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Community structure of the microbiome of ixodes scapularis in relation to sex, stage, lineage, and geography

Christopher Lee Clark
University of Richmond

Community Structure of the Microbiome of *Ixodes scapularis* in Relation to Sex, Stage, Lineage, and Geography

by

Christopher Lee Clark

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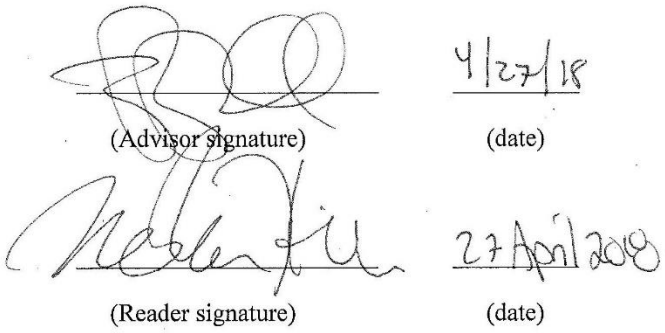
Advisor: Dr. R. J. Brinkerhoff

Community Structure of the Microbiome of *Ixodes scapularis*



This thesis has been accepted as part of the honors requirements in the Department of Biology.

Community Structure of the Microbiome of *Ixodes scapularis*

Introduction

There are at least 44 species of ticks known to exist in the United States (Merten and Duden, 2000) with at least four of these species, which are known to be involved in the transmission of human pathogens, being found in the state of Virginia (VDH, 2016). All four of these ticks are known to transmit human pathogens; however, the most notorious amongst them, and the focus of this study, is the black legged tick, or *Ixodes scapularis*. *I. scapularis* is found along the east coast and mid-west of the United States, from Florida westward into central Texas and as far north as Maine, Minnesota, and Iowa (Patnaude and Mather, 2014). This distribution has been linked to the distribution and abundance of the adult stages' primary host, the white-tailed deer, *Odocoileus virginianus* (Wilson et al., 1985; Wilson et al., 1988). Although *I. scapularis* primarily feeds on white-tailed deer as adults (Piesman et al., 1979; Carey et al., 1980; Wilson et al., 1990), they may also feed on other smaller mammals present in the wild or domesticated livestock (Kierans et al., 1996; Anderson and Magnarelli, 1980). However, while their potential hosts may be diverse, it has been shown that they depend on deer, and in some cases other large mammals, to support large or particularly dense populations (Duffy et al., 1994). This relationship is not surprising, given the female ticks depend on large mammals to reproduce. The range of *I. scapularis'* range contains some of the most densely human populated areas in the United States (Miller et al., 1990) and their range across North America is only growing (Lastavica et al., 1989; Anderson et al., 1990; Godsey et al., 1987; Davis et al., 1984; Ogden et al., 2014; Clow et al., 2016; Ogden et al., 2006; Ogden et al., 2008; Ogden et al., 2009), presenting the United States with a potential future public health crises of spreading tick borne diseases.

Community Structure of the Microbiome of *Ixodes scapularis*

I. scapularis is the primary vector responsible for transmitting a number of the aforementioned tick-borne diseases, including *Borrelia burgdorferi*, the agent of Lyme disease, which infects somewhere between 20,000-30,000 people every year, although the CDC estimates that the actual number of cases is likely 10 times higher than those reported, and is the most commonly reported vector disease in the United States (CDC, 2003; CDC, 2008). Unfortunately, these numbers are only rising with an average of 25,000 cases being reported annually in the united states every year since 2007 at a steadily increasing rate (CDC, 2014). This species of tick also transmits a number of lesser known pathogens including human babesiosis, *Babesia microti*, granulocytic ehrlichiosis, known as HGE, (Des Vignes and Fish, 1997) as well as multiple species of *Rickettsia* (Billings et al., 1998; Kurtti et al., 2015), which are responsible for the spotted fever illnesses, including *Rickettsia helvetica* which may be responsible for multiple life-threatening symptoms and severe infections around the globe (Nilsson et al., 1999; Fournier et al., 2000; Fournier et al., 2004). However, although the range of *Ixodes scapularis* is increasing and cases for various tick-borne diseases are on the rise, new research methods that seek to understand the interactions of the microbial community within these vectors have the potential to open groundbreaking doors to vector management and disease prevention.

The microbiome is a term used to describe a community of organisms living in a shared local space, whether that be in soils, waters, or even the human body. The microorganisms that live inside and on us, often referred to as the microbiota, are believed to outnumber all the cells in our body by a factor of 10 (Turnbaugh et al., 2007). The composition of the human system, including the human cells and their microbial counterparts is described as forming a larger, highly functioning ‘supra-organism’ (Turnbaugh et al., 2007). These microorganisms likely

coevolved with us, and our more ancient ancestors, over thousands, if not millions, of years, providing us with essential resources and pathways that help us survive (Gill et al., 2006; Huttenhower et al., 2012). These communities can be both incredibly diverse, such as in the gastrointestinal system, where they provide assistance in digestion or disease prevention (Turnbaugh et al., 2009; Qin et al., 2010), or equally simple, such as in the vagina, where they prevent urogenital diseases (Fredricks et al., 2005). However, these communities are sensitive as well, and any imbalance in the microbiome anywhere on the human body can lead to both acute and chronic illnesses. For example, in the gastrointestinal system, a lack of diversity in the microbiome has been linked to obesity and inflammatory bowel disease (Turnbaugh et al., 2009; Qin et al., 2010) and an abundance of diversity in the vagina has been linked to bacterial vaginosis (Fredricks et al., 2005). Clearly a healthy microbiome is crucial for sustaining a healthy human; however, the interactions amongst the members of this microbial community may be even more important.

