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Impact of Vector Range Expansion on Pathogen transmission Dynamics of Lyme Disease in Southeastern Virginia.

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Abstract: Blacklegged ticks, *Ixodes scapularis* is the primary vector of Lyme disease in eastern United States. *Borrelia Burgdorfei*, the etiological agent of Lyme disease is transferred by ticks of Ixodes species. In recent years, its congener, *Ixodes affinis* has been expanding its range northwards from its southern population. We were interested in studying how the introduction of this new vector affected the interaction between the pathogen genotype and the host. We hypothesized that differential host use by *I. affinis* and *I. scapularis* would partly explain observed differences in *B. burgdorferi* infection prevalence and genotypic structure in southeastern Virginia. The result from our analysis does not support our hypothesis. Both species of ticks were found on small rodents and mammals. Ticks and mammals were able to acquire wide range of pathogen genotypes. We believe that range expansion of *I. affinis* is driven by various biotic and abiotic factors. Further studies is needed to understand the competition between the two tick species for food and resources and to see how it impacts the spread of Lyme disease.

Introduction:

Direct transmission of pathogens among hosts is a relatively simple process in disease ecology whereas vector-borne transmission, which relies on susceptible hosts as well as competent vectors and their intersection in time and space is a more complex process. The variation in host species, reservoir competence for the pathogen, environmental factors such as climate, pathogens and interactions of these factors widely influences the host availability, vector abundance and determine the trajectory of pathogen distribution and spread of the infection (Aperson et al., 1993).

Tick borne infections are a threat to animal and human health throughout the world (Feunte et al., 2008). Lyme disease, transmitted by blacklegged tick *Ixodes scapularis*, is the most prevalent vector-borne disease in the United States (Bacon et al., 2008). The etiological agent of Lyme disease is the spirochete *Borrelia burgdorferi*, which is an internal parasite maintained in natural cycle by an infection cycle from ticks to mammals and then back to immature ticks (Pal et al., 2004). There is no evidence of vertical transmission of pathogen, therefore this enzootic cycle is important for the survival and proliferation of the spirochete in the natural reservoir (Patrican et al., 1997). Black legged ticks of genus, *Ixodes*, are known for transmitting the pathogen *B. burgdorferi* to mammalian hosts and ultimately to humans. *B.burgdorferi* infect a wide range of vertebrate animals including small mammals and birds (Hanincova et al., 2006).

Transmission of *B.burgdorferi* is dependent on ticks, therefore, tick life cycle and behavior are critical determinant of pathogen transmission. Most ticks go through four life stages: eggs, six-legged larva, eight legged nymph, and adult. After hatching eggs, ticks must eat blood at every stages to survive (Wang et al., 1997). Typically, eggs are laid during early

summer (June and July) and hatch into larvae in late summer. Larvae typically feeds on small vertebrates such as mice, voles and chipmunks and molt into nymphs during late summer. The nymphs then enter diapause and are dormant until the next spring. Then they emerge to feed on rodents, small mammals, birds and humans into the late spring and summer and molt into adults in the fall. Adult female ticks as well as nymphs and larvae feed on larger mammals such as deer, dogs, raccoons, and bite humans whereas males typically do not feed. The adult female tick then drops off these animals and lay eggs in spring, completing the two-year life cycle (Wang et al 1997). All ticks feed at least twice: once as larvae and again as nymphs, while female feeds a third time as adults (Bosler et al., 1993). For non-transovarially-transmitted pathogens, such as *B. burgdorferi* (Patrican et al., 1997), a tick is infected either as a larva or as a nymph and can pass the infection into another mammal only after molting into the next stage. The host range of a parasite, that is, whether it is a generalist or host specialist, is a vital life history trait that will affect both the parasite's population dynamics and its evolutionary trajectory. The level of host specialization of parasites is a key issue in infectious disease research because patterns of cross-species transmission affects parasite dispersal and can facilitate epidemics (Hanincova et al., 2006).

The addition of new species to a tick community has unpredictable impacts on pathogen prevalence and tick species community structure. The new tick may or may not be able to transmit the pathogen, it may have different seasonality, or it may feed on different host types that have different competence. In disease ecology when a new vector is introduced in a similar environment as primary vectors, infection prevalence either amplifies or dilutes. Because some host are incompetent for the pathogens, presence of these species can dilute the pathogen and

hence reduce the prevalence of the infection. On the other hand, if hosts are competent they amplify the pathogens in nature reservoir thus increasing the risk of disease

In the northeastern and midwestern United States, the primary tick species for human disease is *Ixodes scapularis* (the blacklegged tick). Based on climate and landscape modelling, these two regions in United States were identified as two discontinuous Lyme disease foci, with a transitional zone including sites with uninfected *I.scapularis* population (Diuk-Wasser et al., 2012). *Ixodes scapularis* is distributed across the United States, ranging from Florida to Nova Scotia, Canada, and west to North and South Dakota and Mexico (Kierans and Clifford, 1978). Host seeking nymphs are quite uncommon outside these two regions-midwestern and northeastern, that represent the two discontinuous Lyme disease foci (Duik-Wasser et al., 2012). Outside these region a transitional zone including sites with uninfected I.scapularis population exists (Diuk-Wasser et al., 2012).

