Host-Parasite Associations of Small Mammal Communities and Implications for the Spread of Lyme Disease

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HOST-PARASITE ASSOCIATIONS OF SMALL MAMMAL COMMUNITIES AND IMPLICATIONS FOR THE SPREAD OF LYME DISEASE

A Thesis
Presented to
The Faculty of the Department of Biology
Western Kentucky University
Bowling Green, Kentucky

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Master of Science

By
Matthew John Buchholz

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HOST-PARASITE ASSOCIATIONS OF SMALL MAMMAL COMMUNITIES AND IMPLICATIONS FOR THE SPREAD OF LYME DISEASE

Date Recommended 20 April 2016

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First and foremost, I dedicate this thesis to my parents, Bill and Terri Buchholz. Thank you for fostering a love of wildlife and the outdoors in me from a young age. Without your love and support throughout my life none of this would have been possible. I love you.

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PREFACE

This thesis is written as two independent chapters entitled “Host-Parasite Associations of Small Mammal Communities in South-Central Kentucky” and “Ecological Aspects of the Lyme Disease System in South-Central Kentucky”. The decision to present the thesis as two chapters is not suggestive that material covered in each chapter is unrelated. In fact, the biological systems that are described here are entwined. Rather, the decision to separate the two chapters was made because different questions and hypotheses were addressed in each chapter, and joining them may confuse the reader. As a result, there exists some cross-over between sections of both chapters (particularly the materials and methods, figures, and tables). However, the chapters do not reference each other. This thesis is best read as two distinct chapters that emphasize basic (i.e. identifying host-parasite associations and their contributing factors) and applied (i.e. using those associations to understand the ecology of Lyme disease) research while bearing in mind that the subject matter in each chapter could stand on its own but is also related to the other.
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Many zoonotic pathogens of concern to human and veterinary health are maintained in the environment within small mammal reservoirs and vectored to new hosts by ectoparasitic arthropods. While the ecological relationships among small mammals, ectoparasites, and disease-causing symbiotic microorganisms are important to these dynamics, little is known about them across much of North America. The sylvatic cycle of *Borrelia burgdorferi*, the etiologic agent of Lyme disease, is of particular interest because Lyme disease is the most common vector-borne disease of humans in the United States. However, cases of Lyme disease are primarily confined to the northeastern and Midwestern United States, with only sporadic cases extending into the southeast. As a result, much of what is known of the ecology of Lyme disease comes from studies conducted in those regions. The goal of this study was to examine the ecological dynamics of the *B. burgdorferi*/vector/reservoir system in south-central Kentucky and gain insight into the relative paucity of Lyme disease in Kentucky. Small mammals were captured using live traps in three 200x50 m trapping grids within Western Kentucky University’s Green River Preserve from November 2014-October 2015. Captured small mammals were identified to species and standard measurements were recorded. Ectoparasites were removed and retained for identification. Collected blood and tissue were examined for *B. burgdorferi* DNA by polymerase chain reaction with primers specific to the OspA gene. The Bray-Curtis dissimilarity index, Schnabel population
estimates, and the Shannon-Wiener diversity index were used to assess the structure of
the small mammal communities. Parasite infestation was low but was affected by age and
sex of the host, site, and season in different parasite taxa. Infestation by *Ixodes*
*scapularis*, the primary vector for *B. burgdorferi*, was uncommon and prevalence of *B.*
burgdorferi in blood was similar to the lowest prevalence previously observed in the
Lyme disease endemic regions. We found that life history characteristics of hosts and
ectoparasites drive their associations. We also suggest that the lack of an efficient vector
for *B. burgdorferi* is the likely explanation for the few reported cases of Lyme disease in
Kentucky.
Chapter 1: Host-Parasite Associations of Small Mammal Communities in South-Central Kentucky

ABSTRACT

Host-ectoparasite associations are model systems for ecological and evolutionary studies. However, due to the potential complexity of relationships between multiple host and ectoparasite species at any given site these associations are often poorly understood, including in North America. The goal of this study was to elucidate the host-ectoparasite associations of small mammal communities in south-central Kentucky. Small mammals were captured from November 2014-October 2015 using live traps in three 200x50 m trapping grids within Western Kentucky University’s Green River Preserve. Captured small mammals were identified to species and standard measurements were recorded. Ectoparasites were removed and retained for identification. The Bray-Curtis dissimilarity index, Schnabel population estimates, and the Shannon-Wiener diversity index were used to assess the structure of the small mammal communities. Nine species of ectoparasites including three ixodid ticks, five species of Siphonaptera, and one mesostigmatid mite were collected from seven species of small mammals captured during this study and prevalence and mean intensity were calculated for each host-parasite association. Infestation by ectoparasites was generally low, but was affected by age and sex of the host, site, and season in different parasite taxa. The findings presented here provide an inventory of small mammal and ectoparasite species of the WKU Green River Preserve as well as insight into the dynamics of host-ectoparasite associations in south-central Kentucky.
INTRODUCTION

Parasitism

Parasitism is the most common form of lifestyle in the animal kingdom, having evolved independently in almost every animal phylum as well as in many bacterial and fungal lineages, with free-living organisms providing rich environments for parasites to exploit (Roberts and Janovy, 2009). Giving testament to the widespread nature of parasitism, it is suspected that all living organisms serve as host to at least one parasitic symbiont over the course of their lifetime (Roberts and Janovy, 2009).

While the cost of parasitism to the host varies across parasitic taxa, with some associations even approaching a commensalism, all parasites do extract some degree of fitness from their host organism. Such costs usually involve myriad and interactive factors, including ingestion of blood or other bodily fluids/tissues, loss of hair resulting in thermoregulatory stress, metabolic cost of grooming behavior, or immunological response. These costs can further vary from a seemingly insignificant loss of nutrients to diseases resulting in extreme pain or even death. For these and other reasons, parasite ecology and host-parasite associations have become popular fields of inquiry among biological researchers.

Ectoparasites

The term “ectoparasite” covers a broad range of organisms (mostly arthropods) that live outside of the host and get their nutrition by penetrating the host’s skin to feed on blood, lymph, and other bodily secretions (Roberts and Janovy, 2009). Some well-known ectoparasitic taxa include Siphonaptera (fleas), Mesostigmata (mites), and Ixodidae and Argasidae (ticks). The ecology of ectoparasites is an increasing focus for
many researchers as many species are capable of vectoring pathogens relevant to human and veterinary health. The etiologic agents for diseases such as Lyme, plague, Rocky Mountain spotted fever, anaplasmosis, flea-borne spotted fever, and many others are transmitted to humans and non-human animals through the feeding activities of ectoparasites (Bratton and Corey, 2005; Bitam et al., 2010).

Arthropod ectoparasites go through several developmental stages during their life cycle. For example, over a duration of 2-3 years, the tick life cycle includes egg, larva, nymph, and adult stages (Oliver, 1989). During each non-egg stage of their life cycle, ticks will take a single blood meal to prepare for molting and transition to the next stage as well as preparation for egg-laying in adult females (Parola and Raoult, 2001). Presence and abundance of each tick life stage in the environment is highly seasonal, resulting in a host’s tick burden being comprised of mostly one stage at any given time of the year (Figure 1). Fleas also pass through four life stages: egg, larva (three instars), pupa, and adult. At the end of the pupal stage the flea will form a cocoon, lying dormant until a suitable host arrives, potentially extending the flea’s lifespan significantly beyond the standard two weeks (Bitam et al., 2010). Once a suitable host is present, the adult flea will emerge from the cocoon to occupy the habitat of the host or the host itself (Bitam et al., 2010).

The duration of an ectoparasite’s physical contact with a host is variable. Certain permanent and obligate ectoparasitic taxa such as lice (Pthiraptera) pass through all of their life stages on a host (Reed et al., 2007). However, other taxa, such as ticks, are described as temporary ectoparasites, e.g. ectoparasites that spend only about as much time on-host as it takes to acquire a blood meal (Lehane, 2005). Additionally, periodic
ectoparasites, such as most fleas and mesostigmatid mites, spend a significantly longer time on the host than what is required to feed, but the time spent on-host is still a relatively small portion of their total life span (Lehane, 2005). As a result, such taxa are free-living much of the time and must cope with the environmental characteristics of their habitat. At temperate latitudes, ticks require sufficient plant litter to provide protection from cold temperatures while they overwinter as nymphs and adults (Gray, 1998). Additionally, ticks are at risk of desiccation while questing for a new host, generally requiring relative humidity above 80% (Gray, 1998). Fleas are also known to have different rates of pupal cocoon formation and survival rates within the cocoon vary depending on the temperature and humidity of their environment (Silverman et al., 1981). Variation in these microclimatic factors can result in extreme fluctuation of the abundance of ectoparasites in the environment from year to year and site to site (Ostfeld et al., 1996).

**Small Mammal Assemblages**

Small, non-volant mammals (rodents and insectivores < 250g) are consistently the most abundant mammalian taxa in terrestrial landscapes and contribute greatly to the biodiversity present in an ecosystem (Carey and Johnson, 1995; Stephens and Anderson, 2014a). Often unseen but almost always present, small mammalian species such as *Peromyscus leucopus* (white-footed mouse), *P. maniculatus* (deer mouse), *Microtus ochrogaster* (prairie vole), and more visible species such as *Tamias striatus* (eastern chipmunk) can make up large portions of the biomass within an ecosystem. Small mammals have profound impacts on the local floral and faunal communities through their roles as granivores, folivores, and insectivores. Granivores and folivores play significant
roles in the development of floral communities by consuming vegetation and serving as agents of dispersal for seeds and spores (Gibson et al., 1990; Howe and Brown, 2000). Insectivores consume large numbers of insects, greatly influencing the distribution and abundance of primary consumers of a food web (Getz et al., 1992). Small mammals also represent a crucial source of prey for numerous reptilian, avian, and mammalian predators (Carey and Johnson, 1995). As a result of the interactions with other components of an ecosystem’s food web small mammals play an integral role in the structure of an ecosystem.

Assemblages of small mammals are affected in myriad ways by their environment. Habitat effects are often so influential that many small mammal species are associated with specific habitat characteristics. Long-recognized habitat associations include *P. leucopus* with forested habitats in middle North America and *Sorex* and *Blarina* spp. (shrews) in mesic habitats (Morris, 1979; Yahner, 1992). While these associations have been supported by occupancy models that include several measures of biotic and abiotic habitat characteristics (Moore and Swihart, 2005; Stephens and Anderson, 2014a), they do not necessarily describe strict habitat limitations but rather where the associated species may be the most numerous small mammal species present. Small mammal assemblages are consistently composed of several sympatric species occupying different niche space to minimize interspecific competition (Grant, 1972). Sympatric species create a complex community that affects the environment not only through interactions with other small mammals but also many other species that are prey for and predators or parasites of small mammals.

**Host-Parasite Associations**
Host-ectoparasite associations have become model systems for ecological and evolutionary studies. Each host is a well-defined unit of study with a sample community of ectoparasites and each individual of a host species provides a replicate sample (Presley, 2011). Host body size, sex, population density, and other ecological and demographic characteristics can affect the quality and quantity of ectoparasite communities (Presley, 2011). By examining the effects of each individual host’s characteristics on its ectoparasite assemblage, researchers can better understand the associations that ectoparasites have with their hosts and how those associations may have developed over ecological and evolutionary time.

Host-parasite associations range across a spectrum from specific to general. Parasites that fall towards the specific end of the spectrum typically parasitize a single host species while general parasites will parasitize many different hosts. This is also known as “host specialization” or “host breadth”. Many species of ticks and fleas choose rodent species as hosts, without any evident selection of hosts other than mere presence of the host (Krasnov et al., 2002; Brunner and Ostfeld, 2008). Distribution of ectoparasites on their hosts is often highly heterogeneous in that a few host individuals will be heavily infested while the majority of individuals will have few or no parasites (i.e. aggregated on relatively few hosts) (Shaw et al., 1998). Aggregated distribution is quite common in host-parasite associations (Hawlena et al., 2005; Brunner and Ostfeld, 2008). Burdens of certain ectoparasites such as ticks have been suggested to follow the so-called “80-20 rule”. The rule states that 80% of the blood meals or other feeding events within a parasite population will derive from approximately 20% of the available hosts (Brunner and Ostfeld, 2008). The wide host breadth and aggregated distribution of
these ectoparasites culminates in only a few individual hosts of each species typically
hosting the majority of parasites in a given system.