Our skin also has a thriving microbiome, where we come into contact with a significant number of pathogens every day. Certain species of bacteria found within our skin microbiome, including *Staphylococcus epidermidis* and *Propionibacterium acnes* have been shown to have a substantial impact on our skin health. *P. acnes* has been shown to reduce the likelihood of developing a MRSA infection, which has profound impacts on human health; however, an excess of *P. acnes* has been linked to an increased risk for cases of acnes vulgaris. Fortunately for humans, *S. epidermidis*, another skin bacterium, inhibits the growth of *P. acnes*, reducing an individual's likelihood for developing severe acne while also allowing a presence of *P. acnes* to reduce the risk of MRSA infections (Shu et al., 2013; Wang et al., 2014). The interactions amongst the microorganisms in the human microbiome are clearly profoundly important to

sustaining a healthy individual; however, the microbiome is not unique to humans and the interactions within a microbiome may prove to be just as influential in other systems.

Essentially all known organisms have a microbiome living either on their surface or within their structures. However, in terms of human health, the most important microbiome may very well be that of the vectors which carry some of the most severe known human pathogens. Although the pathogens which cause these diseases, from plague and malaria to Lyme disease and spotted fevers, are found within the microbiome of vectors, these organisms also harbor other microorganisms which may work to reduce, eliminate, or potentially facilitate their more pathogenic neighbors. One such example that has been studied significantly in the last decade or so is the interactions between *Wolbachia* bacteria and the malaria parasite, *Plasmodium falciparum*. It has been shown in repeated studies that the presence of this bacteria, and even some other gram negative bacterial species, inhibits the transmission and host susceptibility for malaria infections (Glaser et al., 2010; Hughes et al., 2011; Bahia et al., 2014; Pumpuni et al., 1993; Gonzalez-Ceron et al., 2003). In other cases, it has even been shown that the presence of *Wolbachia* limits the transmission of certain arboviruses as well, including dengue and chikungunya (Moreira et al., 2009). However, it is important to note that not all of these interactions are prohibitive and beneficial to human health. For example in the flea, where the presence of a diverse bacterial community may actually promote the development of plague bacteria, *Yersinia pestis* (Jones et al., 2015). These complex interactions continue in other vectors such as sand flies, *Lutzomyia longipalpis*, where the microbiome is crucial for the survival of *Leishmania infantum* which causes Leishmaniasis (Kelly et al., 2017) and tsetse flies where their microbiome has a profound impact on the transmission of *Trypanosoma brucei* which causes African trypanosomiasis (Weiss et al., 2011). Given interactions in other disease

vectors, it follows that vector species of the genus *Ixodes* may harbor bacteria with similar interactions and that their microbiome should have profound impacts on the diseases they transmit and their competence as a vector.

As with the other vector species, there are studies that show certain bacterial species within ticks influence the transmission and development of other species. For example, a particularly diverse microbiome in ticks has been negatively correlated with the success of *B. burgdorferi* (Narasimhan et al., 2014) and certain rickettsial species alter the transmission of other pathogenic rickettsial species such as *R. rickettsia* (Childs and Paddock, 2002; Ahantarig et al., 2013). However, there is still a significant gap in knowledge as to what factors drive the development of this microbiome and the interactions within it. Although prior field studies have shown a variation in the microbiome of vectors as a function of factors such as species (Hawlena et al., 2013), sex (Williams-Newkirk et al., 2014; Zhang et al., 2014), and region (Carpi et al., 2011), there is a lack of clear impacts of feeding on the vector microbiome (Rynkiewicz et al., 2015; Swei and Kwan, 2017) or the impacts of the local environment inhabited by the vector (Hawlena et al., 2013). These interactions, specifically that of the blood meal and the local environment, have the potential to have profound impacts on the microbiome and it will be increasingly important to identify any potential factors at play for future disease management given the variability in local environments and host blood.

This study sought to fill these gaps of knowledge ticks as a model species. Based on the known information on the microbiome of vectors, and ticks more specifically, I sought to answer two prominent questions: are there relationships between the microbiome of individual ticks and do ticks from different sexes or life stages have variance in their microbiomes? In reference to my first question, I hypothesized that ticks coming from the same lineage, being the males,

females, and resulting eggs, would have a more similar microbiome to each other than to other ticks and that ticks coming form different geographic locations within Virginia would have variance in their microbiomes. For my second question, I hypothesized that females would have a more diverse and abundant microbiome as a result of their increased blood meal size and frequency and the increased time they spend on their host.