Ixodes affinis, also a competent vector for *B. burgdorferi* but one that does not parasitize humans, is found in the southern United States , with established populations from Florida, Georgia, South Carolina, North Carolina and Virginia and appears to be expanding North (Nadolny et al., 2011). *Ixodes scapularis*, the principal vector for the Lyme disease bacterium in the eastern United States has existed in eastern Virginia for decades (Nadolny et al., 2011), but the congener *I. affinis* has only recently been reported in Virginia after expanding from southern source population (Nadolny et al., 2011). This new tick which is a sylvatic vector for *Borrelia burgdorferi* sensu stricto, the bacterial agent of Lyme disease has been expanding northward from historic ranges in the southern United States. (Nadolny et al., 2015)

The outer surface protein C (osp C) interacts with tick cells and salivary proteins (Brisson et al., 2004) and so variation in osp C could be linked with vector competence.The outer surface

protein C gene (ospC) is one of the most highly diverse proteins of the Lyme disease spirochete (Wilske et al., 1993). The genetic diversity of osp C has been classified into major group of alleles. An osp C major group is defined as group of alleles that are different in >8% of the nucleotide sequence from alleles in other major groups and <2% different from other alleles in the same major group (Wang et al., 1999). ospC strain diversity is maintained in the natural reservoir because of the interspecies multiple niche polymorphism.

Oliver et al. (2003) found that *I.scapularis* can transmit both *B.burgdorferi* and *B.bissettii*. Additionally, they also found out that the closely related but usually non-human biting *I.affinis* also experimentally transmitted the *B.burgdorferi* s.s isolate SI-1. Additionally, *I. affinis* and *I. scapularis* are both efficient vector of *B.bissettii* and *B.burgdorferi* and *I affinis* plays an important role as enzootic vectors of *B.burgdorferi* (Oliver et al., 2003). While there is a large amount of literature explaining the effect of *I.scapularis* and endemics of Lyme disease, very little is known and written about the competent vector *I. affinis*. How does the presence of competent vector affect the interaction between host species and *B.burgdorferi* genotypes? How does this affect the fitness and dynamics of the pathogens?

For vector-borne pathogens, shifts in vector or reservoir host species distribution can affect interactions between reservoirs and ultimately impact pathogen transmission dynamics. If enzootic Lyme disease is established by *Ixodes scapularis* ticks, strain diversity typical of this species should occur in the hosts. However if a new species of tick is introduced in the environment, there is a chance that the strains of osp C present will be diverse. The new species may bring new strains of the pathogen in the environment .If certain strains are adapted to particular ticks or hosts, we would expect to see exclusive associations with little mixing of genotypes within vectors or hosts.

The aim of the current study was to sample rodents in an area of southeastern Virginia where both *Ixodes* species are now sympatric to explore differences in: 1) patterns of host parasitism, and 2) infection prevalence with and genotypic variation of *B. burgdorferi* between vector species .We hypothesized that the vector of Lyme disease, *I. affinis* and *I. scapularis*, have different host preference and therefore they are able to keep separate lineages of bacteria apart, or different bacteria genotypes are adapted to each vector type which would partly explain observed differences in *B. burgdorferi* infection prevalence and genotypic structure in southeastern Virginia.

Methods

Tick collection

Ticks were collected from 2010-2014 from fourteen different sites in Southeastern Virginia by Robyn Nadolny and her crew at Old Dominion University using standard tick collecting methods, as previously described (Nadolny et al., 2011).

Tick species identification

All ticks collected were first photographed and identified morphologically using microscopic examinations. A tick ID key was used to identify the two species of *Ixodes* ticks (Kierans et al., 1989). Ticks were flash frozen in liquid nitrogen and pulverized following microscopic examinations. DNA was extracted using the DNeasy Blood and tissue Kit, according to the manufacturer's protocol (Qiagen Inc., Valencia, CA) DNA was eluted at a final volume of 100 µl and stored at -20^{o}C until processing.

