (Steiner et al. 2006) as well as in Texas, Oklahoma, and Virginia (Waldrup et al. 1990). Its widespread infection in human-biting ticks, combined with its genetic relatedness to the human-infectious *Babesia* species, implies that it may cause disease in immune-compromised people (Armstrong et al. 1998). The ubiquity with which we found these microbes across the Midwestern *I. scapularis* range (*B. miyamotoi*, 6 of 10 sites; *Ba. odocoilei*, 8 of 10 sites) underscores the importance of diagnostic assays that differentiate them from the genetically and morphologically similar agents that pose public health risk (i.e., *B. burgdorferi* and *Ba. microti*). The definitive diagnostic test for human babesiosis is identification of parasites on Giemsa-stained thin blood smear (Wormser et al. 2006), and the ability of *Ba. microti* to be specifically identified in the manner is unknown.

2. The location of the greatest variety of forms of the taxon. The number of microbial taxa was greatest in the highly-established tick populations, where all five microbes were

present (*B. burgdorferi, B. miyamotoi, A. phagocytophilum, Ba. microti, and Ba. odocoilei*). *Ba. microti* was not present in intermediate-established or recently-invaded ticks. Similarly, as demonstrated by the population-specific rarefaction curves (Fig. 4), *B. burgdorferi* strain richness was greatest in highly-established tick populations. Given a common sample size of 60 infected ticks in all groups, for example, statistically greater strain richness is found at highly-established sites (13.6±1.3) versus intermediate-established (11.4±0.7) and recently-invaded (9.4±0.4). Nevertheless, the diversity of IGS genotypes that we found within recently-invaded ticks was higher than we had anticipated given the shorter time available for colonization of these diverse strains. Similarly, Wang et al. (1999b) found that the *ospC* variation within a local population was almost as great as the variation of a similar-sized sample of the entire species. It may be that infected ticks are invading from areas of high pathogen prevalence, or that uninfected, invading ticks readily encounter infectious hosts in the areas into which the ticks are invaded – these hosts could be previously infected due to pathogen transmission from other tick species.

Diversifying selection may influence patterns of *B. burgdorferi* strain diversity through at least two mechanisms. First, host associations, facilitated by selective killing of strains by host-specific innate immune factors (Kurtenbach et al. 2002), may maintain diverse populations of Lyme disease spirochetes, at both the inter-species level (e.g. in Europe, *B. burgdorferi* and *B. afzelii* are mammal-associated, whereas *B. garinii* and *B. valaisiana* are bird-associated (Dubska et al. 2009)) and at the intraspecies level of *B. burgdorferi* (Brisson and Dykhuizen 2004). The magnitude of intraspecies host association is, however, debated (Hanincova et al. 2006). Secondly, the antigenic types

that have already infected a host cannot establish a subsequent infection in the same host because of the immune response. Therefore, rare antigenic types are more likely to infect previously-infected hosts, which leads to some hosts acquiring multiple strains and maintenance of high levels of variation at the population level (Wang et al. 1999b).

3. The greatest number of overlapping distributions. Co-infections - acquired from sequential feeding events on infected hosts or from a single feeding event of a co-infected host - are generally expected to occur in highest frequency in sites with well-established transmission as opposed to recently-colonized foci with many naïve hosts and a reduced diversity of strains due to invasion by one or a few founding ticks. Co-infections are epidemiologically important because human infection with multiple pathogens may lead to incomplete diagnosis and insufficient treatment (Swanson et al. 2006). At the inter-species level, we found that ticks co-infected with multiple organisms were indeed most common at long-established sites, where 10.4% of ticks harbored more than one microbe (compared with 6.7 and 6.4% at intermediate-established and recently-invaded sites, respectively). Across all our study groups, *B. burgdorferi-A. phagocytophilum* co-infections occurred more frequently than would be expected given the prevalence of each individual pathogen. Steiner et al. (2008) also found significant co-infection of these pathogens in adult *I. scapularis* from Ft. McCoy, one of our long-established sites.

At the intra-species level, mixed strain *B. burgdorferi* infections were negligibly most common in highly-established ticks, where 5% of *B. burgdorferi*-infected ticks harbored more than one IGS strain (compared with 4 and 3% in intermediate-established and recently-invaded ticks, respectively). There was significant variability in the

proportion of mixed-strain infection within each establishment group, however, including a maximum of 15% at Castle Rock, a highly-established site in Northwestern Illinois. The comparable mixed strain infection prevalence between recently-invaded and highly-established ticks is expected given the near-equivalent *B. burgdorferi* infection prevalences, and suggests that the degree of interaction among infected ticks and hosts is a newly invaded region is high. In comparison, Gatewood et al. (2009) report an overall prevalence of mixed strain infections at the IGS locus of approximately 20% in nymphs from across the range of *I. scapularis* establishment, including sites in both the Northeast and Midwest, with no apparent geographic bias. The comparatively lower prevalence of mixed strain infections in our study, especially in consideration that adults are likely to have more mixed strains given the additional bloodmeal they have had in comparison to nymphs, may be an artifact of the truncated region of the IGS that we analyzed (i.e., signals of mixing at polymorphic sites could have occurred in a region of the IGS that we did not assess), or mixed strain infections may more commonly produce poor quality sequences that we were not able to assess. Using the *ospA* and *ospC* gene targets, Wang et al. (1999b) found 45 and 50% mixed-strain infections in adult *I. scapularis* from the endemic area of Shelter Island, NY (the sites from which *B. burgdorferi* was first isolated), and Guttman et al. (1996) found 60% of adults from the same areas harbored at least two strains of *B. burgdorferi*. IGS appears not to be as sensitive as *ospC* in detecting multiple strains, and therefore our report of mixed strain prevalence should be considered a minimum, and as long as there is no facilitation or exclusion of particular strains, the bias should be the same across sites.

4. The location of the most primitive form. In regard to *B. burgdorferi* genotypes, the RST 1 group is ancestral to RST 2 and 3 (Bunikis et al. 2004a). While strain richness – one measure of genetic diversity – is less in RST 1 than in RST 2 and 3, the nucleotide diversity (i.e., the number of nucleotide difference between strains) in RST 1 is greater than RST 2 and 3 (Bunikis et al. 2004a). A low density of RST 1 strains was present within all three establishment groups, the proportion of which did not relate to tick establishment status (5.4, 8.1, and 7.6% in highly-established, intermediate-established, and recently-invaded populations, respectively). The presence of more than one RST 1 strain, however, was found only within highly-established ticks. Across the Midwest, RST 1 was only represented in 6.2% of infections. Furthermore, the RST 1 had less strain richness (comprised of only 2 strains) as compared to RST 2 and 3 (with a total of 20 strains). Conversely, in a study of *I. scapularis* nymphal infection across its distributional range including both the Midwestern and Northeastern United States, Gatewood et al. (2009) found that RST 1 strains comprised 20.8% of all infected nymphs. The apparent absence of RST 1 at two long-established sites and one recently-invaded site combined with the overall low prevalence of this group in the Midwest may reflect different mechanisms involved in maintaining Midwestern versus Northeastern *B. burgdorferi*.

5. The identification of continuity and directness of individual variations or modifications radiating from a 'centre of origin' along the highways of dispersal. Assessment of the structure of the genetic data could allow for speculation as to the mode or directionality of tick and pathogen dispersal, assuming a center of origin. For

example, if the long-established I. scapularis populations were the most dense, contained the highest prevalence of infection with multiple pathogens and coinfections, and the highest diversity of *B. burgdorferi* strains, and if the intermediate-established and recently-invaded tick populations were characterized as nested subsets of these parameters, this pattern may suggest a linear mode of invasion, with length of establishment a useful predictor of the parameters. Conversely, if recently-invaded ticks were equally or more abundant and infected with diverse pathogens, then a directionality of dispersal from established foci would not be concluded. Across the Midwest, we found marginally significant global population structure ($P = 0.06$). While the majority of strain types found in any establishment group were shared among all three groups, suggesting high rates of gene flow, the presence of indigenous strains within each establishment group, however, suggests some isolation by distance and an emerging evolutionary divergence. Over 99% of the genetic variation that was observed was due to within group differences.

In our dataset, the IGS haplotype frequencies of the recently-established population were significantly different than both the highly-established and intermediate-established populations. The network analysis, exhibited by some star-like topology with low levels of sequence divergence and a high frequency of unique mutations, is indicative of a rapid population expansion, in the absence of selection. Similarly, analysis of mismatch distributions suggests that a recent demographic expansion has occurred in the recently-invaded population ($P = 0.02$) and intermediate-established population ($P = 0.05$), with no evidence for recent expansion in the long-established population ($P = 0.41$). The trend in the molecular diversity index Theta_k was that long-established sites

were the most diverse, and recently-invaded sites were least diverse, but this difference was not significant.