Methods

Ixodes scapularis ticks were collected from the ears of white-tailed deer in New Kent County, Virginia at Burks Farm. The deer were brought to the processing plant by the hunters who took them from various locations around Virginia including James City, Loudon, New Kent, and Prince George County as well as two unknown locations. After obtaining the hunters permission to include their deer in our study, the ears from the deer were removed and tweezers were used to remove any attached ticks from the ears. The ticks removed from these deer were placed in separate tubes based off of the deer they were removed from and were taken back to the University of Richmond. Supplemental samples for this study from Botetourt county were also obtained from Dr. Liz Gleim at Hollins University. All ticks, both from the field and Hollins University, were held in individual tubes at 23 degrees Celsius at a humidity of 90-95% using a saturated solution of potassium sulfate in a humidity cabinet. Females that had a male attached to them were allowed to lay the eggs they were carrying prior to preparation for DNA extraction and amplification. Upon egg laying, females were removed from males and each sample, male, female, and egg mass, were placed in individual tubes and frozen at -20 degrees Celsius. A total of 31 samples were obtained including 12 females, 12 egg clutches, 6 males, and 1 larvae cluster with 2 samples from Botetourt, 2 from James City, 20 from Loudon, 2 from New Kent, 3 from Prince George, and 2 from unknown locations.

Community Structure of the Microbiome of *Ixodes scapularis*

The sampled ticks were frozen in liquid nitrogen and crushed with pestles to release the internal microbiome of the tick. Microbial DNA was extracted using a Macherey-Nagel NucleoSpin soil extraction kit, following manufacturers protocol (Macherey-Nagel, 2017) and amplified through a PCR of the highly variable v3-v4 region of the microbial 16s rDNA. Following the amplification, gel electrophoresis was performed to ensure an adequate level of amplification was obtained in all samples. Amplified DNA was purified using streptavidin beads and were then barcoded using a Nextera DNA library prep kit, following manufacturers protocol (Illumina, 2016) and then analyzed via Illumina MiSeq, following manufacturers protocol (Illumina, 2015) at Old Dominion University. Final analysis of the resulting data was accomplished through a Unix pipeline as well as analysis in JMP. The Unix pipeline consisted of using multiple scripts involving python codes to analyze the raw fastq sequence reads produced from the MiSeq run. These files were uploaded to a cluster located at Juniata College and the forward and reverse reads of each sequence were combined. This product was then filtered so that only reads of an acceptable quality, typically a Q-score of 30 or higher, were included in the final analysis. These filtered samples were then labeled and run through a series of analytical processes to calculate diversity, develop a taxonomical tree, and perform a UniFrac analysis (Lozupone and Knight, 2005). In JMP, a one-way anova was performed to analyze richness and diversity as a function of life stage, or type, and site location. A phylogenetic tree of all *Rickettsia* bacterial sequences found in this study was constructed and referenced against references for known existing strains using BLAST from the U.S. National Library of Medicine to construct the references and identify the found sequences and MEGA7 for analysis and generation of the final phylogenetic tree.

Results

Sequence Data

Over 50 million sequences of DNA were analyzed through MiSeq analysis, resulting in 323 identified OTU's, or operational taxonomic units. There was an average of 96,945 sequences per tick with a minimum of 230, a median of 79, 626, and a maximum of 324, 772.

Richness and Diversity of the Microbiome

A significant correlation between sex and type and the abundance and diversity within the microbiome was noted in this study. Males were noted to have a significantly higher level of OTU, or operational taxonomic unit which is used since we cannot determine the exact species, richness than eggs, females, and larvae. The maximum richness value for each type was 102 unique OTU's for eggs, 84 for females, 64 for larvae, and 181 for males. The median values for each type were 64.5 for eggs, 45.5 for females, 64 for larvae, and 101 for males with minimum values for each type of 8 for eggs, 9 for females, 64 for larvae, and 93 for males. Similar ranges and IQR's were noted for each type and there were no outliers in this analysis (Figure 1a.). The ANOVA analysis showed a significant relationship between type and species richness [$F(3,27)=6.0634$, $p=0.0027$].

Results for the Shannon Diversity index were similar to those of richness. Again, males were found to have a significantly higher level of diversity than eggs, females, and larvae. The maximum diversity value for each type was 1.81 for eggs, 1.64 for females, 0.65 for larvae, and 3.44 for males. The median values for each type were 0.86 for eggs, 0.41 for females, 0.65 for larvae, and 2.66 for males with minimum values for each type of 0.02 for eggs, 0.01 for females, 0.65 for larvae, and 1.08 for males. Similar ranges were noted for each type, with the males having a slightly larger IQR than the other types sampled and the larvae having no range or IQR

due to the fact that there was only one larval sample, and there were no outliers in this analysis (Figure 1b). The ANOVA analysis showed a significant relationship between type and Shannon diversity [$F(3,27)=12.53$, $p<0.0001$].