Single Tube Real time PCR (aff/scapp assay)

Single tube real time PCR was performed following the established protocol (Wright et al., 2013). The primers amplified fragments of 75 and 142-144 bp for *I.affinis* and *I.scapularis*, respectively. Amplifications were performed in duplicate in 15-µL reaction volumes containing 1X Bio-Rad iQ™ SYBR® Green supermix, 0.5 µM of each primer (aff_f8, aff_r8, scap_f2.2, scap_r2.2), and 2 µL target DNA. Reaction conditions were an initial denaturation at 95°C for 3 min, followed by 40 cycles of 95°C for 15 s, 62°C for 30 s, and 72°C for 30 s, with a plate read after the 72°C (extension) step. A melt curve analysis was performed after amplification by cooling samples to 65°C and raising the temperature at 0.5°C intervals to 95°C for 5 s/interval and a plate read at each step. Real-time PCR reactions were performed on a CFX96™ thermocycler (Bio-Rad, Hercules, CA).

A 454 bp fragment of the tick mitochondrial 16S ribosomal RNA gene was amplified on an icycler (Bio Rad Laboratories, Hercules, CA) using 16S-1 (5'-GTCTGAACTCAGATCAAGT- 3') (Macaluso et al. 2003) and 16S+1 (5'-CTGCTCAATGATTTTTTAAATTGCTGT-3'). PCR products for sequencing were purified using Wizard PCR Preps DNA Purification System (Promega, Madison, WI), and sequencing reactions were performed. Blast search was performed to supplement morphological identification.

Screening for *Borrelia burgdorferi*:

To screen for presence of *B.burgdorferi*, a portion of the outer surface protein C (osp C) gener was amplified by nested PCR (Bunikis et al., 2004). Reactions were carried out in 20 µl volumes using 2 X Taq PLUS Master Mix and 2 µl of DNA template. The PCR protocol

consisted of a 2 minute denaturation at 95ºC followed by 45 cycles of 95ºC for 15 s and 60ºC for 30 s. PCR reactions were performed on an Eppendorf thermocycler. Presence of *B.burgdorferi* was determined by agarose gel electrophoresis.

Strain Identification

Positive amplicons were purified using MACHERY-NAGEL Nucleospin® Gel and PCR Cleanup kits and sequenced. BLAST search was performed to determine the osp C variant. Phylogenetic analysis of unknown and previously characterized variants were performed to determine which osp C types were present in our sites (Travinsky et al. 2010, EID).

Statistical Analysis

To determine whether significant differences exist in infection prevalence between the two different tick species a Fisher's exact test was performed. A chi square test was performed to see the relative frequency of *I .affinis* in host and host seeking *I.affinis*.

Results:

In the two years of this study, we sampled 81 small mammals in this system and detected *B. burgdorferi* DNA in 26 mammal tissues (32.1% overall prevalence, Table 1). Among the tissues that have been confirmed *Borrelia*-positive, we detected the highest infection prevalence in shrew (*Blarina* spp) and house mouse (*Mus musculus*) tissues (Figure 2). Highest no of *Ixodes* ticks were collected from these two species of rodents. Ticks were also found in White footed mouse and Cotton Rat but single tube real time PCR did not give us the id of the ticks. Because we were unable to id the ticks, we are not reporting the data from these samples. While both *Ixodes* ticks seem to parasitize a wide group of mammals (some of the sample sizes were

too low and hence have not been reported) certain host species seem to be preferentially parasitized by one or the other species: cotton rats (*Sigmodon hispidus*) and harvest mice (*Reithrodontomys humulis*) were parasitized exclusively by *I. affinis* whereas house mice (*Mus musculus*) were parasitized primarily by *I. scapularis*.

In total, 309 host seeking ticks and 339 host derived ticks were collected from 14 different sites in Southeastern Virginia (Figure 1). Host derived ticks were collected from three different sites (JC, KP, and ST). In general, *I. affinis* is more abundant than *I. scapularis* as a small mammal parasite in southeastern Virginia (Figure 3). Among the 339 host derived ticks, *B.burgdorferi* was detected in 85 ticks (25.1% infection prevalence). Infection prevalence in host-derived ticks of each species was comparable. We did not find any significant difference between the infection prevalence among the two vectors (*I. affinis* = 24.0%, *I. scapularis* =33.3%; G = 0.45, P > 0.5). Notably, *I. affinis* was found more frequently on hosts than the host-seeking *I. affinis* (Chi-sq = 10.8, p = 0.001).

In the 309 host seeking ticks, 25 were found to be infected with *Borrelia* with an infection prevalence of 8.1%. The infection prevalence was 9.7% and 5.5% among *I. scapularis* and *I. affinis* respectively (Figure 5). Of the 309 ticks, 181 (58.6%) were identified as *I. affinis* and 128 (41.4%) were identified as *I. scapularis*.

osp C variants of 62 ticks were determined using the blast search. Osp C variant "A" and "Hb" were detected more often than the rest (Table 2).The relative abundance of "Hb" variant was higher among mammals while the relative abundance of "A" osp C variant was found to be higher among the ticks (Figure 4). The frequency of all the other osp C variant detected in southeastern Virginia was similar. The distribution of osp C variant showed no significance difference between the different species of ticks. A3 and Hb variant were found frequently

among the host derived ticks, however other variant of osp C were fairly distributed among the host derived ticks as well.