The diversity gradient that we hypothesized was in fact quite shallow, as much of our data show that recently-invaded sites harbor equally or only slightly less diverse assemblages of *B. burgdorferi* than long-established sites. Speculation as to the mechanisms that may account for the observed pattern may include the following: (i) There is a high propagule pressure associated with invasion, not only of the tick, but also of the pathogen, which may invade new sites in diverse wildlife reservoirs or in alternative tick species. With many independent introduction events, of which each may constitute introduction of a different strains, founder effects are not apparent. (ii) Even if a limited number of strains are introduced to a new area, in this multi-host, complex system, so many different reservoir species each present different selective pressures, which leads to diversity. (iii) The time scale for build up of diverse populations may be rapid (given our evidence for rapid demographic expansion) such that even if initial invading diversity is low, our sampling occurs too late to appreciate this. (iv) A pre-existing cryptic cycle of the pathogen may be maintained in advance of the invasion front of *I. scapularis*, such that *I. scapularis* may encounter wildlife infected with diverse strains upon its own invasion.

Epidemiological significance. Two main genetic typing schemes are useful in assessing pathogenicity of different *B. burgdorferi* strains in humans: RST (a tripartite classification of the 25+ IGS strains) and outer surface protein C (*ospC*; an antigenic plasmid-borne gene); *ospC* is in positive linkage disequilibrium with IGS (Bunikis et al.

2004a). Some RST 1 strains are associated with a higher frequency of disseminated infection in humans and more invasive disease in experimental animals (Seinost et al. 1999; Wang et al. 2002; Derdakova et al. 2004; Hanincova et al. 2008; Wormser et al. 2008a), and a bias toward the relatively less invasive RST 2 and 3 strains has been found among infected *I. scapularis* in the Midwest (Gatewood et al. 2009). While our data similarly demonstrate a strong bias toward RST 2 and 3 infections at our Midwestern sites, RST 1 strains (IGS 1A and 'Midwest L') were found within ticks of all establishment groups, including recently-invaded ticks, which underscores the rapidity with which newly detected tick populations may pose a public health risk.

The pathogenicity in humans of the large number of novel IGS mutant strains is unknown, though we have no *a priori* reason to believe that single or double nucleotide polymorphisms within the non-coding intergenic spacer region would necessarily correspond to a change in virulence. To begin to assess the epidemiological risk associated with novel strains found within this cryptic cycle, we sent total DNA from 88 *B. burgdorferi* samples with comprising five IGS strains that were not previously reported in the literature or in Genbank (Midwest A, E, J, K, and M) to a laboratory at the University of California-Irvine for direct *ospC* typing (B. Travinsky and A. Barbour, unpublished data). Of these, *ospC* was successfully amplified and sequenced from 87 samples, resulting in detection of 48 single strain infections and 39 mixed-strain infections. Present in single strain infections included 18 *ospC* types, including representatives of the four *ospC* major groups that have been associated with disseminated human Lyme disease (Seinost et al. 1999). The degree to which mixed strain infections were present at the *ospC* locus within these samples (which had no

evidence of mixing at the IGS locus) further underscores the complexity of the maintenance of pathogen diversity across the Midwest.

Data on genetic diversity of *B. burgdorferi* have practical implications for interpretation of serodiagnoses. The IGS marker that we have used to quantify diversity is linked to serodiagnostic antigens (including ospC), and variation has been found in the ability of various diagnostic protocols for detection of diverse strains, with C6 ELISA able to detect more strains than the standard two-tiered approach (Wormser et al. 2008b).

The confirmed cases of Lyme disease and human granulocytic anaplasmosis reflect the patterns we observed based on tick data. In 2007, the national incidence of Lyme disease was 9.2 cases/100,000 people (Hall-Baker et al. 2009), in which highly-established Wisconsin and Minnesota reported incidences of 32.4 and 23.8 cases/100,000 people, respectively. Incidence in Illinois (1.2 cases/100,000 people), Indiana (0.9 cases/100,000 people) and Michigan (0.5 cases/100,000 people; on average, >55% of reported cases in Michigan are from the Lyme-disease endemic hotspot in Menominee County; E. Foster, pers. comm.). The incidence of human granulocytic anaplasmosis reflects a similar trend, but of a lesser magnitude than *B. burgdorferi.* In 2007, the national anaplasmosis incidence was 0.3 cases/100,000 people (Hall-Baker et al. 2009), with Minnesota and Wisconsin reporting incidences of 6.2 and 1.2 cases/100,000 people, respectively. The only other Midwestern state from which we collected ticks that also reported cases was Illinois, with an incidence of 0.05 cases/100,000 people; no cases were reported from Indiana or Michigan.

Table 6.1. Best estimates of the relative dates of *Ixodes scapularis* population establishment at the sub-state level in selected Midwestern areas based on published literature. Field sites sampled in the current study are categorized based on their best estimate relative establishment.

Establishment status	State	Year of reported *Ixodes scapularis* establishment	Citations	Field sites of the current study
Long-established	Wisconsin, NW and central	1967	Jackson and Defoliart 1970	Governor Dodge, Fort McCoy
	Minnesota, E and central	1983	Loken et al. 1985; Drew et al. 1988	St. Croix
	Michigan, S Upper Peninsula	1986	Strand et al. 1992	Menominee North and South
	Illinois, NW	1987	Bouseman et al. 1990; Kitron et al. 1991	Castle Rock
Intermediate-established	Indiana, NW	1990	Pinger et al. 1991; Pinger et al. 1996	Indiana Dunes and Tippecanoe River
Recently-invaded	Michigan, SW Lower Peninsula	2003	Foster 2004; Hamer et al. 2007; 2010	Saugatuck, Duck Lake, and Van Buren
	Illinois, NE	2005	Jobe et al. 2006; Jobe et al. 2007	Chicago North and South

Table 6.2. Index of abundance and infection status of 1595 adult *I. scapularis* adults across levels of *I. scapularis* establishment in the Midwestern United States, 2006-2007. Significant differences among establishment groups are noted with superscript letters. UP = Upper Peninsula; LP = Lower Peninsula.

				I. scapularis adults		Infection prevalence (%)					
Status	Site	State	Meters Dragged	No.	Ticks per 1000m^2	*B. burgdorferi*	*B. miyamotoi*	*A. phago.*	*Ba. microti*	*Ba. odocoilei*	co-infections
Long-established	St. Croix State Forest	MN	5000	61	12.20	85.25	0	14.75	1.64	0	13.11
	Castle Rock State Park	IL	11437	185	16.18	46.49	2.16	8.65	0	5.41	8.65
	Governor Dodge State Park	WI	9400	281	29.89	58.72	2.14	9.96	0	6.41	13.52
	Fort McCoy	WI	8400	341	40.60	42.23	5.57	11.44	1.17	2.64	7.92
	Menominee North and South	MI UP	2200	102	46.36	56.86	0	16.67	0	0.98	11.76
	All		**36437**	**970**	**26.62**	**52.06**	**2.99**	**11.24**[a]	**0.52**	**3.92**	**10.41**[a]
Intermediate-established	Indiana Dunes National Lakesho	IN	8400	76	9.05	38.16	1.32	3.95	0	0	2.63
	Tippecanoe River State Park	IN	4160	221	53.13	52.94	1.36	7.24	0	4.98	8.14
	All		**12560**	**297**	**23.65**	**49.16**	**1.35**	**6.40**[b]	**0.00**	**3.70**	**6.73**
Recently-invaded	Saugatuck and Duck Lake	MI LP	8490	36	4.24	27.78	0	0	0	2.78	2.78
	Van Buren State Park	MI LP	3000	194	64.67	54.12	0.52	4.64	0	8.25	6.70
	Chicago North and South	IL	5400	68	12.59	54.41	0	2.94	0	5.88	7.35
	All		**16890**	**298**	**17.64**	**51.01**	**0.34**	**3.69**[b]	**0.00**	**7.05**	**6.38**[b]

Table 6.3. Matrices of co-infection for each *I. scapularis* establishment group. Infection prevalence (%) with each individual microbe is on the diagonal in bold. Expected co-infection prevalence is in the upper triangle, and observed co-infection prevalence is in the lower triangle. Observed prevalences are not different than expected unless indicated with an asterisk. Bb = *B. burgdorferi*; Bm = *B. miyamotoi*; Ap = *A. phagocytophilum*; Bam = *Ba. microti*; Bao = *Ba. odocoilei*.