There appears to be no correlation between the site which the deer were taken from and the richness or Shannon Diversity of the microbiome. The maximum values for richness were found to be 25 for Botetourt, 43 for James City, 181 for Loudon, 82 for New Kent, 84 for Prince George, and 91 for unknown. Median values were found to be 16.5 for Botetourt, 28.5 for James City, 67 for Loudon, 45.5 for New Kent, 67 for Prince George, and 53 for unknown. Minimum values were found to be 8 for Botetourt, 14 for James City, 31 for Loudon, 9 for New Kent, 64 for Prince George, and 15 for unknown. Significant disparities were noted in the range and IQR of each site due to the variance in the number of samples obtained from each location (Figure 2a). The ANOVA analysis showed no significant relationship between site and OTU richness [$F(5,25)=1.8387$, $p=0.1417$].

A similar pattern was observed in the Shannon Diversity index, with no significant relationship between site and diversity. The maximum values for Shannon Diversity were found to be 0.02 for Botetourt, 0.02 for James City, 3.27 for Loudon, 0.85 for New Kent, 1.81 for Prince George, and 1.13 for unknown. Median values were found to be, 0.02 for Botetourt, 0.01 for James City, 0.89 for Loudon, 0.75 for New Kent, 1.81 for Prince George, and 0.85 for unknown. Minimum values were found to be 0.02 for Botetourt, 0.01 for James City, 0.03 for Loudon, 0.59 for New Kent, 1.27 for Prince George, and 0.57 for unknown. Although some variation is noted amongst sites, the significant of this is negated due to the severe range in the number of samples from each site. There is wide range of diversity and richness noted in the Loudon samples, indicating that this location was not necessarily a significant factor for diversity

and we can therefore attribute the apparent differences in other sites to a lack of a significant number of samples from each site. Significant disparities were noted in the range and IQR of each site due to the variance in the number of samples obtained from each location (Figure 2b). The ANOVA analysis showed no significant relationship between site and Shannon diversity [F(5,25)=1.4469. p=0.2424].

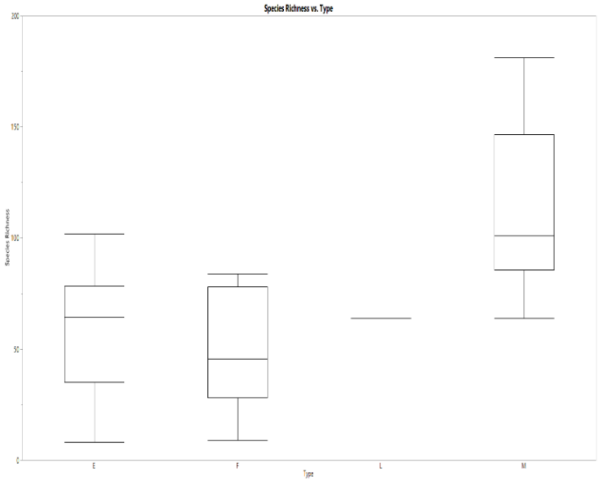


Figure 1a. Species richness versus type was calculated and graphed in a box and whiskers format.

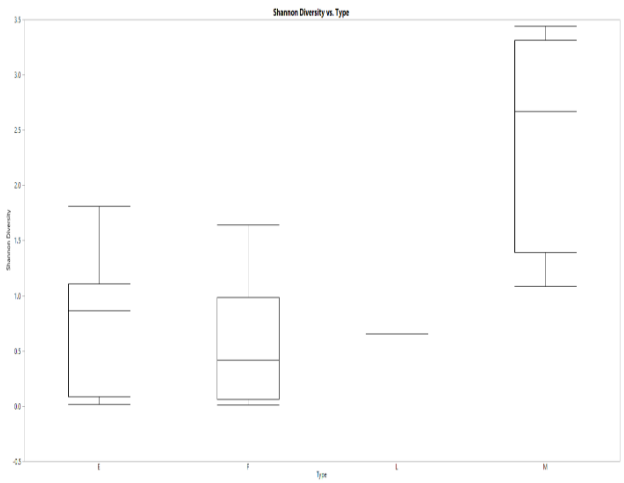


Figure 1b. Shannon Diversity versus type was calculated and graphed in a box and whiskers format.

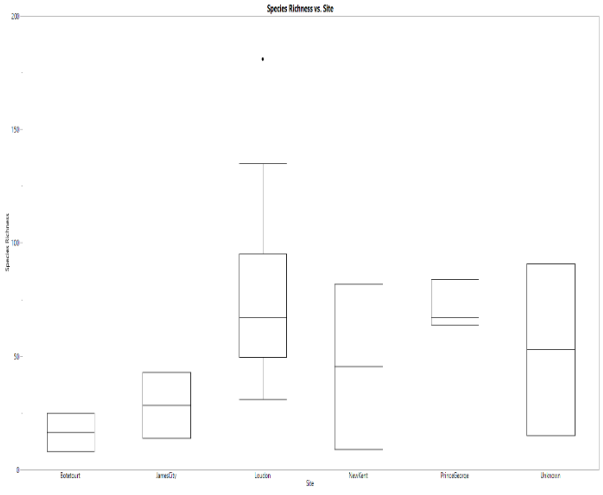


Figure 2a. Species richness versus site was calculated and graphed in a box and whiskers format.