Discussion

Both vectors of Lyme disease, *I.affinis and I.scapularis* were found at more or less same frequency on the different host types in Southeastern Virginia. Although there is no host preference among *I. affinis* and *I. scapularis* for a particular host types, *I affinis* was found to be more abundant than *I scapularis* in southeastern Virginia. *I. affinis* recently expanded northward from its historic ranges in southern United States (Harrison et al., 2010; Nadolny et al., 2011). Our results indicate that *I. affinis* is thriving despite having recently expanded into this new environment. Among both the host seeking ticks and host derived tick, the number of *I. affinis* was higher than the number of *I. scapularis*. This difference in the number of different species of *Ixodes* ticks might be because of our limited sampling of habitat and different communities of wildlife host or simply a sampling artifact. It is also possible that *I. scapularis* is parasitizing different hosts in these environment and therefore the number of *Ixodes scapularis* collected was lower than *Ixodes affinis*. Immature scapularis tend to feed on small mammals such as white-footed mice an Eastern chipmunks that have high reservoir competence for *B.burgdorferi*. Additionally, *I.* scapularis also parasitizes several species of lizards, including *Eumeces laticeps, E. fasciatus, Sceloporus undulatus,* and *S. occidentalis (*Apperson et al. 1993*).* Our sampling effort data does not include ticks and tissues from birds or lizards and this might explain the low sample size for *I.scapularis*.

We hypothesized that different species of ticks fed on different groups of mammals, avoiding competition in the environment where they coexisted. However, our result showed that there was an overlap in the vertebrate host parasitized by the two tick species (Figure 6). The ticks showed no preference in terms of parasitizing the vertebrate hosts. Both species actively parasitized and fed blood-meal from a wide range of mammalian hosts in areas where the two

ticks coexists. Our result does not support the hypothesis that there is host specialization/ host preference among either species of ticks. Since host will be infected with a large and heterogeneous population of *B.burgdorferi*, expressing many different osp C proteins (Wang et al., 1999) it is not surprising that the mammals are able to acquire wide range of *Borrelia* genotypes.

Furthermore, our results indicated that different species of ticks did not carry different strains of pathogen, *B. burgdorferi*, and transmit them to host species. In fact, the results indicated that both tick species as well as the mammals seem to acquire wide range of Borrelia genotypes in southeastern Virginia (Figure 4). Previous sampling efforts from our lab indicated that different patterns of *B.burgdorferi* genetic structure are found in different parts of Virginia (Brinkerhoff et al. unpublished data). The ticks found in Norfolk area exhibited a different pattern which led us to believe that *Ixodes affinis* expanding north from the southern population might be responsible for the introduction of novel genotypes/alleles in these environment. Our data do not support this hypothesis. The different variant of ospC groups are distributed among the different species of the ticks. The distribution of the ospC group seems homogeneous among different tick species and mammals, with each tick species and mammals acquiring a wide range of genotypes.

Although different *ospC* genotypic frequencies was detected between hosts tissues and ticks derived from hosts , it might simply be, because of the failure to sample all important hosts of immature *Ixodes* ticks or failure to thoroughly check all hosts for ticks. Also, because tissue samples were not collected from all hosts, we do not have a complete dataset with which to assess *B. burgdorferi* transmission. There is therefore a need for increased sampling and

additional host taxa to capture a greater representation of the wildlife host community, and to provide tighter probability estimates of the occurrence and transmission efficiencies.

The total number of Lyme disease cases in Virginia increased from 957 registered cases in 2007 to 1346 cases in 2014 (Lantos et al., 2015). At the same time, there were evidence of movement of the new species of ticks in the northern region of Virginia (Nadolny et al., 2011). *Ixodes affinis* is a competent vector for *B.burgdorferi* that has recently been reported in parts of southeastern Virginia (Nadolny et al., 2011) where I. scapularis, the primary vector of Lyme disease is also known to occur. Ticks are the most common agents of vector-borne pathogens in the United States and worldwide they are second only to mosquitoes as arthropod pathogen vectors (Goodman et al., 2005). Borrelia burgdorferi is a bacterial parasite that infects a wide range of vertebrates including mammal, birds and lizards (Aperson et al., 1993) and is transmitted by hard-bodied ticks of the genus *Ixodes*. The zoonotic cycle is well studied, but only as it relates to *I.scapularis*, and little is known about how the presence of additional competent tick species in the same region affects the interaction between the host and pathogen transmission. This study aimed to understand the effect of the presence of competent vector in the interaction between host species and *B.burgdorferi* genotypes.