Long-established (N = 970)

	Bb	Bm	Ap	Bam	Bao
Bb	**52.1**	1.6	5.9	0.3	2.0
Bm	0.9*	**2.9**	0.3	0.02	0.1
Ap	7.1*	0.3	**11.4**	0.06	0.4
Bam	0.2	0	0.1	**0.5**	0.02
Bao	2.2	4.1*	4.1	0	**3.9**

*P = 0.005 - 0.021

Intermediate-established (N = 297)

	Bb	Bm	Ap	Bam	Bao
Bb	**49.2**	0.7	3.1	NA	1.8
Bm	0*	**1.3**	0.1	NA	0.05
Ap	4.7*	0.3	**6.4**	NA	0.2
Bam	NA	NA	NA	**0**	NA
Bao	1.7	0	0	NA	**3.7**

*P = 0.027 - 0.048

Recently-invaded (N = 298)

	Bb	Bm	Ap	Bam	Bao
Bb	**51**	0.2	1.9	NA	3.6
Bm	0	**0.3**	0.01	NA	0.02
Ap	2.3	0	**3.7**	NA	0.3
Bam	NA	NA	NA	**0**	NA
Bao	4	0	0	NA	**7**

Table 6.4. Mixed strain infections and strain richness of single strain *B. burgdorferi* infection in 458 infected adult *I. scapularis* adults across a continuum of *I. scapularis* establishment in the Midwestern united States, 2006-2007. UP = Upper Peninsula; LP = Lower Peninsula.

Status	Site	State	No. sequences	Proportion mixed-strain	No. single-strain sequences	Strain richness	Theta_k
Long-established	St. Croix State Forest	MN	47	0.02	46	10	
	Castle Rock State Park	IL	48	0.15	41	10	
	Governor Dodge State Park	WI	73	0.03	71	15	
	Fort McCoy	WI	94	0.05	89	14	
	Menominee North and South	MI UP	51	0.02	50	9	
	All		**313**	**0.05**	**297**	**19**	**4.37 (2.64-6.97)**
Intermediate-established	Indiana Dunes	IN	19	0.05	18	7	
	Tippecanoe River State Park	IN	58	0.03	56	9	
	All		**77**	**0.04**	**74**	**12**	**3.81 (1.98-7.03)**
Recently-invaded	Saugatuck and Duck Lake	MI LP	5	0.00	5	4	
	Van Buren State Park	MI LP	52	0.04	50	9	
	Chicago North and South	IL	11	0.00	11	4	
	All		**68**	**0.03**	**66**	**10**	**3.04 (1.49-5.89)**

Figure 6.1a. Location of field sites for collection of *I. scapularis* across the Midwestern United States, spring 2006-2007. Sites are categorized by establishment status of documented *I. scapularis* populations: highly-established (red circle); intermediate-established (blue square); recently-invaded (green triangle).

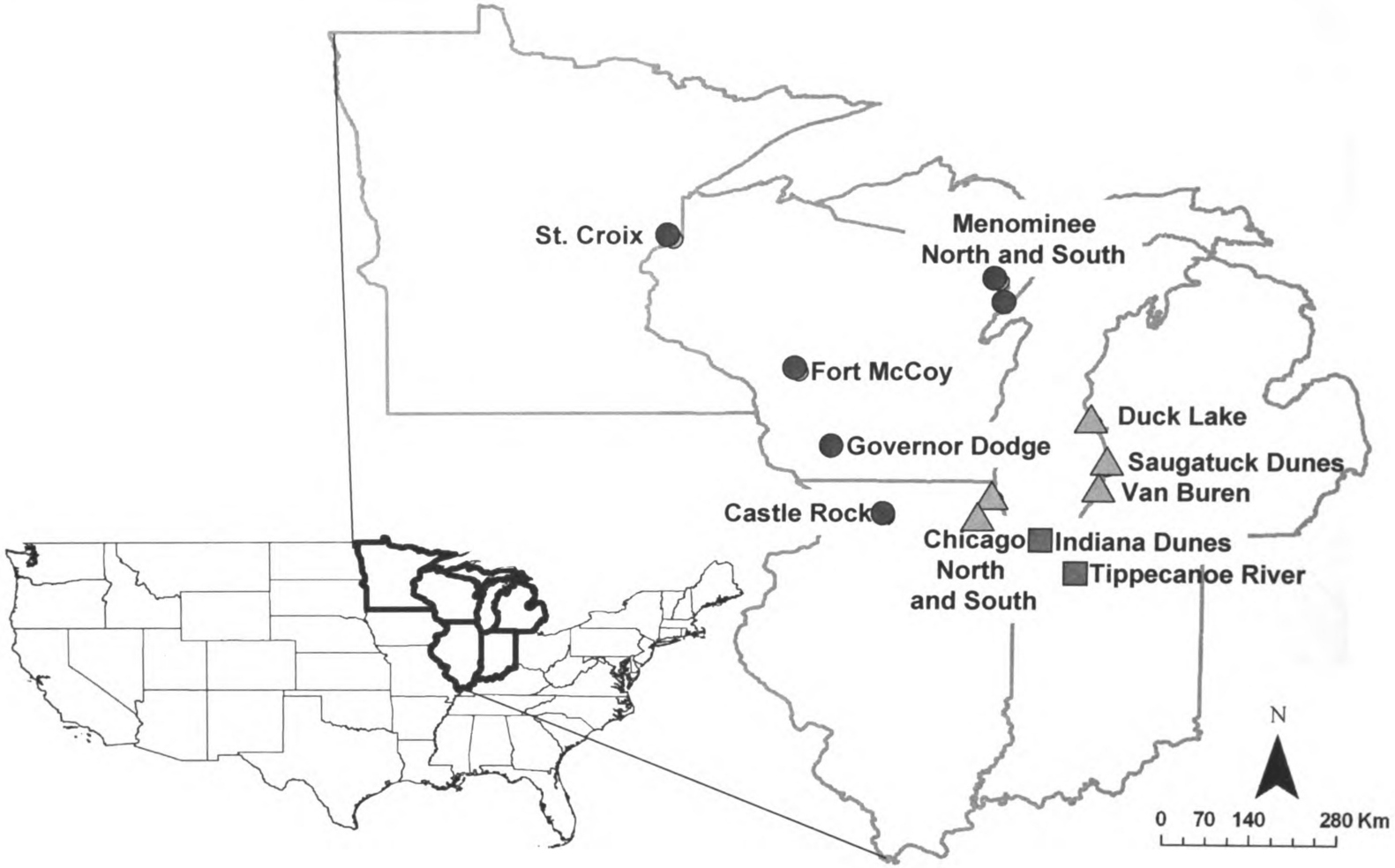


Figure 6.1b. Adult female *I. scapularis* questing on forest understory vegetation.

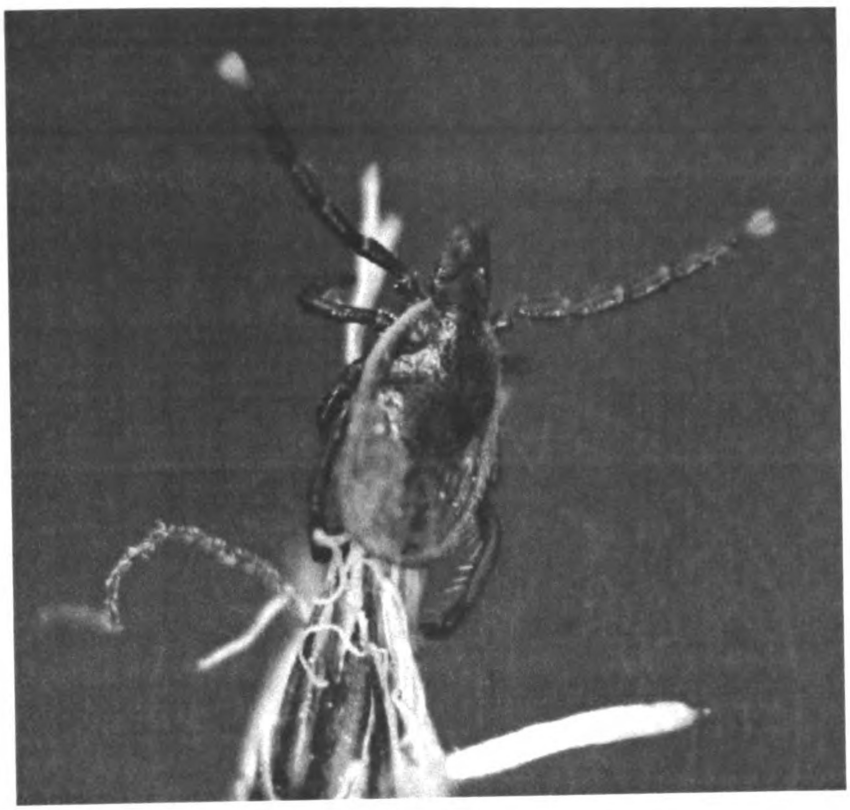


Figure 6.2. Infection of 1595 adult *I. scapularis* with *Borrelia spp., Anaplasma phagocytophilum*, and *Babesia* spp. organisms, and co-infections thereof, from across the Midwestern United States, 2006-2007. Error bars are the standard error of the mean prevalence of all sites within each establishment group.