Successful colonization of a host by an ectoparasite is associated with the
likelihood of the ectoparasite encountering a host. Combes (1991) described this
phenomenon in his development of the concept of an encounter filter. The encounter
filter excludes potential hosts that a parasite would not encounter in the environment due
to behavioral or ecological characteristics of the host and parasite, therefore driving the
evolution and ecology of the parasite to take advantage of the hosts it is likely to
encounter. Tactics such as occupying microhabitats of the host species allow
ectoparasites to wait until a suitable host is present, subsequently allowing the parasite to
colonize the host (Oliver, 1989; Parola and Raoult, 2001; Bitam et al., 2010). Social
behavior of the host species can also facilitate transfer of ectoparasites from one host to
another. Krasnov and Khokhlova (2001) found that fleas were easily transferred between
rodent species when the rodents came into direct contact. The natural history of the host
can also influence the likelihood of becoming infested with ectoparasites. Krasnov et al.
(2011) speculated that the larger home range and wider dispersal of male vs. female
rodents would cause increased occurrence of fleas on males.

Host-parasite associations often drive evolution and ecology of the parasite and
host through a prolonged arms race (Roberts and Janovy, 2009). Parasites continually
adapt to host defenses while the host is ever more adapted toward defense against
parasites. In many systems, these relationships are often extremely complex and can
change with the diversity and distribution of host and parasite species present, as well as
with the varying effects of the system’s abiotic factors. Because of these factors, the host-parasite associations that occur across large systems are often poorly understood.

The degree of parasitism is quantifiable. Two common measures are prevalence and intensity of infestation. Prevalence is the proportion of sampled hosts that were found to be infested with parasites. Intensity is the number of parasites on a host that is infested. When comparing measures of intensity among host demographics and abiotic factors, mean intensity is often calculated and assessed (Bush et al., 1997).

The goals of this project were to investigate host-parasite associations within small mammal communities in south-central Kentucky. We sought to identify and quantify the small mammal species present along with their ectoparasites by conducting a small mammal trapping survey and sampling captured mammals for ectoparasites. Due to the wide host breadth of the ectoparasites likely to be encountered in this study, we hypothesized that prevalence and mean intensity of infestation by ectoparasites would not vary among mammal species but would vary between sexes because males disperse further, therefore increasing encounter rates of ectoparasites (Gaines and McClenaghan, 1980). We hypothesized that ectoparasites would display an aggregated distribution with the majority of the ectoparasites found on only a few individual small mammals. Due to the highly seasonal nature of tick abundance in the environment we hypothesized that the prevalence and mean intensity of ticks would vary by season, while indices of parasitism by fleas would not change by season. As fleas and ticks are not permanent parasites, we hypothesized that sub-adult and adult mammals would have equal prevalence and mean intensity of ectoparasitic infestation because parasites would not accumulate as the host
ages. Finally, we hypothesized that the composition of the small mammal community would vary by season and site, potentially affecting the presence of parasites within sites.
MATERIALS AND METHODS

Small Mammal Trapping and Sampling

Small mammals were trapped from November 2014 – October 2015 on Western Kentucky University’s (WKU) Green River Preserve (GRP) (Figure 2). Trapping grids were established in three different habitat types: young lowland forest, early successional old field, and mixed-age upland forest. The young lowland forest site consisted primarily of *Platanus occidentalis* (American sycamore), *Juglans nigra* (eastern black walnut), and *Fraxinus* spp. (ash trees) with dense ground cover at an elevation of 156 m above sea level (the Green River located adjacent to the site is at 148 m above sea level). Extensive logging of the site occurred approximately 40 years prior to this study removing all large trees. The early successional old field site consisted of a mix of cold and warm season grasses along with a mix of forbs and flowering annuals. Ten years prior to this study, the site was in agricultural production (primarily tobacco) and seven years prior it was replanted in an attempt to restore a prairie habitat. Lastly, the mixed-age upland forest site consisted largely of *Quercus alba* (white oak), *Acer saccharum* (sugar maple), and *Carya* spp. (hickory trees) with a sparse understory and little ground cover at an elevation of 225-235 m above sea level on a westward-facing slope. Selective logging was previously performed on the site leaving many large trees as well as allowing for regeneration of younger trees on the site.

Trapping grids were composed of 100 Sherman live traps (Model LNG, H. B. Sherman Traps, Tallahassee, FL) in a 200x50 m grid with traps placed 10 m apart. Trapping grids were numbered 1 (young lowland forest), 2 (early successional old field), and 3 (mixed-age upland forest) (Figure 2), with each trap within a grid assigned an
identifier (A1 to E20). Traps were baited with rolled oats and peanut butter (Great Value Foods, Bentonville, AR). Trapping grids were checked for captures and closed within two hours of sunrise and reopened approximately two hours before sunset for three consecutive days during each month of the study, except for February 2015 when trapping occurred for two days due to heavy snowfall.

Captured small mammals were transported within the trap to an on-site field laboratory for processing. Locations of all captures and sprung traps without a capture were recorded. Captured small mammals were first identified as either an initial capture or recapture. Small mammals were then identified to species, sex, and age using diagnostic characteristics. Standard measurements including total length, tail length, hind foot length, ear length, and mass were recorded for each initial capture of an individual during a particular month. Reproductive state (testes position for males and vaginal condition and whether lactating for females) was also recorded. Small mammals were then carefully examined with both fine-tipped and entomological forceps for any ectoparasites (attached and unattached) which were subsequently collected into vials of 70% ethanol. Sampling effort was standardized by searching each individual’s pelage, ears, and anal region for two minutes. Additionally, bags used to transfer each small mammal from the trap to hand were searched for any parasites that came off the host. Following parasite sampling, mammals were anesthetized by isoflurane inhalation. Blood was collected by irritation of the retro-orbital sinus with a Pasteur pipet and subsequently transferred into a collection tube containing the anticoagulant K2-EDTA (Terumo Medical Products, Somerset, NJ). Small mammals were then ear tagged (Model 1005-1 Stamped Number, National Band & Tag Company, Newport, KY) with unique numbers
and released at the site of capture. Small mammal handling and sampling protocols were approved by the Western Kentucky University Institutional Animal Care and Use Committee in protocol #14-22.

**Off-host Tick Collection and Quantification of Vegetative Structure**

Off-host ticks were sampled by a tick dragging method. A 1 m$^2$, white corduroy cloth was dragged along three, 100 m transects within each trapping grid. Tick dragging was conducted every three months beginning in January 2015. The drag cloth was inspected every 20 meters and any ticks present on the cloth were collected into vials of 70% ethanol.

Vegetative structure characteristics of each trapping site were collected every three months at the same time as tick dragging. Ten, randomly selected, 1 m$^2$ plots within each of the three trapping grids were used for all measurements of vegetative structure. The number of all combined vegetation stems within each plot was recorded at ground level, 25, 50, 75, and 100 cm from the ground. Tree crown/canopy density was recorded over each plot by using a spherical densiometer facing N, S, E, and W. The four values were then averaged to obtain the crown density over each plot. Depth of plant litter was assessed by measuring the distance from the soil to the top of litter layer at the four corners of each plot and averaging. Density and basal area of trees and woody shrubs were assessed once during June 2015 using a point-quarter sampling method as described by Cottam and Curtis (1956) at each of the plot points.

**Ectoparasite Identification**

All ectoparasites were stored in 70% ethyl alcohol for transport to WKU and sorted and enumerated under 5-12x magnification. Ticks were identified to species and
life stage by following pictorial keys from the University of Rhode Island TickEncounter Resource Center Tick Identification Chart (URI, 2016). Fleas were identified by following the dichotomous key within Ewing and Fox’s “The fleas of North America: Classification, Identification, and Geographic Distribution of These Injurious and Disease-Spreading Insects” (1943). Flea identifications were confirmed by Dr. Ralph Eckerlin (Northern Virginia Community College).

**Data Analyses**

All statistical analyses were conducted using the statistical program R (R Core Team, 2015) and α was set at 0.05. Parasite presence/absence and intensity was determined for each examination of an individual small mammal as individuals were sampled for parasites each month of capture, and all sampling events are considered independent. The mammal species, sex, age (adult or sub-adult), site (trapping grid), and season (Dec-Feb = Winter, March-May = Spring, June-Aug = Summer, and Sep-Nov = Fall) were recorded for each sampling event. Parasite prevalence and mean intensity were calculated for each parasite species individually as well as collectively for the tick and flea taxa.

Prevalence of ticks and fleas was examined by identifying all individuals that harbored at least one tick or flea and creating three-way contingency tables. Age and sex of the mammal were included along with parasite presence/absence for one analysis and season and site were included as variables in another table for a separate analysis. Analyses were prepared in this manner to separate characteristics of the mammal (age and sex) and characteristics of the environment (season and site) to examine how they affect prevalence of ectoparasites. Analyses of the three-way contingency tables were
conducted by comparing generalized linear models involving different interactions with parasite presence/absence. Interactions included the effect of each of the other two variables (age and sex or season and site) independent of the other and the effect of the interaction of the other two variables together. These models were then compared to a base model without any effects added by analysis of deviance using a likelihood ratio test. Follow up comparisons were made using the pairwise.G.test function in the R package “RVAideMemoire” (R Core Team, 2015). To reduce the increased risk of committing a Type I error associated with multiple comparisons a Bonferroni correction was used while performing the post-hoc comparisons.

Parasite prevalence was also examined by a G-test of independence comparing prevalence of an individual parasite species among mammal species. Follow up comparisons were made using the pairwise.G.test function in the R package “RVAideMemoire” (R Core Team, 2015). To reduce the increased risk of committing a Type I error associated with multiple comparisons a Bonferroni correction was used while performing the post-hoc comparisons.

To examine whether mean intensity of flea or tick infestation was affected by host age, host sex, season, or site, two-way analyses of variance testing the interaction of age and sex and the interaction of season and site were performed. Intensities were calculated by adding up all the ticks or fleas present on an individual small mammal regardless of species. Post-hoc comparisons were made using a Tukey’s HSD test. Intensities of each species of ectoparasite were compared using a one-way analysis of variance among the different mammal species. The assumption of normally distributed
data in a parametric ANOVA was tested using the Shapiro-Wilk normality test, and when appropriate a non-parametric ANOVA using resampling was performed instead.

To examine the relationship of mean tick intensity to collected vegetation characteristics, a multiple regression was performed using the data collected from each trapping site during each season.

The structure of the small mammal community was also examined. Population size was estimated for each trapping grid during winter, spring, and summer using the standard Schnabel population estimate (Schnabel, 1938). Diversity of the small mammal community of each trapping grid during each season was calculated using the Shannon-Wiener diversity index (Shannon and Weaver, 1949). Dissimilarities of the composition of the representative small mammal communities between seasons and sites were examined by calculating the Bray-Curtis dissimilarity index for each season-site pairing and using the advanced.procD.lm function within the R package “geomorph” (Adams and Otarola-Castillo, 2013).
RESULTS

A total trapping effort of 10,500 trap nights resulted in 748 captures (7.12% trapping success). Three hundred and thirty-six unique animals were captured, comprising seven species: *Blarina brevicauda* (northern short-tailed shrew), *Microtus ochrogaster* (prairie vole), *M. pinetorum* (woodland vole), *Peromyscus leucopus* (white-footed mouse), *P. maniculatus* (deer mouse), *Reithrodontomys humulis* (eastern harvest mouse), and *Zapus hudsonius* (meadow jumping mouse) (Table 1). Of these, *B. brevicauda*, *M. pinetorum*, and *Z. hudsonius* were so infrequently captured that they were excluded from statistical analyses comparing prevalence and mean intensity of parasite infestation. Prevalence and mean intensities of infestation by all parasite species observed during the study on each mammal species are presented in Table 2.