Ixodes affinis can survive in wide range of environmental conditions and they can feed on wide range of hosts (Harrison et al., 2003). Their ability to thrive in multiple environment and feed on different hosts is driving their expansion to the southeastern region of Virginia which has similar environment conditions and hosts in the natural system (Nadolny et al., 2011, 2015). Although the biotic and abiotic factors that contribute to its survival and dispersion is not known, I believe that the ability of *I. affinis* to survive and thrive in different environmental conditions is driving their expansion towards the southeastern region of Virginia. The risk of infectious

disease varies in space and time. There is contraction and expansion of geographic range of the pathogens and vectors. Therefore, further study is needed to understand how the environmental conditions and the different artifacts may affect the interactions of the vector and the pathogens Our data indicate that *I. affinis* is a generalist's vector, and it is thus able to feed on different hosts potentially allowing it to survive in different environment. Their ability to attach on small mammalian host (Oliver et al., 2003) means they can be easily carried across landscapes by small and large mammals which might be contributing to their geographical expansion. Further study is needed to see what genotypes can be transferred by different ticks to humans and see how the competition for the same hosts drives the survival of the ticks in the areas where they overlap.

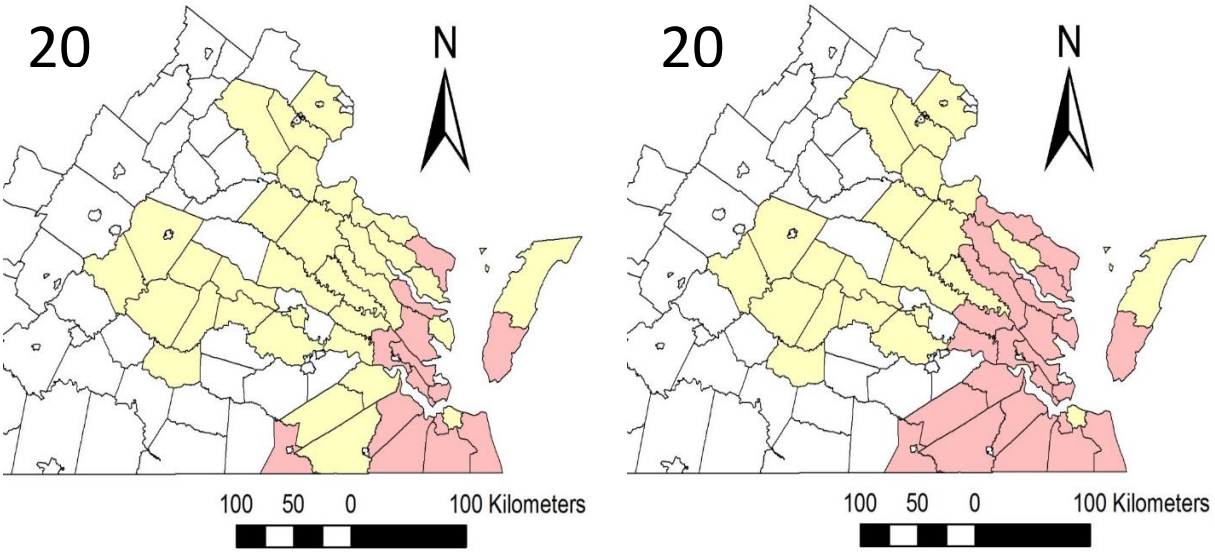

Fig. Spatial distribution by county (red) of *Ixodes affinis* collections in Virginia, USA through 2012 (left) and 2015 (right). Yellow-shaded counties have been sampled annually or biennially since 2011 and have not yielded *I. affinis*.

Figure 1. Locations of study sites in southeastern Virginia. Figure shows 6 of the 14 sites that were used for the collection of ticks.