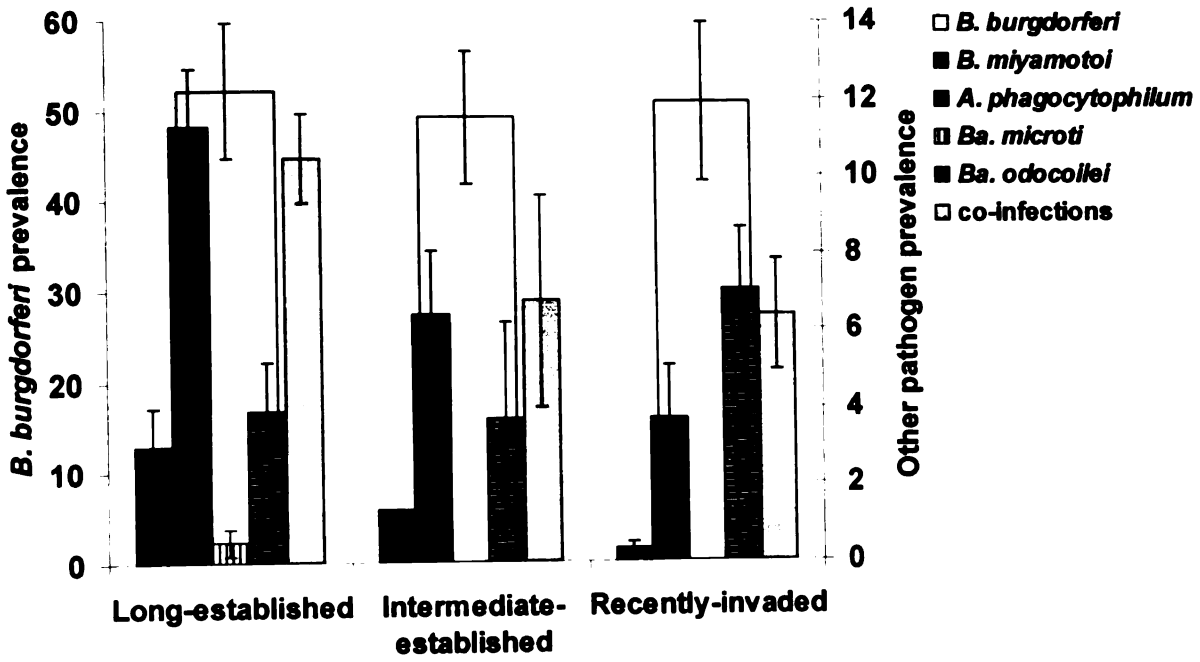


Figure 6.3. Variation in proportion of ribosomal spacer (RST) types 1, 2, and 3 of *B. burgdorferi* across the Midwestern United States, 2006-2007.

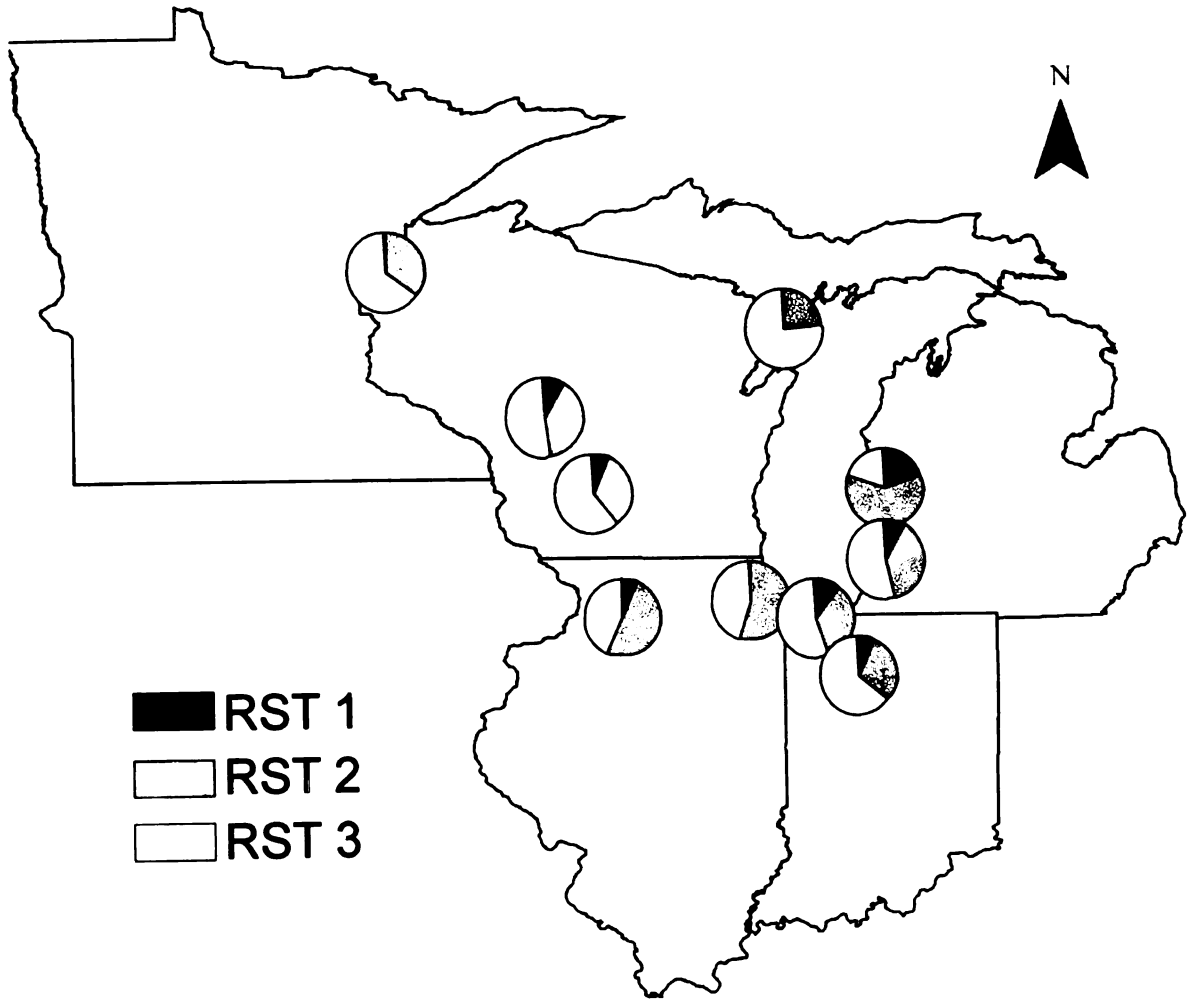


Figure 6.4. Rarefaction analysis of the influence of sample size of detected strain richness of *B. burgdorferi* within adult *I. scapularis* across the Midwestern United States, 2006-2007. Predicted mean strain richness (intervals are standard deviation) is modeled at incremental sampling for each tick establishment group until the observed datapoint is reached. The observed datapoint for each group is plotted as the final datapoint on each curve.