Prevalence of ticks did not vary by host age (Deviance = 0.837, df = 4 & 3, \( p = 0.360 \)), sex (Deviance = 1.427, df = 4 & 3, \( p = 0.232 \)), or by age x sex interaction (Deviance = 8.302, df = 4, \( p = 0.081 \)). However, prevalence of ticks varied by season (Deviance = 64.946, df = 17 & 14, \( p < 0.001 \)), site (Deviance = 13.667, df = 17 & 15, \( p = 0.001 \)) (Figure 3), and by season x site interaction (Deviance = 162.680, df = 17, \( p < 0.001 \)) (Figure 4). Mean intensity of ticks varied by host age (\( F_1 = 5.312, \( p = 0.025 \)) but not by host sex (\( F_1 = 2.463, \( p = 0.122 \)) or by the interaction of age x sex (\( F_{1,54} = 0.898, \( p = 0.348 \)). Mean intensity of ticks did not vary by season (\( F_2 = 2.983, \( p = 0.059 \)), site (\( F_2 = 1.827, \( p = 0.171 \)) (Figure 5), or by the interaction of season x site (\( F_{2,52} = 0.563, \( p = 0.573 \)) (Figure 4). Winter was excluded from the analyses of mean tick intensity by season because no individuals examined during that season harbored ticks.
Prevalence of fleas did not vary by host age (Deviance = 2.290, df = 4 & 3, \(p = 0.130\)) or by age x sex interaction (Deviance = 7.884, df = 4, \(p = 0.096\)), but did vary by host sex (Deviance = 5.490, df = 4 & 3, \(p = 0.019\)). Prevalence of fleas did not vary by season (Deviance = 4.143, df = 17 & 14, \(p = 0.246\)), but did vary by site (Deviance = 9.5989, df = 17 & 15, \(p = 0.008\)) (Figure 6) and by season x site interaction (Deviance = 103.960, df = 17, \(p < 0.001\)) (Figure 4). Mean intensity of fleas did not vary by host sex (\(F_1 = 2.538, p = 0.114\)), age (\(F_1 = 1.499, p = 0.223\)) or by the interaction of age x sex (\(F_{1, 124} = 3.480, p = 0.064\)). Mean intensity of fleas did not vary by site (\(F_2 = 0.554, p = 0.576\)) or season (\(F_3 = 1.156, p = 0.330\)) (Figure 7) but did vary by the interaction of season x site (\(F_{6, 121} = 2.756, p = 0.015\)) (Figure 4). Post-hoc pairwise comparisons showed two statistical differences: mean flea intensity in the lowland forest site during winter was higher than in the early successional old field during spring (\(p = 0.025\)) and the upland forest site during summer (\(p = 0.034\)).

Results of analyses comparing the prevalence and mean intensity of each parasite species on each mammal species are presented in Table 3. For those parasite species (\(D. variabilis\), \(C. pseudagyrtes\), and \(P. hesperomys\)) that showed statistical difference in prevalence among mammal species, comparisons are shown in Figure 8. Due to small sample size, ANOVAs were not conducted to compare mean intensity of \(I. scapularis\), \(A. americanum\), and \(A. fahrenholzi\). Results of Shapiro-Wilk normality tests conducted on the distribution of intensity values showed that all of the parasite species had a non-normal distribution of intensities; as such all ANOVAs conducted were non-parametric ANOVAs using resampling.
Multiple linear regression was conducted to examine the relationship of tick intensity to the density of vegetation stems at ground level, crown/canopy density, and depth of the litter layer. Inclusion of all seasons and sites resulted in insignificant results ($F_{3, 8} = 0.023, p = 0.9951$). The analyses were rerun after removing the observations from the early successional old field as the number of vegetation stems at ground level was much higher in the field site than in either forest site but the results were similarly insignificant ($F_{3, 4} = 0.962, p = 0.492$).

The tick dragging method to collect off-host ticks in the environment resulted in a total spatial area of 3,600 m$^2$ sampled from all sites and seasons combined. From this sampling, a total of 29 ticks were collected. All 29 were collected during April and July 2015, with no ticks collected during either the winter or fall dragging attempts. Of the 29 ticks collected 28 (96.5%) were $A. americanum$ in nymphal and adult life stages. The single other tick collected was an adult female $D. variabilis$.

Schnabel estimates of the small mammal population for each site during winter, spring, and summer are presented in Table 4. Population size was not calculated for fall because the timing of the trapping period did not allow for inclusion of sufficient resampling events to calculate the Schnabel population estimate. Table 5 shows the calculated Shannon-Wiener diversity index values for each season, site, and season-site pairing. Dissimilarity of the composition of the small mammal community as represented by trapping was not significant by season ($F_{9, 6} = 0.631, p = 0.557, r^2 = 0.154$), but was significant by site ($F_{8, 6} = 2.212, p = 0.002, r^2 = 0.359$) and by the interaction of season x site ($Z = 1.789, p = 0.001, r^2 = 0.487$). Post-hoc comparisons of the sites showed that the small mammal composition of the early successional old field site was significantly
different than both the young lowland forest ($P = 0.002$) and mixed-age upland forest ($p = 0.021$) sites. Post-hoc comparisons of the season x site interaction showed differences in the representative small mammal communities in the early successional old field site during summer and fall ($p = 0.038$) and the young lowland forest and early successional old field during winter ($p = 0.018$). Further comparisons approaching significance included the mixed-age upland forest and early successional old field during winter ($p = 0.058$) and the young lowland forest site during winter and summer ($p = 0.065$).
DISCUSSION

Biological systems are driven by interactions among the species that inhabit them. One of the most pervasive forms of symbiosis (sensu lato) in nature is parasitism, occurring in almost every biological system on the planet (Roberts and Janovy, 2009). These host-parasite associations drive the ecology and evolution of both the host and parasite species (Presley, 2011). By observing these relationships we can deduce which host and parasite characteristics are influencing the parasitism symbiosis, reconstruct the evolutionary and ecological history of these systems, and hypothesize possible dynamics of future host-parasite associations.

Rodent-ectoparasite associations provide opportune systems to study parasitic symbioses and how they are affected by and in turn drive the ecology and evolution of rodent and ectoparasite species. The incredible global diversity of rodent species and ectoparasites that infest them results in a wide range of associations being affected by numerous characteristics of the host and parasite species, as well as other biotic and abiotic factors. Additionally, because rodents are relatively easy to capture and sample for ectoparasites, these systems can be consistently studied in a straightforward manner. Studying these systems provides ample information about factors that affect not only the ecology and evolution of host-parasite associations but also contribute to the ecology of disease agents that are vectored and maintained within these systems.

The degree of parasitism that occurs varies widely between different biological systems consisting of different host and parasite species. Overall, the observed prevalence and mean intensity of ectoparasitic infestations on small mammals was relatively low in this study. The highest observed prevalence was 21.9% by
Peromyscopsylla hesperomys on Peromyscus maniculatus and the majority of observed prevalences did not exceed 10%. Furthermore, the mean intensity of any parasite collected in this study did not exceed five. Peromyscus leucopus, P. maniculatus, and Reithrodontymys humulis all belong to the subfamily Neotominae while Microtus ochrogaster belongs to the subfamily Arvicolinae. Our results are consistent with previous field observations that members of the subfamily Neotominae are under parasitized relative to sigmodontines such as Oryzomys spp. (D. Gettinger, Manter Laboratory of Parasitology, University of Nebraska – Lincoln, personal communication). However, the associations varied among parasite and host species and among sites and seasons.

Of the nine species of parasites collected during this study, six showed no difference in prevalence among the four most abundant species of mammals. Interestingly, the three parasite species that did show variation in prevalence were all influenced by the general lack of parasites on Reithrodontymys humulis. Only one flea was collected from a R. humulis during the entire study. While many ticks and fleas are general in their host choice and occur on multiple host species, some fleas and ticks are found more often on certain host species than others (Durden and Kollars, 1997; Krasnov et al., 2002; Brunner and Ostfeld, 2008). Specifically, for this study Peromyscopsylla hesperomys was more often associated with Peromyscus spp. and the relationship between Ctenophthalmus pseudagyrtes and Microtus ochrogaster observed here has previously been recorded in the south-central region of the U. S. (Durden and Kollars, 1997). As hypothesized, the parasites collected during this study had an aggregated pattern of distribution with most of the parasites occurring on only a few host individuals.
(Hawlena et al., 2005; Brunner and Ostfeld, 2008). Furthermore, we found support for the “80-20 rule” (Brunner and Ostfeld, 2008) as 80% of the parasites collected during this study were collected from only 18% of the hosts that were examined. Life history characteristics of ticks and fleas likely contribute to aggregation on relatively few host individuals. Female ixodid ticks deposit between 100 and 18,000 eggs in a single mass (Roberts and Janovy, 2009). Small mammals that are unlucky enough to chance upon one of these masses during larval hatching would likely be infested by many ticks, while those that do not happen upon a hatching egg mass may only encounter and be infested by very few or no ticks from the environment. Additionally, host-seeking behaviors such as occupation of a particular small mammal burrow or nest by ticks and fleas could expose select small mammals to more ectoparasites than others (Oliver, 1989; Parola and Raoult, 2001; Bitam et al., 2010).

Season affected the prevalence and mean intensity of parasitism of small mammals in this study. As hypothesized, tick prevalence was highly seasonal with the vast majority of recorded tick infestations occurring in spring and summer. However, the overwhelming majority of ticks collected from small mammals during sampling were *D. variabilis*, specifically *D. variabilis* larva. Thus, the observed effect of season on tick prevalence and the nearly significant effect on tick intensity are likely better explained by considering only *D. variabilis* instead of all three tick species together. The likely explanation for the seasonal variation in *D. variabilis* prevalence is that the *D. variabilis* life cycle results in high abundance of ticks questing for hosts during particular seasons when each life stage is present in the environment (Wilson and Spielman, 1985; Oliver, 1989). In Kentucky, larval *D. variabilis* activity is highest during March, April, and May.
(Kollars et al., 2000; URI, 2016), which we defined as spring. Additionally, the abundance of *D. variabilis* nymphs and adults is highest during late spring and summer (Kollars et al., 2000; URI, 2016). Additional seasonal patterns would likely have been observed if more *A. americanum* and *I. scapularis* had been collected as the seasonal abundance of life stages of different species of ticks is not consistent (URI, 2016).

Site also affected the prevalence of tick infestation. Prevalence of tick infestation was higher in both forest sites than in the old field site. Deciduous forests provide the microclimatic conditions that best allow ticks to survive throughout their life cycle (Gray, 1998). Additionally, while the old field site was previously planted to restore native prairie habitat, several exotic grass species have invaded the site including *Dactylis* spp. (orchard grasses), *Setaria faberi* (japanese bristlegrass), and *Fescue arundinacea* (Kentucky 31 tall fescue). Civitello et al. (2008) found that invasion by exotic grasses reduces survival of *D. variabilis*, potentially decreasing its prevalence on small mammals in our old field site. Interestingly, the prevalence of both ticks and fleas, as well as mean intensity of flea infestation varied with the interaction of season x site. This suggests that the change in the site’s habitat characteristics throughout the year was directly affecting the parasite species. This supports previous findings that because these ectoparasites spend significant time off-host, they are strongly affected by the environment they inhabit and the changes that occur to that environment over the course of a year (Lehane, 2005; Krasnov et al., 2010).