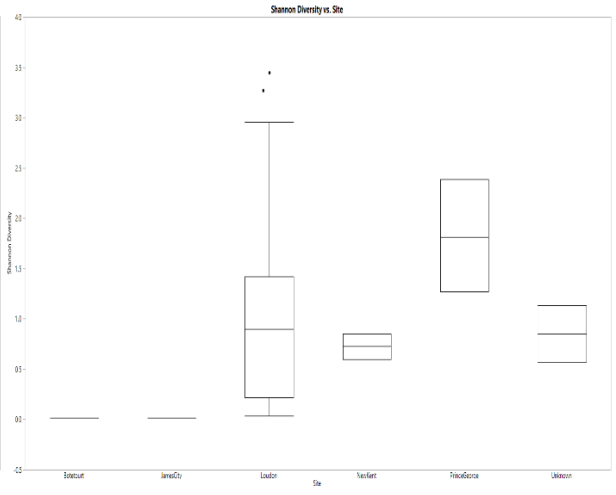


Figure 2b. Shannon Diversity versus site was calculated and graphed in a box and whiskers format.

Community Structure of the Microbiome of *Ixodes scapularis*

Proportion of Rickettsia in the Microbiome

The proportion of *Rickettsia* bacteria in the microbiome appeared to be dependent on life stage and sex. Eggs, females, and larvae were found to have the largest proportion of *Rickettsia* bacteria in their microbiome, with eggs having a maximum value of 99%, females of 99%, larvae of 88%, and males of 16%. Median values were found to be 77% for eggs, 91% for females, 88% for larvae, and 5% for males with minimum values of 5% for eggs, 5% for females, 88% for larvae, and 2% for males. Eggs and females were noted to have significantly larger ranges and IQR's than the males and larvae, although the larvae lack these values due to the fact that there was only one larval sample (Figure 3). The ANOVA analysis showed a significant relationship between the proportion of *Rickettsia* and stage and sex [$F(3,27)=6.9624$, $p=0.0013$].

Rickettsia abundance within a tick was not dependent on the site of the sample. The maximum values for each site were 99% for Botetourt, 99% for James City, 99% for Loudon, 87% for New Kent, 5% for Prince George, and 58% for unknown. Median Values were found to be 99% for Botetourt, 99% for James City, 71% for Loudon, 80% for New Kent, 5% for Prince George, and 36% for unknown with minimum values of 99% for Botetourt, 99% for James City, 2% for Loudon, 74% for New Kent, 5% for Prince George, and 14% for unknown. Although there do appear to be differences amongst counties, all of the variation for this sample can be accounted for in Loudon county alone, indicating that location likely does not have a significant impact on the proportion of *Rickettsia* bacteria in the microbiome (Figure 4). The ANOVA analysis showed a slight relationship between the proportion of *Rickettsia* and sample site; however, these results are difficult to verify due to the lack of a significant number of samples from each site [$F(5,35)=2.6731$, $p=0.0455$].

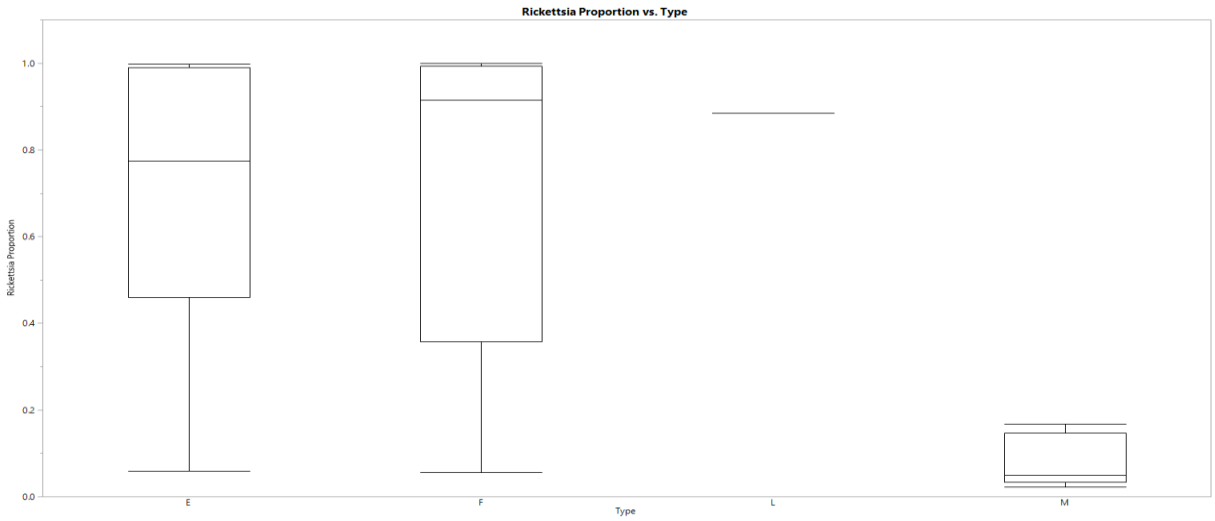


Figure 3. Proportion of *Rickettsia* in the microbiome versus type was calculated and graphed in a box and whiskers format.