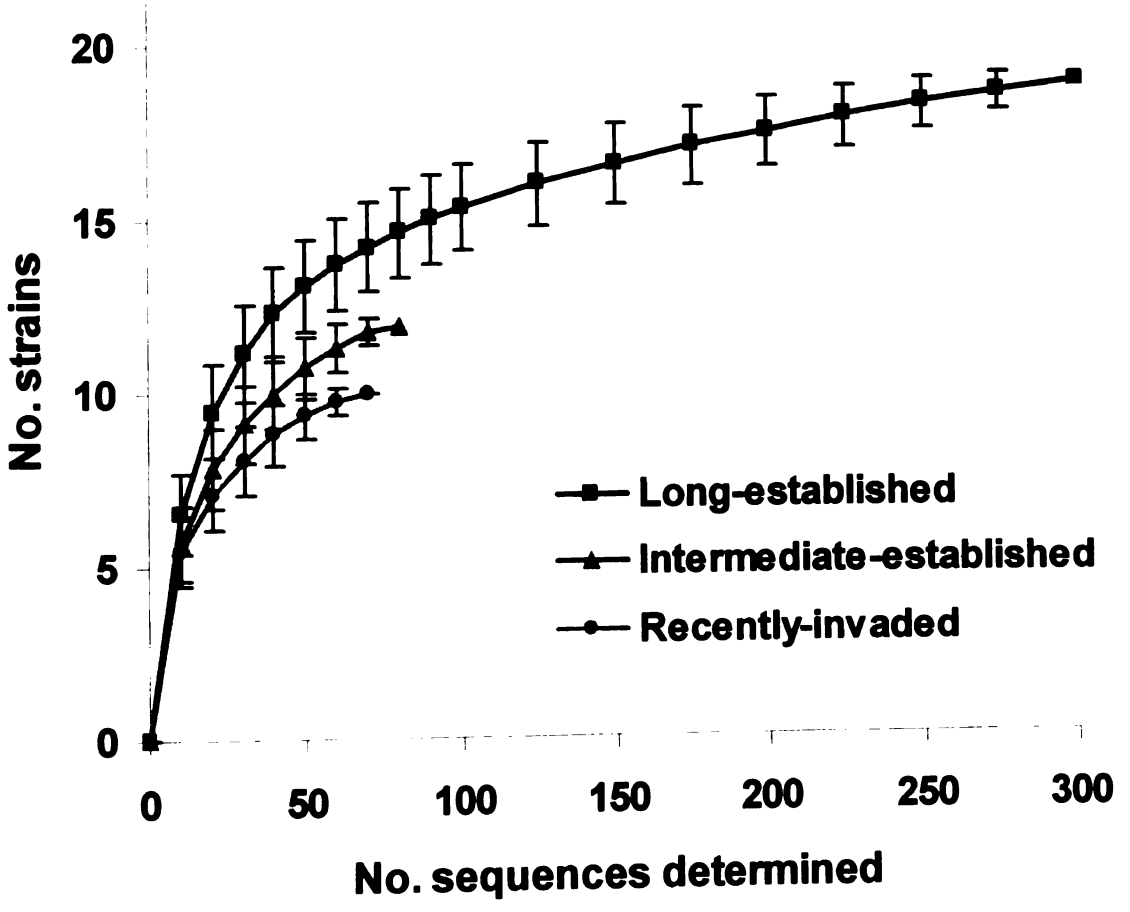
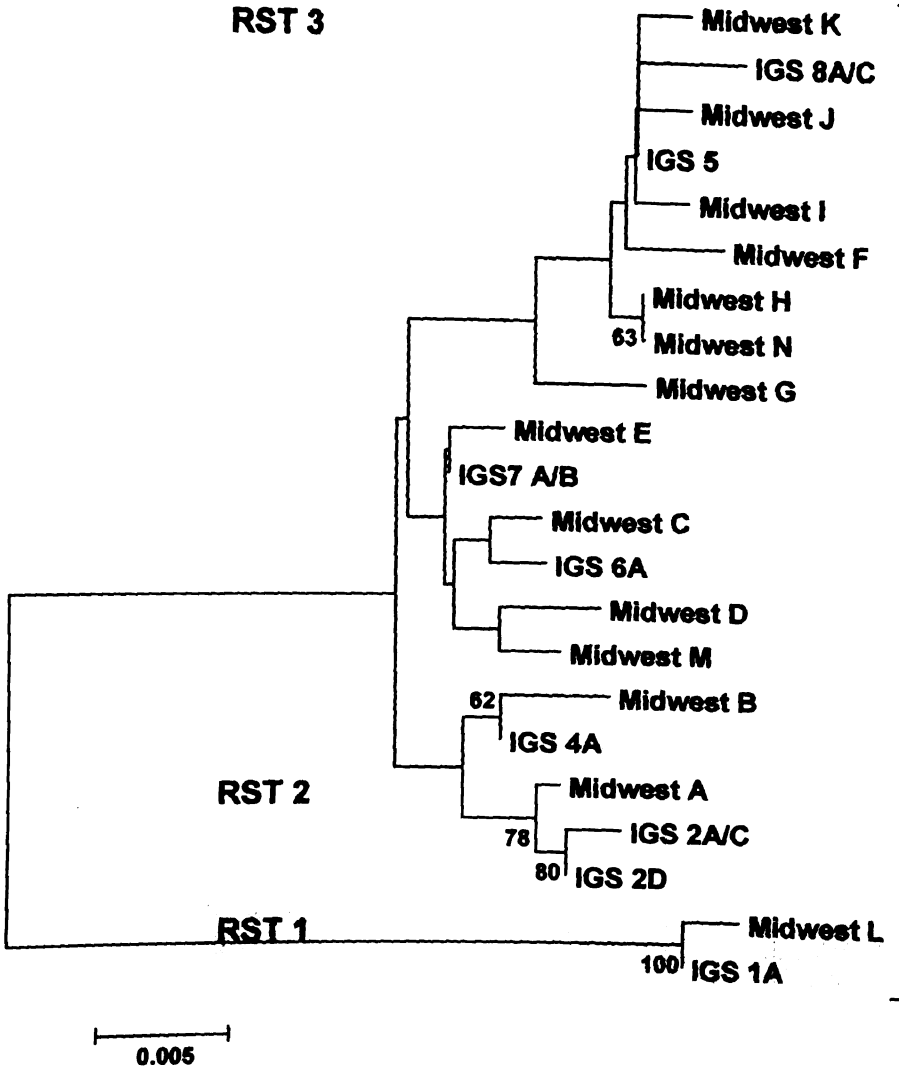
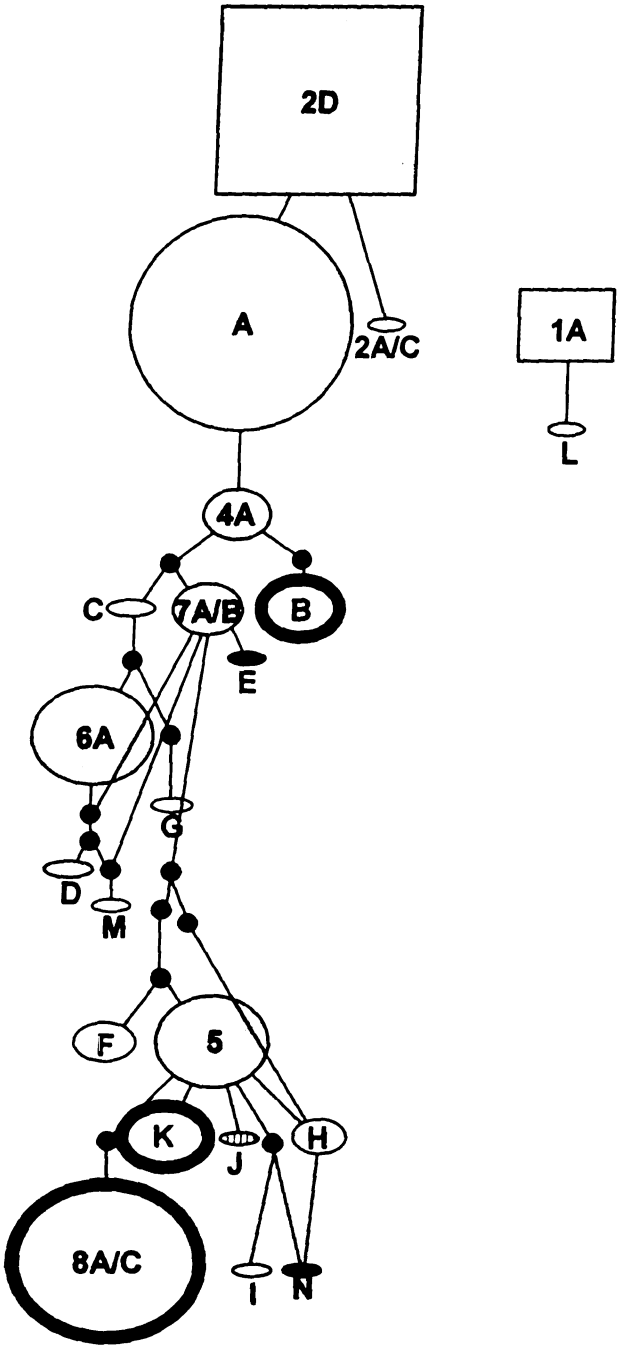


Figure 6.5. Unrooted neighbor-joining phylogram and frequency distribution of *B. burgdorferi* IGS haplotypes collected from across the Midwestern United States, 2006-2007. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches when 60 or above. Haplotypes previously reported in Bunikis et al. 2004 begin with 'IGS'; all strains beginning with 'Midwest' were not previously reported. Strains conforming to the 16S – 23S rRNA RST group 1, 2, and 3 designations by the criteria of Liveris et al. (1995) are demarcated by the labels at the top left of shaded or unshaded groups. The representation of each strain is expressed as a percent within each establishment group.