Demographic characteristics of hosts have been known to influence the prevalence and intensity of parasitic infestation. In the present study, mean intensity of tick infestation was the only measure of parasitic infestation to vary with age of the host.
The tick and flea species recorded in this study are all temporary or periodic feeders (Lehane, 2005; Krasnov et al., 2010). Conventional wisdom dictates that temporary parasites like ticks should not accumulate over the lifespan of the host, as would be expected with permanent parasites because they spend only a limited amount of time on the host and then drop off. However, variation in prevalence and intensity of temporary parasites by host age has been previously recorded. Krasnov et al. (2006) found variation in flea prevalence and species richness of fleas parasitizing several species of rodents by age, hypothesizing that the life history characteristics of the host could be the cause of older individuals being more heavily parasitized. One life history characteristic affecting parasitism by ectoparasites could be that increased age of the host is associated with increased body size therefore creating a larger “target” or resource for parasites to find and colonize (Krasnov et al., 2006). Additionally, dispersal of rodents that have matured to adults could still influence the host encounter rate for temporary ectoparasites. 

*Peromyscus californicus* has been observed to stay very close to its natal home range as juveniles and not disperse widely until adult age (Ribble, 1992).

Sex-biased parasitism has been recorded in numerous host-parasite systems involving arthropod, helminth, and unicellular parasites (Moore and Wilson, 2002). While sex-biased parasitism has been recorded in systems with female-biased and male-biased parasitism, the majority of the recorded instances show bias toward male hosts (Moore and Wilson, 2002; Seeman and Nahrung, 2004; Krasnov et al., 2005). In particular, many arthropod parasites, such as ticks and fleas, display male-biased parasitism (Moore and Wilson, 2002). Factors such as dispersal and home range size, activity levels, and sexual size dimorphism have all been suggested to influence sex-
biased parasitism. Ticks quest for new hosts by climbing vegetation and waiting for a suitable host to wander past, while many fleas will occupy the most suitable microhabitat for the host, waiting for a host to approach (Parola and Raoult, 2001; Bitam et al., 2010). Both strategies rely on the host coming to the parasite instead of the parasite searching out a host. As a result, more active hosts that cover larger spatial areas are more likely to encounter parasites (Combes, 1991). In the present study we hypothesized that males would have higher prevalence and mean intensity of flea and tick infestation due to male rodents displaying larger dispersal areas (Gaines and McClenaghan, 1980). Our results showed that prevalence of flea infestation was the only statistically significant instance of male-biased parasitism in this study. However, all three of the other host-parasite indices calculated (mean intensity of flea and tick infestation and prevalence of ticks) were skewed towards males, just not to a statistically significant degree. Therefore we suggest that the rodent-ectoparasite system observed in the present study likely is biased toward male hosts. Interestingly, daily activity rates of female rodents have been recorded as between 20% to over 50% higher than males (Lightfoot, 2008). Higher daily activity rates in females would seemingly expose them to more ectoparasites over the short period of time these parasites remain on-host. However, it is possible that because males have a larger dispersal area and home range, females are encountering only the ectoparasites that are present in their limited home range while males are encountering more parasites as they are covering more area over the two-to-four day span that temporary parasites are present on the rodent. Another explanation for the observed male bias could be that males are simply larger “targets” for ectoparasites. Many species of rodents display male-biased sexual size dimorphism (Schulte-Hostedde, 2008). Larger host body size could result in
ectoparasites being more likely to find and colonize the host as well as more space for ectoparasites to spread out to avoid localized competition. However, in the present study the variation in mass between sexes did not exceed 2 g (M. ochrogaster males were 7.2% heavier than females on average). The only statistically significant sexual dimorphism in mass was in P. maniculatus with males being 1.2 g (6.4%) heavier than females (Two tailed T-test, t = -2.167, df = 199, p = 0.031). These small variations in mass may or may not have contributed to the male-biased parasitism observed in this study.

Density of hosts in the environment can affect the distribution and composition of ectoparasite communities. Higher density of hosts has been recorded to correlate with increased species richness of ectoparasites (Krasnov et al., 2002). Increased density of hosts allows for species of parasites that may normally be out-competed by other parasites or have minimal dispersal ability to find and colonize hosts. Additionally, high host density can result in a form of a “dilution” effect (Kiffner et al., 2011). The overabundance of potential hosts with a set population of ectoparasites could dilute the prevalence and intensity of parasitism as the parasites have many hosts to choose from. The present study calculated density of the entire representative small mammal community at each trapping site. Small mammal density in the early successional old field site was calculated between 185-250/ha in different seasons. This high density of hosts and a “dilution” effect could further explain the lower prevalence of tick infestation than in either of the two forest sites where small mammal density did not exceed 89/ha.

Results of the dissimilarity analyses showed that the composition of the representative small mammal community was different in the early successional old field site compared to either forest site. The difference in composition of the small mammal
community is directly related to the abundance, species richness, and evenness of the community at each site. Diversity of the small mammal community (factoring in species richness and evenness) was generally low at all three sites but small mammal species diversity was highest in the field site. These findings are likely related to the composition of the vegetation at each site as different species of vegetation provide different habitat niches for small mammal species. Previous studies have found higher diversity of small mammals in fields is associated with standing vegetation and that diversity in forests was not affected by stand age but by other vegetative characteristics such as vegetative species diversity and density of understory and ground vegetation (Carey and Johnson, 1995; Huntly and Inouye, 1987). Species richness was also low during this study, R = 4, 5, and 3 for the lowland forest, old field, and upland forest sites respectively. While low host species richness would normally result in low parasite richness, this correlation is likely offset by the wide host breadth of the ectoparasites observed in this study (Hechinger and Lafferty, 2005). However, some parasite and host species present in the community may have been underrepresented in our study due to the sampling method. Stephens and Anderson (2014b) found that pitfall and Sherman traps capture different subsets of the small mammal community; in particular pitfall traps were more successful capturing shrews. Only a single shrew was captured during this study using Sherman traps. Inclusion of more shrews in this study may have found other parasite species, which may have altered patterns of observed host-parasite associations.

Additional studies are needed to further elucidate the host-parasite associations of small mammals across the state of Kentucky. This study was limited to three trapping grids at one location and is therefore of limited geographical coverage. Patterns
uncovered here may or may not be extrapolated to other sites in the state. Furthermore, future studies that utilize multiple capture techniques may be able to obtain a more complete representative sample of the small mammal community, allowing researchers to more thoroughly describe the host-parasite associations. By further monitoring the host-parasite associations of small mammals and how they may change over time, we can not only learn about the ecology and evolution of these systems but also understand the risk of humans contracting pathogens that are maintained and vectored through these systems.
LITERATURE CITED


Chapter 2: Ecological Aspects of the Lyme Disease System in South-Central Kentucky

ABSTRACT

The incidence of tick-borne zoonoses such as ehrlichiosis, Rocky Mountain spotted fever, and Lyme disease has steadily increased in the southeastern United States in recent years. According to the U.S. Centers for Disease Control and Prevention (CDC), the southeastern states accounted for 1,200 of the over 25,000 confirmed cases of Lyme disease reported in the U.S. during 2014. *Borrelia burgdorferi*, the etiologic agent of Lyme disease, is maintained in small mammal reservoirs and vectored to new hosts by ixodid ticks. The purpose of this study was to examine the ecological relationships of the *B. burgdorferi/vector/reservoir* system and understand dynamics of Lyme disease in Kentucky. Small mammals were captured using live traps in three 200x50 m trapping grids within Western Kentucky University’s Green River Preserve from November 2014-October 2015. Captured small mammals were identified to species and standard measurements were recorded. Ticks were removed and retained for identification. Collected blood and tissue from small mammals was screened for *B. burgdorferi* DNA by polymerase chain reaction with primers specific to the OspA gene. Prevalence of *B. burgdorferi* in Kentucky small mammals was comparable to the lowest recorded prevalence in regions in which Lyme disease is endemic. Moreover, infestation of small mammals by *Ixodes scapularis*, the primary vector of *B. burgdorferi*, was rare, while *Dermacentor variabilis* comprised the majority of ticks collected. These findings provide ecological insight into the relative paucity of Lyme disease in Kentucky.
INTRODUCTION

Tick-borne diseases and Lyme disease

Tick-borne diseases have become a major public health concern as ticks are the leading vector of infectious disease agents in the United States (Hill and Wikel, 2005). A diverse group of etiologic agents including spirochetes, gram-negative bacilli, intracellular rickettsia, Ehrlichia spp., protozoa, and viruses cause a wide range of diseases including Rocky Mountain spotted fever, erlichiosis, babesiosis, anaplasmosis, and Lyme disease (Walker, 1998; Bratton and Corey, 2005). While each tick-borne disease presents unique challenges for public health officials, Lyme disease is the most common vector-borne disease of humans in the United States, having been reported in all 48 contiguous states and Alaska with extensive surveillance and awareness in endemic regions (Guerra et al., 2002; CDC/DVBD, 2014). Although the disease was first described from cases centered on Cape Cod, MA during the 1960s, it was not until 1982 that the etiologic agent, a spirochete bacterium, was first isolated by Burgdorfer and colleagues, who later named it Borrelia burgdorferi (Burgdorfer et al., 1982; Steere et al., 2004). Specifically, Lyme disease in North America is caused by B. burgdorferi sensu stricto (hereafter referred to as Borrelia burgdorferi). Borrelia burgdorferi has successfully been isolated from tick specimens collected during the late 1800s and may have been widely distributed in forested regions of North America for thousands of years (Persing et al., 1990; Steere et al., 2004).

While prevention of Lyme disease may be facilitated in part by informing the public about transmission risk, the complex ecological dynamics comprising the B. burgdorferi/vector/reservoir system presents significant challenges to the control of the
disease. Complex multi-species associations among different tick species that vector the bacterium from one host to another, numerous reservoir species (across a wide range of mammalian and avian taxa) that maintain the presence of *B. burgdorferi* in the environment, and numerous other biotic and abiotic factors create a dynamic system that presents health professionals and ecological researchers with many different challenges and questions.

**Vectors**

*Borrelia burgdorferi* is transmitted to new hosts through the bite of an ixodid tick. All ixodid ticks share a common life cycle comprised of four stages: egg, larva, nymph, and adult (Figure 1). Transition through the complete life cycle is typically completed in 2-3 years and is influenced by environmental conditions including temperature, humidity, and photoperiod (Oliver, 1989; Parola and Raoult, 2001). During each non-egg stage of the life cycle, ticks will take a single blood meal to prepare for molting and transition to the next stage (Parola and Raoult, 2001). During a blood meal, ticks may take up *B. burgdorferi* from an infected host or transmit it to a naïve host. The larva is the only stage not capable of transmitting *B. burgdorferi* to a new host as they have not fed previously and transovarial transmission of *B. burgdorferi* from mother tick to offspring is understood to be rare (Ostfeld and Keesing, 2000; Rollend et al., 2013). Due to seasonal timing of emergence and their small size preventing detection, nymphs are understood to be the commonest source of new infections to humans and non-human animals (Ostfeld and Keesing, 2000) (Figure 1).

In the Eastern United States, tick assemblages are generally made up of three species: *Ixodes scapularis* (black-legged or deer tick) *Dermacentor variabilis* (American
dog tick), and *Amblyomma americanum* (lone star tick). *Ixodes scapularis* is recognized as the primary vector of *B. burgdorferi* between hosts, but spirochetes have been isolated from all three tick species (Clark et al., 2002). Piesman and Sinsky (1988) found that *D. variabilis* and *A. americanum* are able to acquire *B. burgdorferi* as larvae but infection was short-lived and unlikely to be passed on to a new host during nymphal feeding.