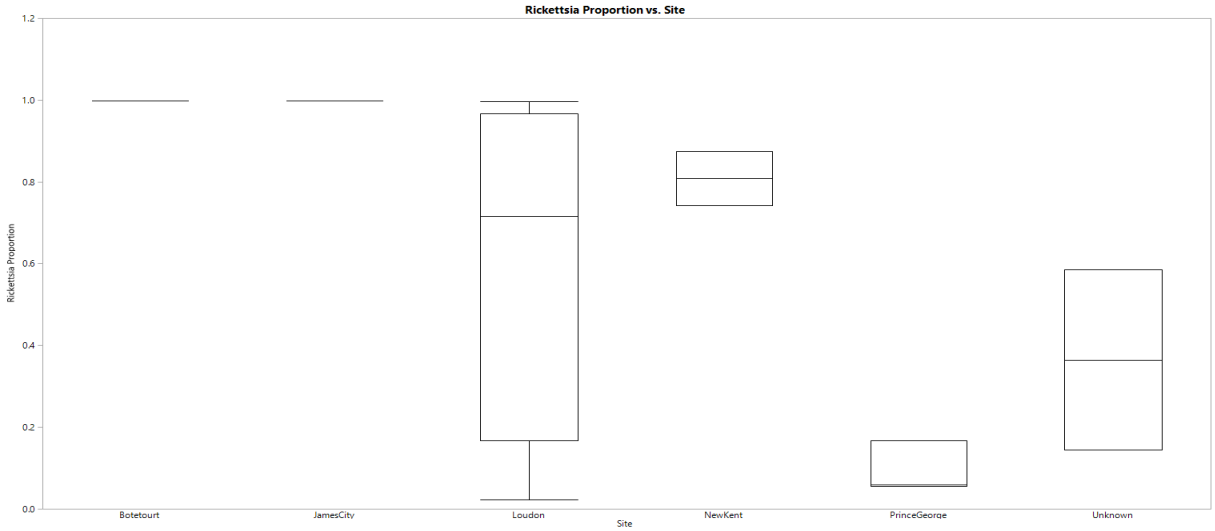


Figure 4. Proportion of *Rickettsia* in the microbiome versus site was calculated and graphed in a box and whiskers format.

Rickettsia Phylogenetic Tree

An analysis of the phylogeny of the *Rickettsia* bacteria OTU's found in this study indicated that potentially new, previously unidentified, strains of bacteria from the *Rickettsia* genus may be present. OTU's 1 and 457 appear to be closely related to *R. buchneri, R. monacensis, R. monteiror,* and *R. japnoica* as well as an isolate from an endosymbiont of the

bacteria typically found in *Ixodes scapularis*, a species of tick known to be present on the west coast. However, the remaining strains of *Rickettsia* identified in this study appear to be previously unidentified, potentially recently-derived lineages, especially OTU's 279 and 573 which appear to be newly identified and closely related (Figure 5). This is a potential answer to this presentation, given their isolation on the tree from previously identified species and the fact that they are more closely related to one another than to any other species on the tree (Figure 5). However, it is also possible that our sequence data was simply not accurate enough to properly group these OTU's or they are more closely related to some other known rickettsial species that was not included on this tree.