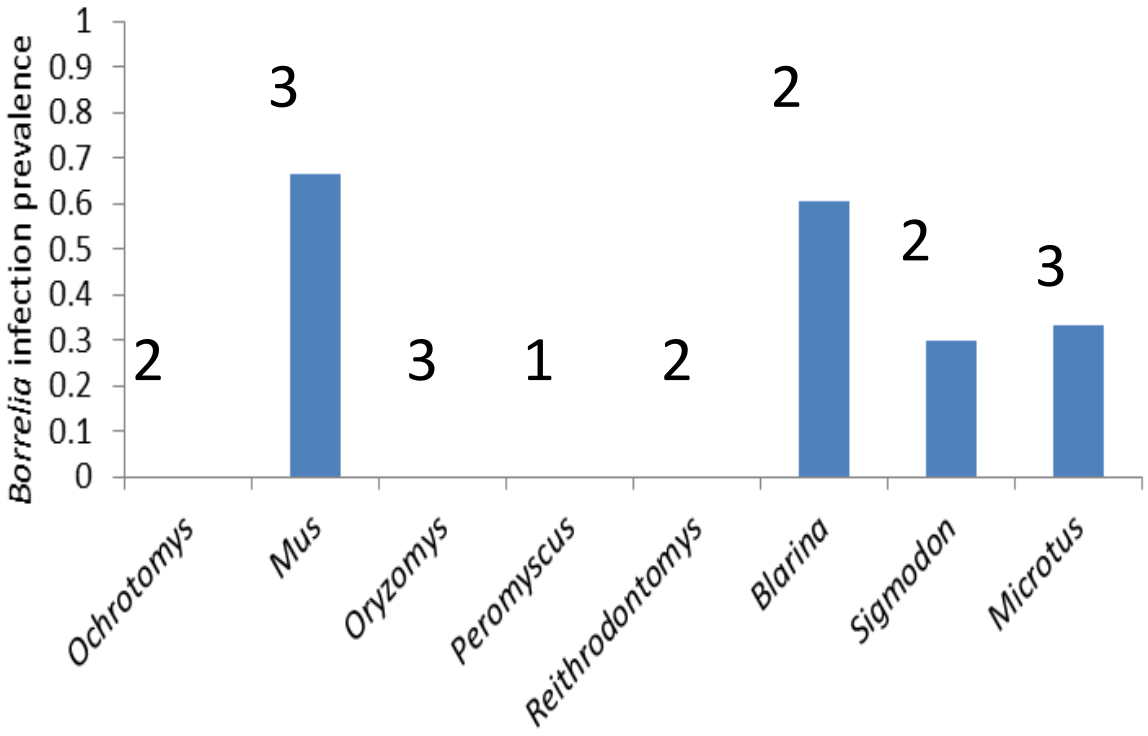

Figure 2. *Borrelia* infection prevalence among the mammal species identified in this study. Sample sizes are indicated above each column.