Because transmission of *B. burgdorferi* through its enzootic cycle requires at least two feeding events, one to take up the bacterium and one to pass it to a new host, the host-seeking behavior of ticks contributes greatly to the spread of Lyme disease. Typically, ticks display two different kinds of search behaviors: ambush and attack (Oliver, 1989; Parola and Raoult, 2001). The ambush behavior (also known as “questing”) consists of climbing vegetation in the general environment and waiting for a host to pass by, while the attack behavior is when a tick actively moves toward its potential host. While both strategies ostensibly are effective means of reaching a host, *I. scapularis* employs a third strategy of waiting in the nests of host species to feed on them (Oliver, 1989; Parola and Raoult, 2001). This additional host-seeking behavior may increase the probability of locating a host, therefore allowing *I. scapularis* to more consistently move through its entire life cycle and subsequently transmit *B. burgdorferi* to new hosts.

Successful transmission of *B. burgdorferi* from a tick to the mammalian host is directly associated with the mechanism of tick feeding. Prior to attachment, ticks move through the host’s hair for several hours before inserting their mouthparts into the host skin and releasing salivary substances that anchor the tick to the host, as well as anesthetic, vasodilation, anticoagulant, and immunosuppressive compounds (Oliver,
The injected compounds aid in feeding success by diminishing host defenses and increasing blood flow to the attachment site. However, it has been shown that during the first 24-36 hours of attachment, there is very little ingestion of host blood. It is not until approximately three days after the initial attachment that the tick rapidly engorges itself on host blood (Oliver, 1989; Parola and Raoult, 2001). The timing of the feeding sequence affects the transmission of *B. burgdorferi* from tick to host. Hojgaard et al. (2008) found that *B. burgdorferi* is never transmitted from tick to host before 24 hours, and rarely during the 24-48 hour period. Prior to transmission *B. burgdorferi* displays increased expression of outer surface protein (Osp) A compared to OspC (Walker, 1998; Salyers and Whitt, 2002). Following ingestion of a blood meal by the tick, expression of OspC increases in response to the presence of certain blood components and an increase in temperature (Walker, 1998; Hojgaard et al., 2008). This change in relative expression releases *B. burgdorferi* from adherence to the tick midgut, passing it through the salivary compounds to the host circulatory system (Walker, 1998; Hojgaard et al., 2008).

**Reservoirs**

Numerous species have been recorded as infected by *B. burgdorferi* including mammals such as *Peromyscus leucopus* (white-footed mouse), *Odocoileus virginianus* (white-tailed deer), *Tamias striatus* (eastern chipmunk), *Sciurus carolinensis* (eastern gray squirrel), *Didelphis virginiana* (Virginia opossum), and *Procyon lotor* (northern raccoon), as well as birds including *Turdus migratorius* (American robin), *Dumetella carolinensis* (gray catbird), *Melospiza melodia* (song sparrow), and *Cardinalis cardinalis* (northern cardinal) (Ginsberg et al., 2005; Brunner et al., 2008). While all of these
species (and likely others) are capable of being infected, they vary greatly in competency as reservoirs (i.e. the likelihood of transmission of the bacteria to a feeding tick and the ability for the bacteria to replicate within the reservoir) (Ginsberg et al., 2005; Brunner et al., 2008).

Of the known reservoir taxa, rodents are understood to include the greatest number of species that can serve as competent reservoirs (Kollars, 1993). The primary reservoir for *B. burgdorferi*, largely responsible for maintaining its presence in the environment, is *P. leucopus* (Ostfeld et al., 1995). Additionally, *P. leucopus* is one of the principal hosts of *I. scapularis* (Ostfeld et al., 1995), creating an association that results in increased likelihood of successful progression through the *B. burgdorferi* sylvatic cycle. Studies have shown that 40-90% of the larval ticks that feed on an infected *P. leucopus* take up *B. burgdorferi*, subsequently enabling them to pass the bacteria to a new host (Fish and Daniels, 1990; LoGiudice et al., 2003). Other rodent species such as *T. striatus* and *Microtus pennsylvanicus* (meadow vole) have been found to have intermediate levels of reservoir competency (Brunner et al., 2008) further contributing to the maintenance of *B. burgdorferi* in an ecosystem.

Questions remain about the ability of reservoir species to clear *B. burgdorferi* through an immune response (Hofmeister et al., 1999) but the ability to infect a vector is understood to decline over the course of infection in some species, including *M. pennsylvanicus* (Markowski et al., 1998). However, *B. burgdorferi* is to some degree capable of evading the host immune system (Steere et al., 2004). This suggests that some other mechanism, such as sequestering the bacterium in tissue, may enable an infected reservoir to reach a state where they are no longer infective to a feeding tick.
While *O. virginianus* is capable of being infected, they do not contribute to the dispersal of *B. burgdorferi* as transmission from *O. virginianus* to ticks is extremely unlikely, making them an incompetent reservoir (Telford et al., 1988). However, they do contribute to the system by maintaining populations of *I. scapularis* that heavily parasitize deer throughout their range (Hanincova et al., 2006). The role *O. virginianus* plays in the ecology of Lyme disease is so important that the discovery of the disease in the late 20th century was coincident with the expansion of deer populations following their near extinction from over-hunting (Walker, 1998; Steere et al., 2004). With the multitude of hosts that are at least capable of intermediate competency as a reservoir for *B. burgdorferi*, it is likely that several enzootic cycles are occurring within endemic regions that together facilitate maintenance and transmission of the bacterium throughout the environment.

**Lyme disease Incidence**

The incidence of Lyme disease is on the rise in the United States. In 1991, Lyme disease became a Nationally Notifiable Disease, meaning that all human cases are required by law to be reported to the U.S. Centers for Disease Control and Prevention (CDC). This has facilitated the accrual of substantial data on the occurrence of the disease in humans. The number of cases reported annually has steadily increased during the last twenty years from 9,000 in 1992 to approximately 19,000 in 2006, to as high as almost 30,000 cases in 2009, and over 25,000 cases in 2014 (Bacon et al., 2007; CDC/DVBD, 2014). The disease appears to be heavily influenced by geography, with 96% of the cases reported in 2014 coming from 14 states that make up two separate endemic regions (CDC/DVBD, 2014) (Figure 9). One endemic region is the upper
Midwest including Wisconsin and Minnesota while the other largely comprises Connecticut, Delaware, Maine, Maryland, Massachusetts, New Hampshire, New Jersey, New York, Pennsylvania, Rhode Island, Vermont, and Virginia in the Northeast where the incidence of Lyme disease reached as high as 87.9 cases per 100,000 people in Maine during 2014 (CDC/DVBD, 2014).

In response to the high number of reported cases, much of the research into the ecology of *B. burgdorferi* has been conducted in the Northeast where the disease is common. However, *B. burgdorferi* has been isolated from different reservoir species (both avian and rodent) and ticks in southern states such as Florida, Georgia, South Carolina, and North Carolina, where the disease is not thought to occur endemically (Oliver et al., 1995; Oliver et al., 2000; Ryan et al., 2000). These patterns invoke questions regarding why there are so few cases reported in the Southeast relative to the Northeast, even though the etiological agent has been isolated. Several hypotheses have been proposed to explain this pattern. Anthropologic hypotheses such as underreporting of cases due to a lack of physician awareness of the disease and lower human population density in the Southeast provide one approach to explaining the lack of cases. However, another approach involves explanations at the biological and ecological level. One of the most prevalent ecological hypotheses suggests that Lyme disease is rare in the Southeast due to the relative absence of *B. burgdorferi* and tick vectors readily capable of transmitting the bacterium to humans. However, numerous studies have indicated that *B. burgdorferi* is present throughout the Southeast (Kollars, 1993; Levine et al., 1993; Sonenshine et al., 1995; Norris et al., 1996; Ouellette et al., 1997; Lin et al., 2001; Clark et al., 2002; Clark, 2004; Oliver et al., 2008). Therefore, it seems likely that factors other
than *B. burgdorferi* presence are influencing the maintenance and transmission of *B. burgdorferi* throughout the southern United States. Past studies have indicated that factors such as imperfect synchrony of the tick life cycle with reservoir population spikes preventing infection of new hosts, increased availability of incompetent reservoirs (e.g. herpetofauna) as potential food sources for ticks, and differing feeding behavior in nymphal ticks may be minimizing transmission of *B. burgdorferi* in the southern United States (Oliver 1996; Walker, 1998; Jacobs et al., 2003).

The purpose of this study was first to determine whether *B. burgdorferi* is present within rodent reservoirs in south-central Kentucky. Additionally, the study sought to identify any ecological explanations for the lack of reported cases in Kentucky by investigating the relationship between *B. burgdorferi*, rodent reservoir communities, and the ticks that vector the bacteria throughout the environment. We hypothesized that *B. burgdorferi* would be present in different rodent reservoirs, but at a lower prevalence than what is observed in the endemic regions. We also hypothesized that the prevalence of *B. burgdorferi* would change throughout the year with periods of higher prevalence not aligning with periods of peak *I. scapularis* abundance and human activity in the outdoors (i.e. summer). It was suspected that much of the rodent assemblage would be comprised of *P. leucopus*, providing an ample abundance of competent reservoirs. Therefore, in order to explain the diminished prevalence of *B. burgdorferi*, we hypothesized that *I. scapularis* would be observed parasitizing rodents at a substantially lower prevalence than other tick species.
MATERIALS AND METHODS

Small Mammal Trapping and Sampling

Small mammals were trapped from November 2014 – October 2015 on Western Kentucky University’s (WKU) Green River Preserve (GRP) (Figure 2). Trapping grids were established in three different habitat types: young lowland forest, early successional old field, and mixed-age upland forest. The young lowland forest site consisted primarily of *Platanus occidentalis* (American sycamore), *Juglans nigra* (eastern black walnut), and *Fraxinus* spp. (ash trees) with dense ground cover at an elevation of 156 m above sea level (the Green River located adjacent to the site is at 148 m above sea level). Extensive logging of the site occurred approximately 40 years prior to this study removing all large trees. The early successional old field site consisted of a mix of cold and warm season grasses along with a mix of forbs and flowering annuals. Ten years prior to this study, the site was in agricultural production (primarily tobacco) and seven years prior it was replanted in an attempt to restore a prairie habitat. Lastly, the mixed-age upland forest site consisted largely of *Quercus alba* (white oak), *Acer saccharum* (sugar maple), and *Carya* spp. (hickory trees) with a sparse understory and little ground cover at an elevation of 225-235 m above sea level on a westward-facing slope. Selective logging was previously performed on the site leaving many large trees as well as allowing for regeneration of younger trees on the site.

Trapping grids were composed of 100 Sherman live traps (Model LNG, H. B. Sherman Traps, Tallahassee, FL) in a 200x50 m grid with traps placed 10 m apart. Trapping grids were numbered 1 (young lowland forest), 2 (early successional old field), and three (mixed-age upland forest) (Figure 2), with each trap within a grid assigned an
identifier (A1 to E20). Traps were baited with rolled oats and peanut butter (Great Value Foods, Bentonville, AR). Trapping grids were checked for captures and closed within two hours of sunrise and reopened approximately two hours before sunset for three consecutive days during each month of the study, except for February 2015 when trapping occurred for two days due to heavy snowfall.

Captured small mammals were transported within the trap to an on-site field laboratory for processing. Locations of all captures and sprung traps without a capture were recorded. Captured small mammals were first identified as either an initial capture or recapture. Small mammals were then identified to species, sex, and age using diagnostic characteristics. Standard measurements including total length, tail length, hind foot length, ear length, and mass were recorded for each initial capture of an individual during a particular month. Reproductive state (testes position for males and vaginal condition and whether lactating for females) was also recorded. Small mammals were then carefully examined with both fine-tipped and entomological forceps for any ectoparasites (attached and unattached) which were subsequently collected into vials of 70% ethanol. Sampling effort was standardized by searching each individual’s pelage, ears, and anal region for two minutes. Additionally, bags used to transfer each small mammal from the trap to hand were searched for any parasites that came off the host. Following parasite sampling, mammals were anesthetized by isoflurane inhalation. Blood was collected by irritation of the retro-orbital sinus with a Pasteur pipet and subsequently transferred into a collection tube containing the anticoagulant K2-EDTA (Terumo Medical Products, Somerset, NJ). Small mammals were then ear tagged (Model 1005-1 Stamped Number, National Band & Tag Company, Newport, KY) with unique numbers
and released at the site of capture. Small mammal handling and sampling protocols were approved by the Western Kentucky University Institutional Animal Care and Use Committee in protocol #14-22.