Establishment of *I. scapularis*			
Long-established	Intermediate-established	Recently-invaded	All
6.4	4.1	0	**5.0**
14.1	29.7	0	**14.6**
0	0	1.5	**0.2**
7.1	2.7	18.2	**8.0**
0.3	0	0	**0.2**
4.4	0	0	**3.0**
3.7	0	0	**2.5**
0	1.4	0	**0.2**
0.7	0	0	**0.5**
0	1.4	0	**0.2**
3.7	5.4	4.5	**4.1**
1.7	0	0	**1.1**
8.1	2.7	18.2	**8.7**
1.0	1.4	1.5	**1.1**
0.3	0	0	**0.2**
6.1	0	3.0	**4.6**
1.7	13.5	3.0	**3.9**
19.5	13.5	21.2	**18.8**
0.3	0	0	**0.2**
15.5	16.2	21.2	**16.5**
0.3	0	0	**0.2**
5.1	8.1	7.6	**5.9**

Figure 6.6. Minimum spanning network (MSN) of *B. burgdorferi* IGS haplotypes collected from across the Midwestern United States, 2006-2007. Each black circular node connecting haplotypes represents one mutational change; a 7 base pair indel that occurs within the IGS is considered as one change. The size of each haplotype is proportional to its frequency within the sampled population. The rectangles represent the haplotypes with the highest outgroup weight, which correlates positively with haplotype age. The two haplotypes within RST 1 (IGS 1A and Midwest L) are linked to each other by one mutational change and are unlinked to the rest of the network due to a high degree of divergence. Strains are categorized as to their occurrence within each of the three *I. scapularis* establishment groups: highly-established only (white); intermediate-established only (black); recently-invaded only (striped); two groups (white with thick border); all three groups (gray).



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CHAPTER 7

SYNTHESIS AND FUTURE DIRECTIONS

This dissertation illustrates an emerging multi-host, multi-vectored transmission network involving a suite of pathogens that impact both human and veterinary health, and is a contribution to the emerging 'One Health' discipline which promotes a holistic approach for improving human and animal health (King et al. 2004). The emergence of Lyme disease in the United States in the 1970s, now the leading vector-borne disease of humans and also a significant cause of dog morbidity, has served as a model system for the study of vector-borne zoonoses ecology and control. To date, at least 14,414 research articles have been published with 'Lyme disease' as a topic (www.isiknowledge.com), starting with the original description of the disease within a cluster of children in Connecticut in June, 1977, in the journal Arthritis and Rheumatism (Steere et al. 1977). For a comparison, a search of the phrase 'West Nile virus' yielded 7,508 publications, over 83% of which occurred since the 1999 New York City outbreak.

This dissertation has contributed to our understanding of the Lyme disease system in its description of a local focus of tick invasion and establishment into a landscape that may already harbor the pathogen at a low-level. This unfolding scenario is analyzed in a broader context of endemicity of the system across the Midwest. In Chapter 1, I provide a review of the steps of species invasions and describe the recent molecular and geographic analytical tools and their importance for advancing the field of vector-borne zoonotic diseases through a review of four emerging diseases. In the rest of my dissertation, I elucidate the status of blacklegged ticks and their infection with multiple

pathogens in zones of endemicity as well as emergence, with a particular interest in the Lyme disease pathogen due to its significance in human health.

In Michigan, *Ixodes scapularis* is endemic in a focal area of the Upper Peninsula and is currently invading the southwestern Lower Peninsula. Using the long-standing ticks in Menominee County and the recently-detected ticks in southwestern Michigan (Foster 2004) as study populations, my early work (Chapter 2) demonstrated that recently-invaded adult ticks were infected with *Borrelia burgdorferi* (47.3%), *Anaplasma phagocytophilum* (1.1%), and *Babesia odocoilei* (4.4%), but at prevalences significantly less than that of the endemic population (60.7, 14.3, and 7.1%, respectively). This chapter emphasized the need for heightened awareness by human and veterinary health professionals for tick-borne infections, and also raised question as to the mechanisms and relative speed of tick versus pathogen invasion and establishment. This prompted a larger study across gradients of *I. scapularis* establishment in five states of the Midwestern United States (Chapter 6).

In Chapter 3, I demonstrate that an invasion of *I. scapularis* has occurred in lower Michigan using a five-year dataset (2004-2008). In comparing data collected from small and medium mammals, birds, and drag sampling, I found surveillance of white-footed mice to most clearly show tick colonization of new sites, as well as increases in tick density within sties over time. Specifically, along Michigan's west coast, I found high densities of ticks on mice in all years at Van Buren State Park, in the southwestern corner (where 75-100% of mice were infested each summer, with no change over time). At sites further north, lower yet significantly increasing infestations were found. For example, at Duck Lake State Park near Muskegon, mouse infestation was approximately 10% in the

first year of sampling, and reached a peak of approximately 75% as the study progressed. In comparison, I did not detect a pattern of invasion across an inland transect, where blacklegged ticks were found in very low densities and only in occasional years at some inland sites, with no appreciable build-up after first year of detection. Surprisingly, low-prevalence *B. burgdorferi* infection was detected in other tick species and in wildlife at inland sites, as well as at northern coastal sites in years prior to the arrival of *I. scapularis*. These infections suggest that cryptic *B. burgdorferi* transmission by other vector-competent tick species may be occurring in the absence of *I. scapularis*, and this sparked a detailed look for a vector that may be responsible for cryptic pathogen maintenance (Chapter 5). Other *Borrelia* spirochetes, including *B. miyamotoi* and *B. andersonii*, were present at a low prevalence within invading ticks and local wildlife.

With funding from Michigan State University College of Veterinary Medicine Companion Animal Fund, I carried out a side project to address emerging Lyme disease in relation to a non-wildlife host: the pet dog. Specifically, I asked whether surveillance for ticks or exposure to tick-borne pathogens in pet dogs can provide an early warning of an invading disease system. This project formed collaboration with 18 veterinary clinics that operated along the coastal and invasion transects used for wildlife sampling. I worked with veterinarians to acquire dog blood samples and ticks removed from dogs. In comparison to our simultaneous wildlife studies, I found that dog surveillance was insensitive to tick invasion. Only two of 353 dogs (0.6%), both from the southwestern tick focus, were exposed to *B. burgdorferi*. A small number of dogs over a broader spatial scale harbored *I. scapularis*, which raised questions not only about potential undocumented travel histories of these animals, but also about the existence of

populations of this tick outside the known invasion zone. While I concluded that veterinarians can contribute to our understanding of blacklegged tick distribution through opportunistic collections of ticks from dogs that visit their clinics, it seems that the use of anti-tick prophylaxis among dogs across the Lower Peninsula (average of 50 % of dogs are treated) is likely to reduce the utility of pet dogs for tick-borne disease surveillance.

The broader impact of this side project was the *Conference for Veterinary and Medical Practitioners on Emerging Lyme Disease in Michigan* that I organized along with those who co-wrote the grant with me. Held in November, 2005, participants included veterinarians that contributed samples to our study, human physicians, wildlife biologists, public health officials, university researchers, and others. This outreach opportunity provided information for medical practitioners about emerging disease risk, and provides an example of how we can use principles of the 'One Health' paradigm for dissemination of research results.

Given the hints that *B. burgdorferi* was present in the landscape in the apparent absence of *I. scapularis* (Chapter 3), I investigated in detail the existence of a cryptic cycle of pathogen maintenance at a focal site outside the southwestern Michigan invasion zone in Chapter 5. At this site, eleven percent of 19,631 wild birds of 105 species were found to harbor ticks from 2004-2007, and *B. burgdorferi* was present in all years at a low prevalence (3.5%) within ticks of two species removed from birds – *I. dentatus* and *H. leporispalustris*. The former species has been demonstrated in laboratory trials to be a competent vector for *B. burgdorferi*. I also detected infection in ticks and ear biopsies removed from rabbits at this site. Importantly, intensive small mammal trapping at this site indicated that no sampled white-footed mice or eastern chipmunks harbored *I.*

scapularis, when simultaneous trapping at the southwestern Michigan hotspot indicated that 73% of small mammals were infested. This study represents the first time that significant levels of *B. burgdorferi* have been found concurrently in birds, rabbits, and their specialist ticks in the apparent absence of *I. scapularis*.

Bird-associated *B. burgdorferi* comprised 25 unique rDNA intergenic spacer strains, the majority of which have not been previously reported from the Lyme disease endemic northeastern or Midwestern US. At least three strains in cryptic transmission have previously been associated with disseminated Lyme disease in humans. If *I. scapularis* invades such zones of cryptic pathogen transmission, it is possible that novel *B. burgdorferi* strains may be introduced to human and canines. Furthermore, cryptic cycling may reduce the time lag between *I. scapularis* invasion and the build-up of infection prevalence, this accelerating disease risk.

In my final data chapter, Chapter 6, I tested the center of origin theory in relation to the establishment of *I. scapularis* in the Midwest and its infection with diverse species and strains of microorganisms. Through analysis of 1565 adult *I. scapularis* ticks from 13 sites across five Midwestern states, I found that tick infection prevalence with multiple microbial agents, co-infections, and strain diversity of *B. burgdorferi* were all positively correlated with the duration of establishment of tick populations. The interpretation of strain diversity was, however, hindered by a positive relationship between number of strains identified and sample size. Furthermore, while lower than in areas of tick establishment, a significant level of diversity of *B. burgdorferi* was present among recently-invaded ticks, suggesting that factors other than time since establishment of the tick population are responsible for explaining pathogen diversity. I conclude that the

invasion of ticks and emergence of various tick-borne diseases may reflect processes that are more complex than the scenario whereby infected, invading ticks are the only means of introduction of pathogens to naïve host communities.