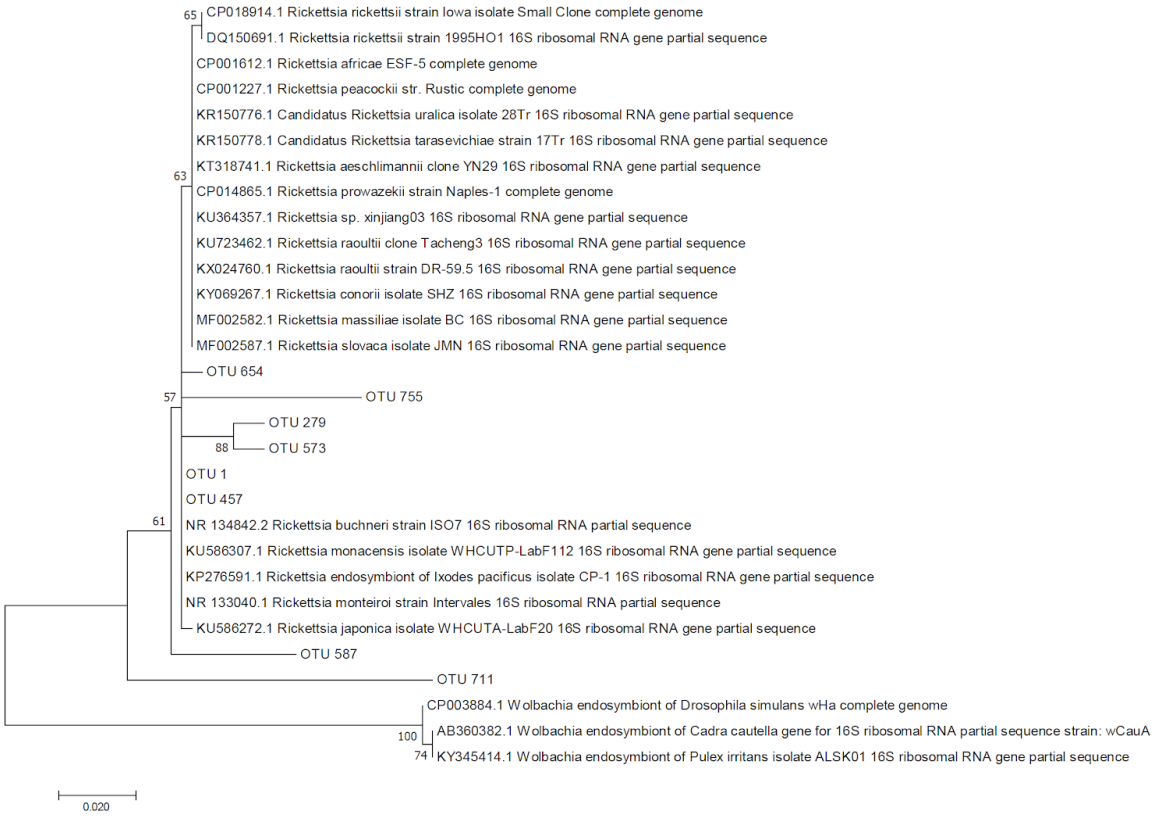


Figure 5. Phylogenetic analysis of identified *Rickettsia* species was performed and a phylogenetic tree containing references form BLAST was created

Community Structure of the Microbiome of *Ixodes scapularis*

UniFrac Analysis

UniFrac analysis is a form on statistical analysis used to graph samples in a three-dimensional planed based off of the similarities in their microbiome. The more similar two samples are in terms of diversity, which is calculated through raw diversity as well as examining the taxonomical distance between bacterial samples, the closer they will appear on the graph. UniFrac analysis through the Unix pipeline revealed a highly accurate model able to account for a total of 79.41% of the samples variance, both phylogenetically and microbiologically, in three-dimensional space. Through this analysis, clear trends were noted in the community and phylogenetic similarities amongst eggs and females with clear dissimilarities between these two groups and males (Figure 6). Although difficult to portray in two dimensions, a clear cluster of eggs, females, and larvae, portrayed as blue and red dots, was noted in the graph. Males, indicated by green dots, were isolated from the rest of the samples in the front left portion of the graph, with one outlier closer towards the egg and female clusters (Figure 6).

When samples were coded based on sampling location, there was no discernable pattern between sample site and the similarities between the microbiome or taxonomy of the samples. Samples from Loudon county could be found across the plane and, although other locations appear to be clustered together, they lack the sufficient samples to determine a distinct relationship (Figure 7).

A further analysis was run to compare the relationships described above amongst individual mating units, including the male and female pairs and their subsequent larvae or eggs. A clear pattern of females and their resulting eggs being closely related was observed in multiple samples while the males were typically significantly isolated from their female mates and resulting eggs (Figure 8). Although some of the female and egg pairs are less closely related

than others, there is a clear trend of females being much more closely related to their egg clutches than males are related to either the eggs of the females (Figure 8).

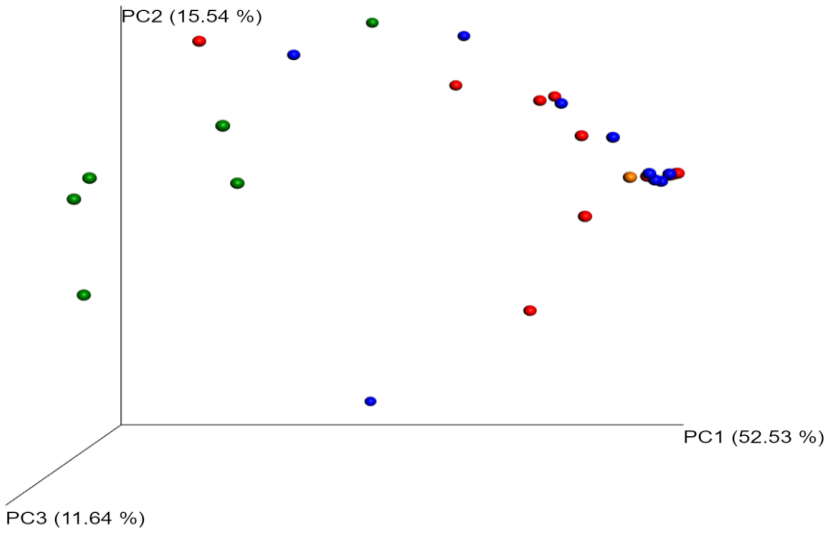


Figure 6. UniFrac analysis of females, eggs, larvae, and males was performed to identify the variance amongst the microbiome and taxonomical structure of each sample. Red dots are eggs, blue females, orange larvae, and green males.