**Collection of off-host Ticks and Ticks on White-Tailed Deer**

Off-host ticks were sampled by a tick dragging method. A 1 m², white corduroy cloth was dragged along three 100 m transects within each trapping grid. Tick dragging was conducted every three months beginning in January 2015. The drag cloth was inspected every 20 meters and any ticks present on the cloth were collected into vials of 70% ethanol.

Ticks were also collected from white-tailed deer in November 2014 and November 2015. Deer harvested by hunters on the GRP were examined for attached and unattached ticks by searching through hair and on the ears, nape of neck, and anal region. Any ticks present were collected into vials of 70% ethanol.

**Tick Identification**

All ticks were stored in 70% ethyl alcohol for transport to WKU, sorted and enumerated under 5-12x magnification. Ticks were identified to species and life stage by following pictorial keys from the University of Rhode Island TickEncounter Resource Center Tick Identification Chart (URI, 2016).

**DNA Isolation**

Genomic DNA was isolated from 50-100 µl of collected small mammal blood, 25 mg of renal tissue, and 10 mg of splenic tissue. Renal and splenic tissues were collected during necropsy of any individual that died during the study. DNA was isolated using a DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) according to the manufacturer’s
instructions, with the exception of the incubation period of tissue extended to overnight. DNA concentration was measured using a Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA). DNA samples were stored at -80°C.

**Polymerase Chain Reaction (PCR)**

DNA samples were analyzed for the presence of *B. burgdorferi* DNA by polymerase chain reaction using oligonucleotide primers specific to the OspA gene (F, 5’-TATTTATGGGAATAGGTC-3’ and R, 5’-GACTCAGCACCTTTTTG-3’; Integrated DNA Technologies, Coralville, IA) (Bunikis et al., 2004). For each sample a 25 µl reaction mixture was prepared containing 12.5 µl of GoTaq Hot Start Colorless Master Mix, 2 µl (10 µM) of each primer, 50 ng of template DNA, and sterile nanopure water to bring the volume to 25 µl. Each PCR plate contained a negative control with sterile nanopure water in place of template DNA and a positive control containing *B. burgdorferi* strain 31 genomic DNA (ATCC, Manassas, VA) as a template. PCR plates were loaded into an automated thermal cycler (Mastercycler pro, Eppendorf, Hauppauge, NY). Reaction conditions were as follows: 40 amplification cycles of denaturation at 95°C for 30 seconds, primer annealing at 55°C for 60 seconds, and extension at 72°C for 90 seconds. PCR products were stored at -20°C until they were analyzed by agarose gel electrophoresis on 1% agarose gels using ethidium bromide staining. To prevent contamination, all PCR procedures were carried out in a biological safety cabinet using a dedicated set of pipettes with aerosol barrier pipette tips. Additionally, *B. burgdorferi* strain 31 genomic DNA was added to the positive control in each PCR plate in a separate room with a different pipette than the one that was used to prepare the sample reactions.

**Sequencing**
Six samples were selected for genetic sequencing to confirm identification of *B. burgdorferi*. Selected samples included two PCR-positive samples each of blood, renal, and splenic tissue collected from small mammals. The selected samples were run through a new PCR reaction using the same conditions as previously described. The resultant PCR products were then frozen at -20°C until being shipped on dry ice overnight to the Genomic Sciences Laboratory (GSL) at North Carolina State University (Raleigh, NC, USA). Samples were sequenced by Sanger Sequencing on an ABI 3730 DNA Analyzer using the Platinum Sequencing option offered by the GSL. Generated sequences were analyzed by BLAST analysis and compared to sequences in the National Center for Biotechnology Information (NCBI) GenBank database. Sample ID and maximum identity were recorded for each of the sequenced samples.

Data Analyses

All statistical analyses were conducted using the statistical program R (R Core Team, 2015) and α was set at 0.05. Prevalence of *B. burgdorferi* was assessed among mammalian species, age, sex, and season (Dec-Feb = Winter, March-May = Spring, June-Aug = Summer, and Sep-Nov = Fall) in both blood and tissue samples in separate analyses. Because multiple blood samples were collected from several individual small mammals, the definition of an observation differed by test. When comparing prevalence among mammal species, a single observation was the combination of all collected blood samples from an individual animal throughout the study. For assessing prevalence by mammal age and sex an observation was defined as all collected blood samples from an individual when it was identified as either an adult or sub-adult. For assessing prevalence by season an observation was defined as all samples collected from an individual during a
particular season. A single PCR positive result within any of the blood samples that were combined to make an observation resulted in the observation being labeled as a positive for *B. burgdorferi*. An individual was determined to be positive for *B. burgdorferi* in tissue if a PCR positive result was obtained from either the spleen or kidney collected from that individual.

Prevalence of *B. burgdorferi* in blood and tissue was compared among mammal species and seasons by chi-squared goodness of fit tests. Follow-up comparisons were made using the chisq.post.hoc function in the R package “fifer” (Fife, 2014). To reduce the increased risk of committing a Type I error associated with multiple comparisons, a Bonferroni correction was used while performing the post-hoc comparisons.

Prevalence of *B. burgdorferi* in blood and tissue was compared by age and sex of the mammal by creating a three-way contingency table. Analysis of the three-way contingency table was conducted by comparing generalized linear models involving different interactions with the presence or absence of *B. burgdorferi*. Interactions included were the effect of age independent of sex, sex independent of age, and the interaction of age and sex together. These models were then compared to a base model without any effects added by analysis of deviance using a likelihood ratio test.

The difference in prevalence of *B. burgdorferi* between small mammal blood and tissue was compared by a chi-squared goodness of fit test using the same observation definition as was used when assessing prevalence by species.

Exposure to *B. burgdorferi* was compared among mammal species, age, and sex. Exposure was defined as a PCR-positive result in any sample that was collected from an individual throughout the study regardless if the positive result was from tissue or blood.
It was reasoned that a PCR positive result in any sample meant that the individual small mammal had previously been fed on and infected by an infected tick. Differences in exposure among species were assessed by a chi-squared goodness of fit test. Follow up comparisons were made using the chisq.post.hoc function in the R package “fifer” (Fife, 2014). To reduce the increased risk of committing a Type I error associated with multiple comparisons a Bonferroni correction was used while performing the post-hoc comparisons. Any potential differences in exposure by mammal age and sex were assessed by a three-way contingency table as described previously.

Tick prevalence was assessed for differences in mammal age, sex, season, and site by creating three way contingency tables and analyzing them in the same manner as B. burgdorferi prevalence by sex and age. Age and sex were assessed in one table and season and site in another. Follow up comparisons were made using the pairwise.G.test function in the R package “RVAideMemoire” (R Core Team, 2015). To reduce the increased risk of committing a Type I error associated with multiple comparisons a Bonferroni correction was used while performing the post-hoc comparisons.

Tick prevalence was also examined by a G-test of independence comparing prevalence of an individual tick species on each mammal species. Follow up comparisons were made using the pairwise.G.test function in the R package “RVAideMemoire” (R Core Team, 2015). To reduce the increased risk of committing a Type I error associated with multiple comparisons a Bonferroni correction was used while performing the post-hoc comparisons.

To examine whether mean intensity of tick infestation was affected by age, sex, season, or site, two-way analyses of variance testing the interaction of age and sex and
the interaction of season and site were performed. Intensities were calculated by adding up all the ticks present on an individual small mammal regardless of species. Post-hoc comparisons were made using a Tukey’s HSD test. Mean intensities of each tick species were compared using a parametric one-way analysis of variance between the different mammal species. The assumption of normally distributed data in an ANOVA was tested using the Shapiro-Wilk normality test and when appropriate a non-parametric ANOVA using resampling was performed instead.
RESULTS

A total trapping effort of 10,500 trap nights resulted in 748 captures (7.12% trapping success). Three hundred and thirty-six unique animals were captured comprising seven species: *Blarina brevicauda* (northern short-tailed shrew), *Microtus ochrogaster* (prairie vole), *M. pinetorum* (woodland vole), *Peromyscus leucopus* (white-footed mouse), *P. maniculatus* (deer mouse), *Reithrodontomys humulis* (eastern harvest mouse), and *Zapus hudsonius* (meadow jumping mouse) (Table 1). Of these, *B. brevicauda*, *M. pinetorum*, and *Z. hudsonius* were so infrequently captured that they were excluded from statistical analyses of prevalence of *Borrelia burgdorferi* or prevalence and mean intensity of ticks.

Optimization of the PCR reaction with the OspA primer set resulted in a *B. burgdorferi* positive PCR result of ~790 bp. Of 381 blood samples analyzed, 63 were positive. Those 63 blood samples were collected from 57 individual small mammals. Over the course of the study, 85 individuals died during trapping and renal and splenic tissue was collected and analyzed from all of them. Twenty-seven renal samples and 36 splenic samples were positive for *B. burgdorferi*. Nine individuals were positive in both tissue types. Of the 54 individuals with a PCR positive from tissue, 28 had a blood sample taken during the two days prior to their death. Of those 28, four individuals had a positive result from the blood sample taken during that time as well as a tissue sample, with two of the four having positive results from blood, renal, and splenic tissue.

Attempts to sequence *B. burgdorferi* from the selected samples were unsuccessful. However, a sequence was generated from the positive control *B. burgdorferi* strain 31 genomic DNA (Figure 10). The generated sequence was identified
as the *B. burgdorferi* OspA gene by BLAST analysis. While the unsuccessful results from the samples are confounding, the successful attempt to sequence the positive control DNA allows us to conclude that the primer set used in the PCR reaction was targeting the OspA gene of *B. burgdorferi*. Through this we are able to infer that all other PCR positive samples did include *B. burgdorferi* DNA.

Prevalence of *B. burgdorferi* in collected small mammal blood varied by season (n = 298, $X^2 = 33.132$, df = 3, $p < 0.001$) but not by mammal species (n = 262, $X^2 = 7.113$, df = 3, $p = 0.068$), age (n = 283, Deviance = 2.053, df = 4 & 3, $p = 0.152$), sex (n = 283, Deviance = 0.462, df = 4 & 3, $p = 0.497$) (Figure 11) or by age x sex interaction (n = 283, Deviance = 3.113, df = 4, $p = 0.539$) (Figure 12).

Prevalence of *B. burgdorferi* did not vary between collected small mammal renal and splenic tissue ($X^2 = 1.614$, df = 1, $p = 0.204$), therefore for further analyses of prevalence in tissue a positive PCR result in either the kidney or spleen of an individual was considered a positive in the analyses (i.e. “tissue positive”). Prevalence of *B. burgdorferi* in small mammal tissue did not vary by species ($X^2 = 4.591$, df = 3, $p = 0.204$), season ($X^2 = 4.496$, df = 3, $p = 0.213$), age (Deviance = 0.026, df = 4 & 3, $p = 0.872$), sex (Deviance = 0.152, df = 4 & 3, $p = 0.697$) (Figure 13) or by age x sex interaction (Deviance = 3.246, df = 4, $p = 0.518$) (Figure 12).

Prevalence of *B. burgdorferi* varied between collected small mammal blood and tissue ($X^2 = 49.576$, df = 1, $p < 0.001$). Prevalence in tissue was 63.5% while the prevalence in blood was 21.8%.

Exposure to *B. burgdorferi* varied among species (n = 301, $X^2 = 16.741$, df = 3, $p < 0.001$) and mammal age (n = 322, Deviance = 6.971, df = 4 & 3, $p = 0.008$) but did not
vary by sex (n= 322, Deviance = 0.056, df = 4 & 3, p = 0.813) (Figure 14) or by the interaction of age x sex (n = 322, Deviance = 7.185, df = 4, p = 0.126) (Figure 12).