Evidence for cryptic cycles

The mechanisms by which vector-borne zoonoses may be transmitted are not exclusive to vector-borne transmission. For example, transmission of West Nile virus, typically mosquito-borne, has been demonstrated through organ transplantation (Iwamoto et al. 2003) and blood donation (Pealer et al. 2003), the latter which lead to WNV nucleic acid amplification tests for screening of all donations. Furthermore, mother-to-baby transmission though breast milk cannot be ruled out (Hinckley et al. 2007). While the main mechanism of transmission of *Leishmania* species is through the bite of a sand fly, which serves as a biological vector, a number of other species may serve as mechanical vectors (fleas, ticks), and transmission has been demonstrated through blood transfusion (Cardo 2006), and through direct contact among dogs (Schantz et al. 2005). With regard to Lyme disease, all scientific evidence to date supports only transmission of *B. burgdorferi* through means of infectious tick vectors. In my dissertation, I do not suggest that transmission of *B. burgdorferi* is occurring in the absence of a tick vector, but my results do challenge the well-established co-occurrence of *B. burgdorferi* and *I. scapularis* in my detection of a cryptic pathogen maintenance cycle.

The ecology of Lyme disease is complex, as hundreds of vertebrate species are able to serve as reservoirs for the pathogen and/or hosts for the blacklegged tick. Furthermore, many tick species are competent vectors for the pathogen, and still others

may test positive for the pathogen simply due to infected bloodmeal fragments from an infectious host, which does not indicate vector competency. The vast majority of human cases are, however, attributed to *I. scapularis*. Thus, research which detects infection in alternative tick species or wildlife hosts must clarify if such infection implies a zoonotic risk, and this determination is largely based upon vector competency and host feeding patterns of the cryptic vector. In my work, I identified *I. dentatus* as a cryptic vector, and its strong associations with birds and rabbits, albeit with occasional records of human-biting, suggests that direct zoonotic risk from this cryptic cycle is low. Nonetheless these findings are medically-significant, because the continued invasion of *I. scapularis* may allow for bridging of the cryptic pathogen into humans.

The detection of a pathogen in areas, ticks, or hosts where it is not expected to occur can be met with skepticism, especially when the pathogen has been identified using only molecular assays as opposed to culture-based methods. Even given the sophisticated tools of the molecular revolution, one school of thought is that the presence of a pathogen is most rigorously demonstrated only upon growing the organism in pure culture. Thus, the organism is proven to be alive, and the whole organism is available for future analyses. In the case of the detection of *B. burgdorferi* in bird-associated ticks in the apparent absence of *I. scapularis* (Chapter 5), I did indeed subsequently attempt to culture this cryptic organism. My efforts, however, were unfruitful. Given the low prevalence of infection detected in bird-associated ticks at this site over the years (3.5%), combined with the fastidious nature of the organisms which render them susceptible to out-competition by sympatric bacteria or contaminants, I did not observe any spirochete growth in approximately 50 samples collected from the Pitsfield Banding Station in 2009.

Given unexpected pathogen findings, a skeptic may argue that contamination may be the cause of the some or all of the cryptic infection that I have found. For example, human error may result in contamination of truly negative field samples with pathogen DNA from either positive control or truly positive field samples. This contamination could take place at many stages in the sample diagnostic process, including early on during the DNA extraction phase, during PCR when tiny amounts of contaminating DNA can be amplified millions of times, or the DNA sequencing reaction. The data presented herein, however, should be free from such false positives as demonstrated by at least two lines of evidence. First, proper use and performance of negative controls was maintained throughout all steps in the diagnostic process. Second, the sheer level of diversity that I found in *B. burgdorferi* across the studies supports a dataset that in which contamination does not play a major role. We have found many novel mutations among cryptic, establishing, and endemic *Borrelia*, whereas the preponderance of contamination in the dataset would result in the contaminating strain type being found more ubiquitously.

Given the sequence diversity that I detected at a single genetic locus, including many novel mutants present in the population with a sample size of one, a skeptic may wonder about the presence of (*i*) PCR error or (*ii*) sequencing error in the dataset, resulting in spurious nucleotides that could inappropriately be interpreted as a novel strain. (*i*) Regarding PCR error, the argument is that infidelity of DNA replication may result spurious nucleotides being incorporated into DNA strands, which may later be interpreted as novel mutations. Whereas *in vivo* cell-based DNA cloning involves DNA replication and a very high fidelity of copying due to proofreading mechanisms, *in vitro* DNA replication (as in the case of PCR) is associated with considerably higher rates of

copying error (Strachan and Read 1999). This rate of error is a property of the various types of heat-stable DNA polymerases used in the PCR reaction. The most widely used is *Taq* DNA polymerase, derived from *Thermus aquaticus*, and this enzyme has no associated 3′ → 5′ exonuclease for proofreading. Thus, there is a high error rate due to misincorporation of bases during DNA replication (Strachan and Read 1999). Using this enzyme, a 1 kb sequence that has gone through 20 cycles of duplication is estimated to have an incorrect nucleotide resulting from a copying error in 40% of the newly generated DNA strands. The final PCR amplicons will therefore be a mixture of very similar, but not identical, DNA sequences (Strachan and Read 1999). Despite this PCR error, however, DNA sequencing of the total amplicons is likely to yield the correct sequence because the incorporation of incorrect bases in the final sequence is at random. Thus, for each base position in the sequence, the contribution of one spurious base on one or more strands will be overwhelmed by the contributions of the majority of strands which have the proper base (Strachan and Read 1999). Furthermore, I have employed in recent years a high-fidelity DNA polymerase with associated proofreading activity, such that PCR amplicons have fewer spurious apparent mutations (Cline et al. 1996).

(ii) Regarding sequencing error, nearly all of the *B. burgdorferi* strains reported herein have had a sequencing reaction run at least twice (forward and reverse strands, or forward strand twice). The resulting sequences were aligned, and all of the unique polymorphisms that set each novel strain apart from the common strains were apparent on both sequences. If the polymorphism was introduced as a sequence error, the chance of that same error occurring at the same site within independently-generated sequences would be very unlikely.

Implications for the Lyme disease system in Michigan

The Vectors

Continued monitoring of 9 core sites along the coastal and inland transects will allow for detailed description of the process of establishment. Our standardized and repeated sampling at sites not only where *I. scapularis* was found, but also areas well beyond its apparent distribution, has allowed for us to detect invasion and establishment of ticks into new areas. This dataset provides a rare example of actual tracking of a biological invasion as it occurs, as most medically important species invasions are detected only once disease results. The 9 sites include a longstanding hotspot of invasion (Van Buren), sites which have experienced measurable annual increases in tick density (Duck Lake, Orchard Beach), sites which have been colonized but with no detectable increase yet (Sleeping Bear Dunes, Fort Custer, Ionia), and sites which were not colonized as of the 2008 field season (Lux Arbor, Rose Lake, and Proud Lake; although additional 2009 sampling detected low-density *I. scapularis* at Lux Arbor). Though not described in this dissertation, limited sampling at Proud Lake near Detroit in 2009 resulted in no *I. scapularis* findings. The inclusion of this site in future standardized surveillance is suggested so as to stay ahead of the detected invasion front, as well as to accumulate Detroit-area data that will be of use in addressing the concerns of a growing number of people in that area who are interested in the local presence of ticks.

I also suggest an assessment of blacklegged tick density at additional intermediary sites within the coastal invasion zone as well as paired sites at intervals in the eastward, inland direction from these sites. These coastal data will be useful in determination of a rate of invasion of ticks. Such data can then be modeled across a series of different

scenarios of host-mediated invasion (i.e. migratory bird dispersal of ticks; deer dispersal; small mammal dispersal) to identify a most likely mechanism (or combination thereof) to explain the field observations. A series of biotic and abiotic environmental features (including host densities and climactic conditions) at each site can be measured and compared as a first step in addressing differences between coastal and inland landscapes that may facilitate or hinder tick invasion and establishment.

Evidence of blacklegged ticks not associated with the southwestern invading population or the Menominee County population has arisen in our checks of deer harvested during the mid-November shotgun season (data not reported herein). In 2007, were found three individual deer that each harbored a single adult *I. scapulars* from unexpected areas: one infested deer was harvested in St. Charles of Saginaw County in the east-central part of the state, and two infested deer were harvested in Gladwin County in the northeastern Lower Peninsula. Epidemiologically, there is a difference between an established population (defined by the Centers for Disease Control and Prevention by detection of at least six individuals or at least two life stages) and a reported population (one to a few ticks reported from a focal area, which alone does not indicate that the environment is suitable for tick survival and reproduction). Whether the infested deer or other such observations reflect ticks that have dropped off from mobile hosts into unsuitable habitats (failed invasion), an early-invasion process in suitable habitats, or established, yet previously undetected, populations is yet to be determined.