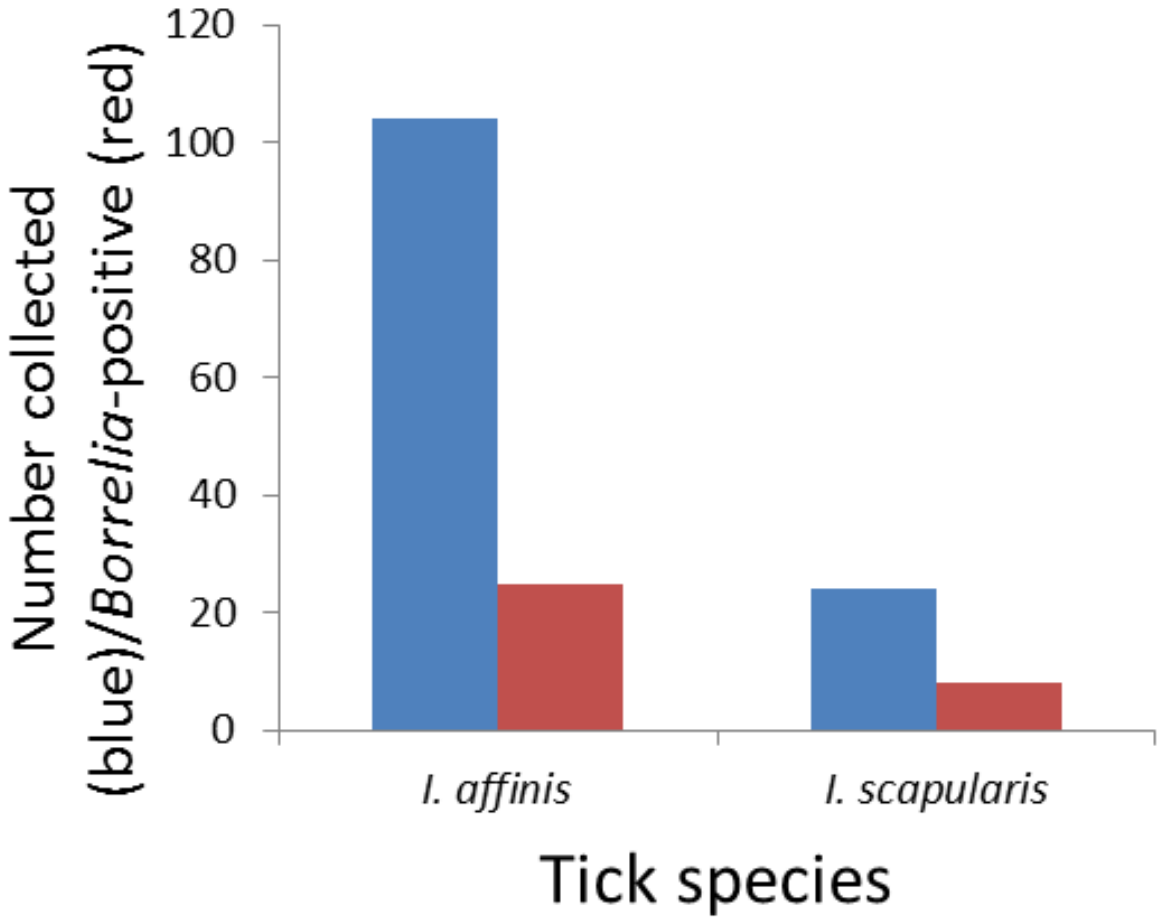

Figure 3. Numbers of each tick species collected from rodents and that tested positive for *Borrelia* spp. By PCR. Tick species identity was determined by single-tube qPCR.

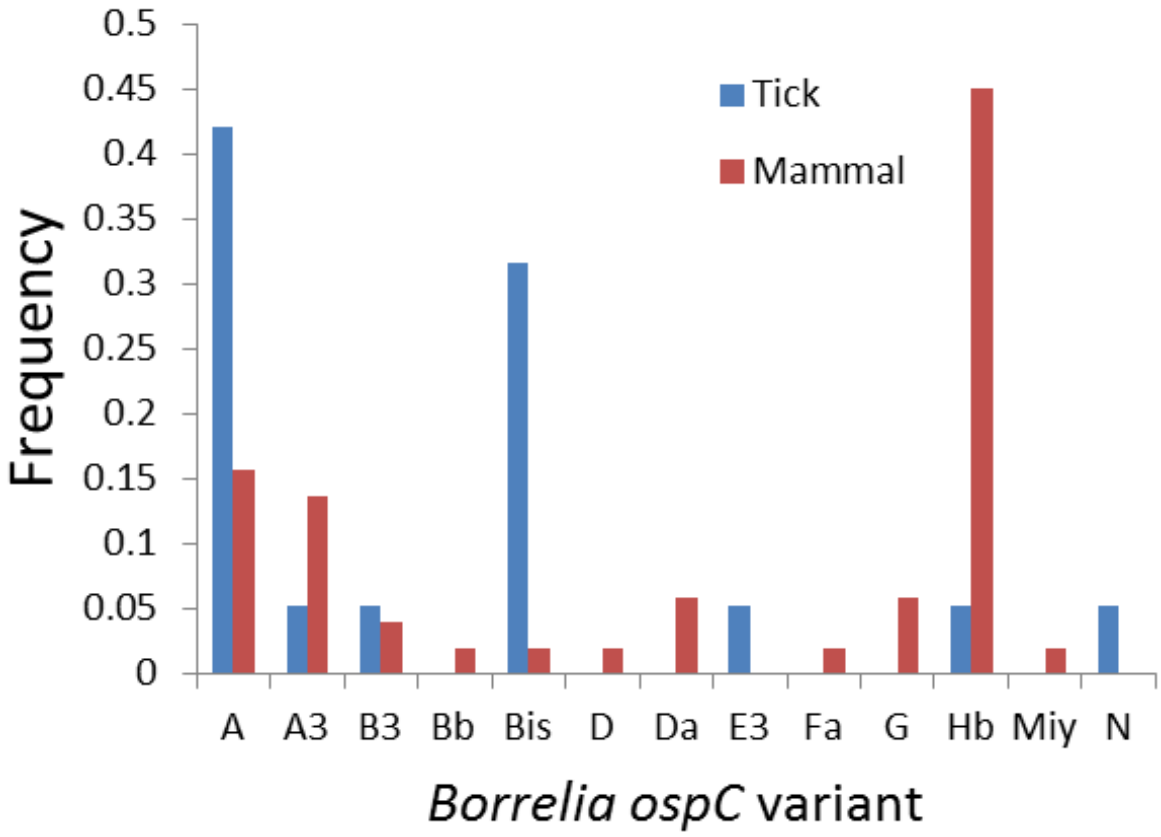

Figure 4. Relative abundance of different ospC genotypes by sample type. Type 'Bis' indicated *B. bissettii* and type 'Miy' indicates *B. miyamotoi*. Frequencies differed significantly by Fisher's Exact Test ($P<0.001$ after 10000 Monte Carlo runs)