Prevalence of ticks did not vary by host age (Deviance = 0.837, df = 4 & 3, p = 0.360), sex (Deviance = 1.427, df = 4 & 3, p = 0.232), or by age x sex interaction (Deviance = 8.302, df = 4, p = 0.081). However, prevalence of ticks varied by season (Deviance = 64.946, df = 17 & 14, p < 0.001), site (Deviance = 13.667, df = 17 & 15, p = 0.001) (Figure 3), and by season x site interaction (Deviance = 162.680, df = 17, p < 0.001) (Figure 4). Mean intensity of ticks varied by host age (F₁ = 5.312, p = 0.025) but not by host sex (F₁ = 2.463, p = 0.122) or by age x sex interaction (F₁, 54 = 0.898, p = 0.348). Mean intensity of ticks did not vary by season (F₂ = 2.983, p = 0.059), site (F₂ = 1.827, p = 0.171), (Figure 5), or by the interaction of season x site (F₂, 52 = 0.563, p = 0.573) (Figure 4). Winter was excluded from the analyses for mean tick intensity by season because no individuals examined during winter harbored ticks.

Results of the analyses comparing the prevalence and mean intensity of each tick species on each mammal species are presented in Table 3. *Dermacentor variabilis* was the only tick species with prevalence that varied between mammal species (Figure 8A). Due to small sample size, ANOVAs were not conducted to compare mean intensity of *I. scapularis* and *A. americanum*. Results of the Shapiro-Wilk normality test conducted on the distribution of intensity values showed that *D. variabilis* had a non-normal distribution of intensities; as such the ANOVA conducted for *D. variabilis* mean intensity was a non-parametric ANOVA using resampling.

The tick dragging method to collect off-host ticks in the environment resulted in a total spatial area of 3,600 m² sampled from all sites and seasons combined. From this
sampling, a total of 29 ticks were collected. All 29 were collected during April and July 2015 with no ticks being collected during either the winter or fall dragging attempts. Of the total 29 ticks collected 28 (96.5%) were A. americanum in nymph and adult life stages. The single other tick collected was an adult female D. variabilis. A total of 13 white-tailed deer were also examined for ticks, six in November 2014 and seven in November 2015. All ticks collected from deer were adults. In 2014, a total of 39 ticks were collected from three of the six deer examined. Total ticks collected of each species were 5 (12.8%) I. scapularis, 3 (7.7%) A. americanum, 28 (71.8%) D. variabilis, and 3 (7.7%) Rhipicephalus sanguineus (brown dog tick). In 2015, a total of 29 ticks were collected from five of the seven deer examined. Total ticks collected of each species were 2 (6.9%) I. scapularis and 27 (93.1%) D. variabilis.
DISCUSSION

Cases of Lyme disease have been reported to the CDC throughout the southeastern U. S. Presence of *B. burgdorferi* in reservoir species has been documented across much of the Southeast including North Carolina, South Carolina, Georgia, Tennessee, Florida, and Virginia (Kollars, 1993; Levine et al., 1993; Sonenshine et al., 1995; Norris et al., 1996; Ouellette et al., 1997; Lin et al., 2001; Clark et al., 2002; Clark, 2004; Oliver et al., 2008). Despite the presence of *B. burgdorferi* and the potential for transmission to humans, questions still remain about the tick vectors and reservoir hosts that maintain the sylvatic cycle of Lyme disease in the southeast. Additional challenges such as the similarity of other diseases like southern tick-associated rash illness (Master’s disease) to Lyme has complicated unequivocal identification and confirmation of Lyme disease. Much of what is known about the ecology of *B. burgdorferi* in the Southeast has been inferred from studies conducted in the northeastern U. S. where the majority of human cases have occurred.

In this study we investigated the *B. burgdorferi/vector/reservoir* system in the southeast U.S. by sampling small mammals and ticks in south-central Kentucky to assess *B. burgdorferi* ecology and the potential for transmission of *B. burgdorferi* to humans. Ticks were collected from the environment and hosts and small mammals were screened by PCR for *B. burgdorferi* presence to determine if the components of the *B. burgdorferi* ecologic cycle are present in south-central Kentucky.

We identified *B. burgdorferi* DNA in the blood and tissue of small mammals captured at the WKU Green River Preserve. Overall prevalence of *B. burgdorferi* in mammals examined by blood sampling was 21.8%, while prevalence in mammals
examined by tissue sampling was 63.5%. These results are consistent with other studies that found prevalence of *B. burgdorferi* to be higher in tissue than in blood. Anderson et al. (1985) sampled mice from Connecticut where 71.5% of collected spleens were positive for *B. burgdorferi* while only 31.3% of blood samples were positive. Callister et al. (1989) also found this pattern in wild *P. leucopus* in Wisconsin where *B. burgdorferi* was isolated from 57% of the spleens and 48% of the left kidneys examined, while only 39% of the blood smears analyzed were positive. *Borrelia burgdorferi* has no effect on the survival of rodent reservoirs so it is unlikely that higher prevalence in tissue is related to increased trap mortality associated with infection (Voordouw et al., 2015). Together, these results suggest that *B. burgdorferi* is present in the ecosystem of south-central Kentucky, ostensibly with the potential for *B. burgdorferi* to move through rodent and avian reservoirs to cause Lyme disease in humans.

To determine if an individual small mammal was exposed to *B. burgdorferi* at some time before or during this study all samples collected from an individual were analyzed by PCR. One hundred and seven of the 301 (35.6%) individuals examined were positive in at least one sample of any type. The results of this study fall within the observed prevalence ranges of *B. burgdorferi* in other studies conducted in the southeastern U.S. Oliver et al. (2003) examined *B. burgdorferi* prevalence in *Peromyscus gossypinus* (cotton mouse), *Sigmodon hispidus* (hispid cotton rat), and *Neotoma floridana* (eastern wood rat), all of which are potentially competent reservoirs. These species were sampled at several sites in Georgia, Florida, and South Carolina, resulting in a combined prevalence of 11.3% in Georgia, 6.5% in Florida, and 41.8% in South Carolina. Additionally, Sonenshine et al. (1995) found that 25-37% of small mammals in Virginia
had been exposed to *B. burgdorferi*. However, exposure to *B. burgdorferi* is often higher in the Lyme endemic regions of the northeastern and upper Midwestern U.S. Prevalence of *B. burgdorferi* in *P. leucopus* has been recorded as high as 88.2% in Wisconsin and 86.4% in Connecticut (Anderson et al., 1985; Anderson et al., 1987). Our results support our hypothesis that *B. burgdorferi* is present in the environment but likely at a prevalence that is comparable to the lowest observed prevalence in the Lyme disease endemic regions and substantially lower than the highest observed prevalence in highly endemic localities within the endemic regions. They also suggest that the difference in reported cases of Lyme disease in the endemic regions and non-endemic regions could be related to the differences in prevalence of *B. burgdorferi* in the environment which is affecting the likelihood of a potential vector becoming infected to pass the bacterium to a human. *D. variabilis* (which is an inefficient vector (Piesman and Sinsky, 1988)) was the most common tick collected. However, *D. variabilis* only occurred on up to 15% of the hosts and the prevalence of *B. burgdorferi* was relatively low in the blood of small mammals at 21.8%. Together this would result in a diminished likelihood of a *D. variabilis* encountering an infected vector and then taking up *B. burgdorferi* and later passing it on to a new host.

A wide variety of mammal species are known to be infected with *B. burgdorferi* and to serve as reservoirs for the bacterium. Small mammal species such as *T. striatus*, *S. hispidus*, *N. floridana*, *Mus musculus* (house mouse), *Cryptotis parva* (least shrew), and *M. pennsylvanicus* are among the many species capable of serving as reservoirs and infecting potential vectors of *B. burgdorferi* (LoGiudice et al., 2003; Oliver et al., 2003; Brunner et al., 2008). It has long been known that the most efficient reservoir for *B.*
Borrelia burgdorferi is *P. leucopus* with the closely related *P. maniculatus* not far behind in its competency as a reservoir (Rand et al., 1993). *Peromyscus maniculatus* occurs across a wide range of habitats and *P. leucopus* is almost ubiquitous throughout its range. The small mammal survey conducted in this study resulted in 76.5% of the individuals captured being either *P. leucopus* or *P. maniculatus* at a roughly even distribution (Table 1) implying that much of the small mammal community at the trapping locality consists of species understood to be very competent reservoirs. This would suggest that the reason for the relative lack of human cases of Lyme disease is not the lack of competent reservoirs, but rather some other factor. It would also suggest that the relatively low prevalence of *B. burgdorferi* observed during the study is not due to a dilution effect. The dilution effect is in play when high species diversity prevents a pathogen from becoming fully established in a community as the relative abundance of suitable hosts is low (Ostfeld and Keesing, 2000). The calculated Shannon-Wiener diversity index values (Table 5) show that each of the trapping grid sites had minimal small mammal diversity throughout the year. With the high number and proportion of competent reservoirs and subsequent low species diversity, it is likely that if all other components of the Lyme disease ecological cycle were present that the bacterium would be common in the environment.

Prevalence of *B. burgdorferi* among small mammal species did not vary in either blood or tissue collected during this study. Prevalence of *B. burgdorferi* infection in a mammal species is likely related to the species’ competency as a reservoir. *Peromyscus leucopus* is the most competent reservoir of *B. burgdorferi* in the wild and it has been noted that a sole infected tick feeding on *P. leucopus* is sufficient for the mouse to
develop infection (Donahue et al., 1987). *Peromyscus maniculatus* has also been reported as an efficient reservoir; up to 80% of *P. maniculatus* fed on by an infected tick for 96 hours become infected with *B. burgdorferi* (Peavey and Lane, 1995). The potential role of *M. ochrogaster* as a competent reservoir for *B. burgdorferi* has been noted previously. Zeidner et al. (2000) found that *M. ochrogaster* in Colorado had an 86.6% prevalence of *B. burgdorferi*; although similar to the present study this was not statistically different from other small mammal species in their study. The high prevalence of *B. burgdorferi* may be because *M. ochrogaster* is a competent reservoir. However, similar to the present study, their sample size was small (n = 17 for the present study, and n = 15 for Zeidner et al., (2000)), potentially skewing results. However, the likelihood of developing infection after being fed on by an infected tick is not the only characteristic of a competent reservoir. A competent reservoir is also highly efficient at remaining infective to feeding ticks so that a vector may become infected by feeding on them. An infected *P. leucopus* is capable of passing infection to nearly 100% of ticks that feed on it during the first 2-3 weeks of infection with infectivity decreasing, but still present, up to 200 days post infection (Donahue et al., 1987). *Peromyscus maniculatus* has been recorded as only infecting 33% of nymphal *Ixodes pacificus* that fed on an infected mouse (Peavey and Lane, 1995). Furthermore, the importance of other potentially competent reservoirs for *B. burgdorferi* has been identified as being minimal in locations where *P. leucopus* is abundant (Markowski, 1998). In this study *P. leucopus* accounted for 39% of the unique individuals captured. Even though the prevalence of *B. burgdorferi* infection did not differ among small mammal species it is likely that the high abundance of the extremely
competent reservoir, *P. leucopus*, would offset any effect of other species with varying reservoir competency.