I therefore suggest that future research include a standardized survey for established ticks at a level of resolution that is sub-county level to produce a very useful tick distribution map. This project could be conducted with the assistance of groups

including the Michigan Department of Community Health (MDCH) and Michigan Department of Natural Resources and Environment (MDNRE) in order to provide fine-scale field sampling over a broad area but within a short period of peak tick activity. This exercise may also identify other independent foci from which invasion may be studied.

The Pathogen

A unique ecological scenario is apparent in a focal area in southwestern Michigan field site, where *I. scapularis* has not yet been observed, but repeatable *B. burgdorferi* infections of bird-associated ticks, rabbit-associated ticks, and rabbit tissues have been detectable. This work has been made possible through our collaborations with bird banders at Pitsfield Banding Station, who process tens of thousands of birds per season, and provide us with thousands of ticks. The effort to obtain an equivalent sample size without this collaboration would be near impossible. While this dissertation reports on samples acquired in 2004-2007, we have already logged and begun identifying samples collected in 2008-2009, and 2010 sample collections are underway. I suggest that future research maintain this collaboration as a priority. This long-term data set will be quite interesting, as this focal site is on the trajectory of invasion by *I. scapularis*. We have the potential to determine the response of the current tick, wildlife, and pathogen communities to the invasion and establishment by *I. scapularis*. Most interesting, we may be able to understand how invading strains of the pathogen interact with cryptic strains. In the meantime, while labor intensive, it may be quite valuable to attempt to meet the gold standard of demonstrating pathogen presence in this area by obtaining the cryptic pathogen in culture. To do this, I suggest that not only should we attempt to

culture from bird-associated ticks, but also ticks and ear biopsies obtained from rabbits, as these sample types are associated with higher infection prevalence at the cryptic site.

Across our studies of invading, cryptic, and established *B. burgdorferi* in Michigan and the Midwest, we detected unprecedented amounts of diversity at the IGS locus, and rarefaction curve analysis suggests that true population diversity is greater than what we detected. Characterization of additional genes of *B. burgdorferi* using an approach such as multi-locus sequence typing will allow for better characterization of the diversity of strains, and relationships of our strains to those reported from endemic foci elsewhere, which may provide insight as to the origin or strains and mechanisms of invasion. Furthermore, given the diversity of strains in the Midwest, future research should address the efficacy of currently available human and canine diagnostic assays for detection of the panel of Midwestern strains.

Implications for Public Health

There are direct human and canine health implications of this invading disease system. A significant increase in confirmed cases of human Lyme disease ($R^2 = 0.40$; 1-tailed $P = 0.015$) has occurred within the zone of active *I. scapularis* invasion in southwest Michigan, and this rate of increase is greater than that reported from the state of Michigan as a whole (Figure 7.1). From 1996 (when Lyme disease became reportable in Michigan) through 2009, incidence in the 14-county region peaked at 1.4 cases per 100,000 people (E. Foster, Michigan Department of Community Health, pers. comm.). Though increasing, incidence in the invasion zone remains substantially less than the average annual incidence in the 10 endemic states from which 93% of human Lyme

disease across the U.S. is reported (Connecticut, Delaware, Massachusetts, Maryland, Minnesota, New Jersey, New York, Pennsylvania, Rhode Island, and Wisconsin). In these states, an average of 29.2 cases per 100,000 population was reported for 2003-2005 (Bacon et al. 2007), and reported annual rates for a majority of these reference states were relatively stable during 1992—2006 (Bacon et al. 2008). Given the low reported incidence in the invasion zone, one must also be cognizant of the 'iceberg phenomenon' of human medicine, which is used to describe the common pattern whereby human patients that report their clinical symptoms of disease represent only a small fraction of the true extent of disease in the population (Lynch et al. 1987). Thus, the dynamic status of blacklegged ticks and their infection reported in this dissertation from 2004-2008 in lower Michigan thus represents an invasion in its early stages, and dissemination of these results now can have a positive effect in reducing disease risk in the future.

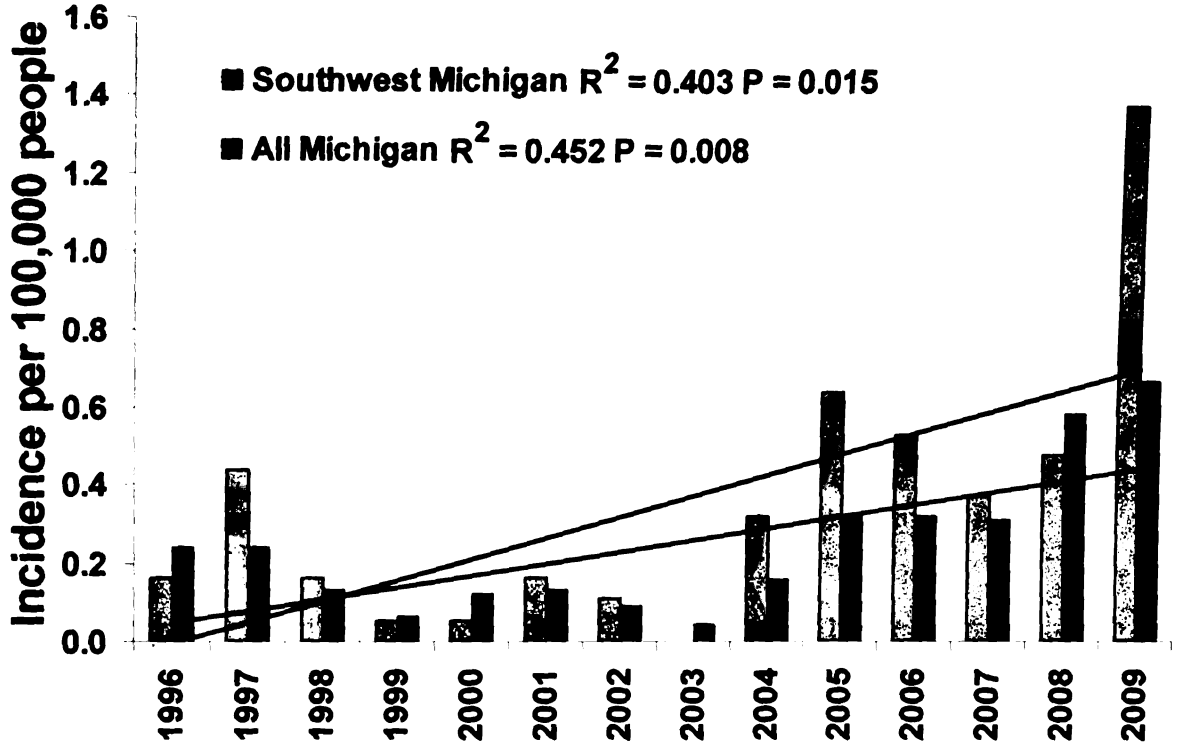
I predict that blacklegged ticks are currently more widespread than we know. The future may include not only a continued eastward expansion of ticks as a continuation of the invasion we have monitored in western Michigan, but also expansion from other yet-undocumented foci. I predict that human anaplasmosis will soon be diagnosed for the first time in Michigan, as will babesiosis and Powassan encephalitis. We have detected the pathogen responsible for anaplasmosis within the invading ticks, though its detection within humans may be delayed due to a lack of specific testing when a human presents with the generic symptoms it causes.

While the density of blacklegged ticks in the invasion hotspot at Van Buren is as high as that which characterizes Lyme disease endemic areas of the Northeast, the infection prevalence of ticks is lower, and nymphal infection prevalence (NIP; an index

of human risk) is therefore lower. In contrast with the significant peridomestic exposures that occur in the Northeastern US, recreational exposure is probably common in the Michigan invasion zone, which is dominated by recreational areas, camps, vacation homes, and rural communities. Michigan is thus likely to export cases of *B. burgdorferi*-infected visitors that were exposed recreationally in the invasion zone (as has been documented at a children's summer camp in summer 2009, E. Foster, pers. comm.), and there may be a time lag before the true public health impact of this invasion is realized.

Vigilance for ticks must be emphasized now so we may be prepared as risk increases. Local or community-scale strategies to reduce disease risk should be discussed, including educational outreach campaigns to teach personal protection measures, and also tick management techniques that are currently being used on a limited scale in the northeast, such as acaricide application to vegetation, manipulation of vegetation to create tick-free transition zones around homes, and methods to reduce tick abundance on deer or mice. Such strategies must be implemented in a manner to preserve the strong interest in outdoor recreation in the state. In the end, Lyme disease is preventable given adequate education of the public and medical community, and so outreach campaigns should be a priority.

Figure 7.1. Incidence (number of reported cases per 100,000 population) of Lyme disease in humans in southwest Michigan (light bars) and the whole state of Michigan (dark bars) in 1996-2009. Case numbers shared by E. Foster, Michigan Department of Community Health (pers. comm.).



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