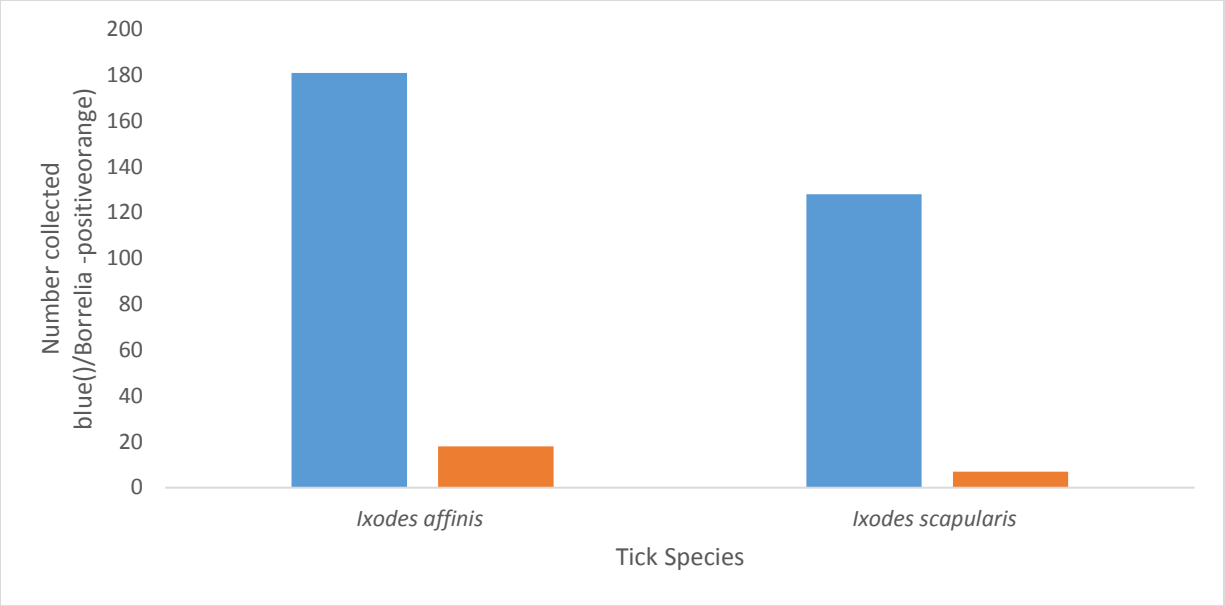

Figure 5: Numbers of host seeking ticks and ticks that tested positive for *Borrelia* spp. By PCR. Tick species identity was determined by single-tube qPCR.

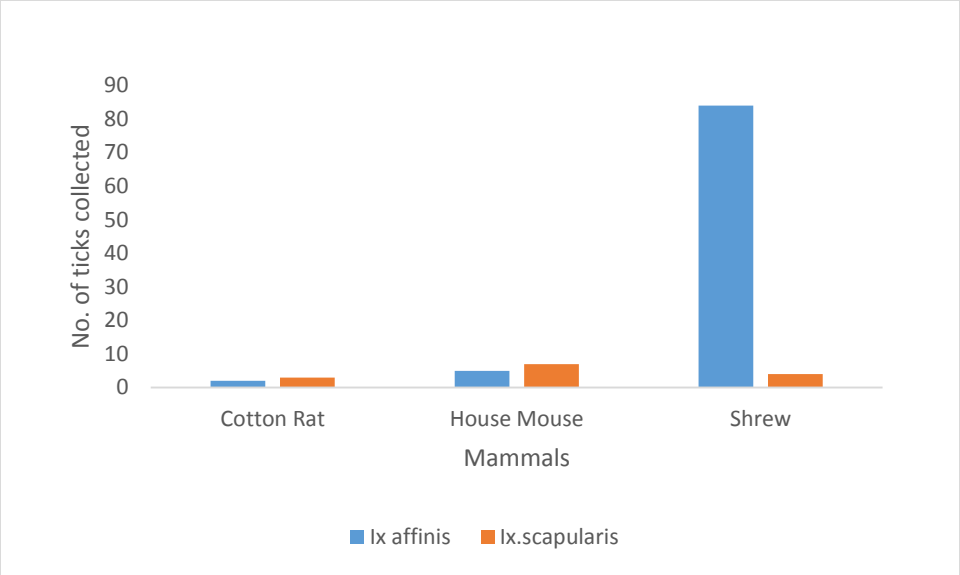

Figure 6: Number of *Ixodes* species collected from different mammals. Tick species id was determined by using single tube real time PCR. Note that ticks were collected from other mammals but these did not yield definitive identities by qPCR.

Total no. of mammals	**81**
Borrelia positive tissue	26
Infection prevalence	32.1%

Table 1: Infection prevalence among rodents

osp C Group	No. of isolates	(%)
A	8	13%
A3	7	11%
B3	2	3%
D	1	2%
Da	3	5%
Fa	1	2%
G	3	5%
Hb	26	42%
mixed	2	3%
Messy	7	12%

Table 2: Percentage distribution of ospC group in host derived ticks. Note that some of the sequences were messy: they failed to align and give consensus sequence. Further, blast search did not confirm any osp C groups.

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