Of the 57 mice identified as blood-positive for *B. burgdorferi*, only six were positive in two separate months over the course of study. These results contradict Hofmeister et al. (1999) who found that of 77 mice included in a longitudinal study of *B. burgdorferi* infection in Maryland, all 77 remained infected from the time of their first capture to their last (28-435 days). Our results suggest that some unidentified physiological mechanism may reduce infection of *B. burgdorferi* in the rodents observed during this study. One potential explanation is varying levels of pathogenicity between isolates of *B. burgdorferi* occurring in different regions. Significant genetic diversity exists within *B. burgdorferi* throughout North America, with substantial variation even occurring within regions (Bunikis et al., 2004; Mechai et al., 2016). Furthermore, genetic diversity of *B. burgdorferi* has been linked to different aspects of pathogenicity (Oliver, 1996; Baranton et al., 2001). Strains of *B. burgdorferi* that occur in Kentucky may differ genetically from strains in the endemic regions of the northeast and Midwestern U. S. In turn, this could result in decreased pathogenicity preventing long term infection in reservoirs as well as a potential explanation for the few cases of human Lyme disease in Kentucky. Additionally, infected reservoirs may in some way be clearing infection in their blood. Laboratory studies have found that mice are capable of clearing *B. burgdorferi* infection through an immune response orchestrated by macrophages (Lasky et al., 2015). Our results of higher prevalence in tissue also raise the question of whether infected reservoirs are capable of clearing infection in their blood but maintaining infection by sequestering spirochetes in their tissues. This might serve to limit the
likelihood of *B. burgdorferi* uptake during a tick’s blood meal. However, Anderson et al. (1985) concluded that individuals positive for *B. burgdorferi* in their spleen and kidneys were still infectious to feeding ticks.

The majority of ticks collected during the present study were *Dermacentor variabilis*, followed by *Amblyomma americanum*, and *Ixodes scapularis*. *Ixodes scapularis* is widely recognized as the primary vector of *B. burgdorferi* between hosts in the eastern U.S. (Gray, 1998). Infection of *I. scapularis* larvae through feeding on an infected reservoir has been recorded at 75%, with transstadial nymphal infection rates of 80% (Dolan et al., 1997). They are also extremely efficient at passing infection to new hosts (Piesman and Sinsky, 1988). However, the relative lack of *I. scapularis* in this study suggests that another vector may be responsible for the majority of *B. burgdorferi* transmission in the system. The contribution of *A. americanum* to the spread of Lyme disease is probably minimal even though they are present as suggested by tick dragging. *Amblyomma americanum* is known to have borreliacidal agents in its saliva preventing *A. americanum* from serving as efficient vectors for *B. burgdorferi* (Ledin et al., 2005).

*Dermacentor variabilis* is capable of becoming infected with *B. burgdorferi* as a larva but the infection is inefficient and often short-lived (Piesman and Sinsky, 1988; Dolan et al., 1997). In Indiana, a state which has an incidence rate for Lyme disease over seven times higher than Kentucky, *I. scapularis* comprised approximately 40% of the ticks collected from rodents (CDC/DVBD, 2014; Rynkiewicz and Clay, 2014). This would suggest that what few *I. scapularis* are present on rodents in Kentucky are likely contributing to the transmission of *B. burgdorferi* between hosts but their diminished abundance relative to
the high abundance of the capable, but inefficient vector, *D. variabilis* may contribute to the relative paucity of Lyme disease cases in the region.

The peaks of *B. burgdorferi* prevalence observed in this study occurred during spring and fall. These results are contrary to the accepted paradigm that peak prevalence occurs during summer when nymphal *I. scapularis* and human activity are both high (Hofmeister et al., 1999; CDC/DVBD, 2014). These results support a hypothesis developed by Oliver (1996) that there is diminished synchrony of the *I. scapularis* life cycle and the peak prevalence of *B. burgdorferi* in rodent reservoirs. Additionally, it is interesting that rather than coinciding with the *I. scapularis* life cycle, the observed seasonal peak prevalence is more closely associated with the life cycle of *D. variabilis*. Spring was defined as the months of March, April, and May which is also the time of peak abundance of *D. variabilis* larva and nymphs in South-Central Kentucky (URI, 2016). The coincidence of peak prevalence of *B. burgdorferi* and *D. variabilis* abundance supports our conclusion that *D. variabilis* is largely serving as the tick vector of *B. burgdorferi* in South-Central Kentucky.

In summary, *B. burgdorferi* does appear to be present and moving through a sylvatic cycle in south-central Kentucky. However, prevalence of the bacterium in the region is comparable to the lowest observed prevalence in the Lyme disease endemic regions of the northeast and Midwestern U.S. Additionally, there appears to be adequate abundance of efficient reservoirs in *P. leucopus* to maintain the presence of *B. burgdorferi* in the environment. We suggest that a likely explanation for the few reported cases of human Lyme disease in Kentucky is the relative lack of an efficient vector.
While *I. scapularis* was collected during this study the numbers were paltry compared to *D. variabilis*, a capable but inefficient vector of *B. burgdorferi*.

Additional studies of the ecology of *B. burgdorferi* in Kentucky are needed to determine the prevalence of and risk of contracting *B. burgdorferi* infection. This study was limited to three trapping grids at one location and is of limited geographical coverage. Patterns uncovered here may or may not be extrapolated to other sites in the region. Additionally, the recent discovery of a new species of bacteria (*Borrelia mayonii*) capable of causing Lyme borreliosis adds another level of complexity to the ecology of Lyme disease. While *B. mayonii* appears to be limited to only a few cases in Minnesota and Wisconsin (Pritt et al., 2016), additional field studies may determine its range is larger than currently known. Future studies are also needed to more accurately describe the vector competence of *D. variabilis* and *A. americanum* to determine the role they are playing in the spread of *B. burgdorferi*. Finally, the dynamics of climate change have caused an increase in cases of infectious diseases, specifically diseases caused by tick-borne pathogens as *Ixodes* ticks increase their range (Ostfeld and Brunner, 2015).

Continued surveillance for *B. burgdorferi* in rodents and other reservoir species in regions not previously considered to be Lyme endemic will be important to further understand the risk of humans contracting Lyme disease as the climate continues to change.
LITERATURE CITED


Voordouw, M. J., S. Lachish, and M. C. Dolan. 2015. The lyme disease pathogen has no effect on the survival of its rodent reservoir host. PloS ONE 10: e0118265.


Figure 1. Generalized life cycle of Ixodidae (hard ticks). (CDC/DVBD, 2014)
Figure 2. Aerial view of a portion of the WKU Green River Preserve. Trapping grids are outlined in white and numbered. The yellow bar depicts 100 m. (© 2015 Google)
Figure 3. Prevalence of ticks by (A) host sex, (B) host age, (C) season, and (D) site. Lowercase letters depict statistically significant differences in prevalence at $\alpha = 0.05$. 
Figure 4. Season x site interactions of (A) prevalence of ticks, (B) prevalence of fleas, (C) mean intensity of ticks, and (D) mean intensity of fleas. Lines with square points depict young lowland forest, circles depict early successional old field, and triangles depict mixed-age upland forest.
Figure 5. Mean intensity of ticks by (A) host sex, (B) host age, (C) season, and (D) site. Lowercase letters depict statistically significant differences in mean intensity at $\alpha = 0.05$. Error bars depict the standard error.
Figure 6. Prevalence of fleas by (A) host sex, (B) host age, (C) season, and (D) site. Lowercase letters depict statistically significant differences in prevalence at $\alpha = 0.05$. 
Figure 7. Mean intensity of fleas by (A) host sex, (B) host age, (C) season, and (D) site.

Error bars depict the standard error.
Figure 8. Prevalence of (A) *Dermacentor variabilis*, (B) *Ctenophthalmus pseudagyrtes*, and (C) *Peromyscopsylla hesperomys*. Abbreviations of small mammal species are PL (*Peromyscus leucopus*), PM (*P. maniculatus*), MO (*Microtus ochrogaster*), and RH (*Reithrodontomys humulis*). Lowercase letters depict statistically different prevalence at $\alpha = 0.05$. 
Figure 9. Map of confirmed human cases of Lyme disease during 2014 (CDC/DVBD, 2014).
**B. burgdorferi OspA gene**

Sequence ID: `emb|X69605.1`  Length: 822  Number of Matches: 1

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Figure 10. Sequence alignment of the generated sequence from positive control *Borrelia burgdorferi* strain 31 genomic DNA. Lower case “a” within the query sequence represents a low complexity sequence that was filtered by BLAST to prevent any artificial hits between sequences that were not truly related.
Figure 11. Prevalence of *B. burgdorferi* in small mammal blood by (A) sex, (B) age, (C) season, and (D) species. Lowercase letters depict statistical significance at $\alpha = 0.05$.

Abbreviations of small mammal species are PL (*Peromyscus leucopus*), PM (*P. maniculatus*), MO (*Microtus ochrogaster*), and RH (*Reithrodontomys humulis*).
Figure 12. Age x sex interactions of *B. burgdorferi* in (A) small mammal blood, (B) tissue, and (C) either blood or tissue. Dashed lines represent females and solid lines represent males.
Figure 13. Prevalence of *B. burgdorferi* in small mammal tissue by (A) sex, (B) age, (C) season, and (D) species. Abbreviations of small mammal species are PL (*Peromyscus leucopus*), PM (*P. maniculatus*), MO (*Microtus ochrogaster*), and RH (*Reithrodontomys humulis*).
Figure 14. Exposure of an individual to *B. burgdorferi* by (A) sex, (B) age, and (C) species. Lowercase letters depict statistical significance at $\alpha = 0.05$. Abbreviations of small mammal species are PL (*Peromyscus leucopus*), PM (*P. maniculatus*), MO (*Microtus ochrogaster*), and RH (*Reithrodontomys humulis*).
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<th>Individuals Captured</th>
<th>Individuals recaptured</th>
<th>Average Number of Recaptures</th>
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<td><em>Peromyscus leucopus</em></td>
<td>318 (42.51)</td>
<td>131 (38.99)</td>
<td>68 (44.16)</td>
<td>2.75</td>
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<td><em>Reithrodontomys humulis</em></td>
<td>65 (8.69)</td>
<td>45 (13.39)</td>
<td>11 (7.14)</td>
<td>1.82</td>
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<td><em>Microtus ochrogaster</em></td>
<td>32 (4.28)</td>
<td>29 (8.63)</td>
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<td>1 (0.30)</td>
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<td><em>Blarina brevicuada</em></td>
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<td>1 (0.30)</td>
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<td><strong>Total</strong></td>
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Table 1. Total captures, unique individuals captured, individuals recaptured, and the average number of recaptures of recaptured individuals of the seven small mammal species captured during the study. Numbers in parentheses depict the percentage of the total for each column.
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<th>Mites</th>
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<th>Ticks</th>
<th>Peromyscus leucopus</th>
<th>Peromyscus maniculatus</th>
<th>Microtus ochrogaster</th>
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<td><em>Androlaelaps fahrenholzi</em></td>
<td><em>Ctenophthalmus pseudagyrtes</em></td>
<td><em>Amblyomma americanum</em></td>
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<td><strong>Prevalance</strong></td>
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Table 2. Prevalence and mean intensity of the nine species of ectoparasites collected during the study on *P. leucopus, P. maniculatus, M. ochrogaster*, and *R. humulis*, the four most common mammal species captured.
Table 3. Results of G-tests of independence and non-parametric ANOVAs comparing prevalence and mean intensity of parasite infestation among mammal species. G-tests of independence were all conducted with 3 degrees of freedom. Significant results are marked with an asterisk.

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<td><strong>Ticks</strong></td>
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<tr>
<td><em>Dermacentor variabilis</em></td>
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<td><strong>Fleas</strong></td>
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<td>9.675</td>
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<tr>
<td><em>Peromyscopsylla hesperomys</em></td>
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<td>&lt;0.001*</td>
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<td><strong>Mites</strong></td>
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<td><em>Androlaelaps fahrenholzi</em></td>
<td>1.977</td>
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Table 4. Schnabel population estimates of each trapping site during winter, spring, and summer. The numbers outside the parentheses depict the calculated population size and the numbers inside the parentheses depict the 95% confidence interval using a Poisson distribution.

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<td>Upland Forest</td>
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<td>250 (157-417)</td>
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<td>Summer</td>
<td>83 (55-136)</td>
<td>231 (146-427)</td>
<td>89 (63-135)</td>
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Table 5. Shannon-Wiener diversity index values for each trapping site during each season and the total index values for the trapping sites and seasons.

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<th>Mixed-Age Upland Forest</th>
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