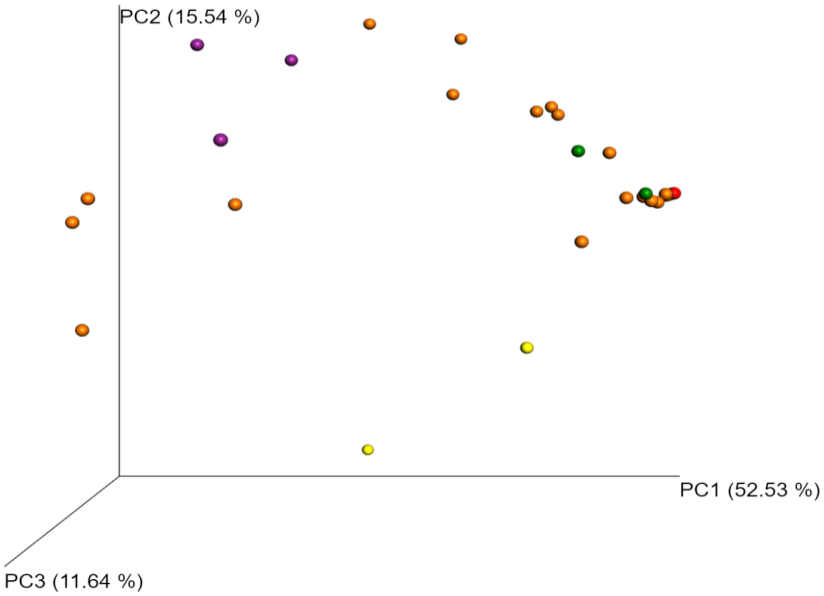


Figure 7. UniFrac analysis of site location was performed to identify the variance amongst the microbiome and taxonomical structure of each sample. Red dots are Botetourt, blue James City, orange Loudon, green New Kent, purple Prince George, and yellow unknown.

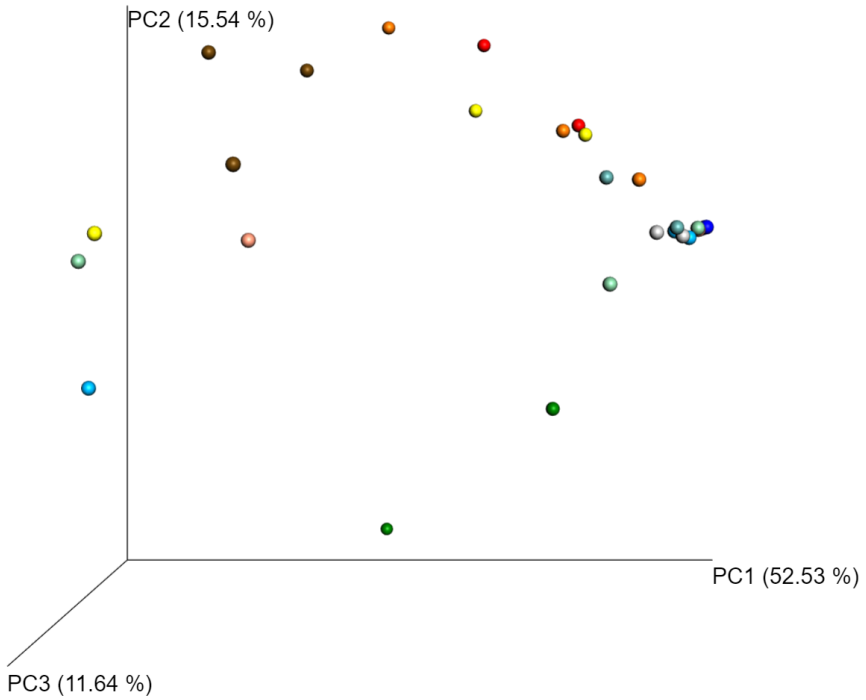


Figure 8. UniFrac analysis of family relationships was performed to identify the variance amongst the microbiome and taxonomical structure of each sample. Each color represents one family unity of females, eggs/larvae, and males. In situations where there was no male present, only the females and eggs share a color.

Discussion and Conclusions

In this study I showed that fed female ticks and their resulting egg clutches and larvae have significantly different structure and diversity of their microbiome when compared to male ticks. Females, eggs, and larvae were significantly less diverse than that of their male counterparts, both in terms or richness and Shannon diversity (Figure 1a; Figure 1b). This finding is consistent with previous studies in which the microbiome of male ticks was shown to be significantly more diverse than that of females. Interestingly, Swei and Kwan (2017) found that larval ticks had significantly higher levels of richness and diversity than adult ticks. Although these findings seem to contradict our own, their study did not separate male and female adult ticks and was conducted on *Ixodes pacificus* rather than *Ixodes scapularis* (Swei and Kwan,

2017). These differences, both in methodology and geographical location may account for the differences noted among studies. Further study regarding the differences of these two species of ticks and their geographical location in the United States may provide further insight into these findings and could produce an explanation for the apparent differences in their respective microbiota.

Further analysis of the microbiome in this study indicated that these differences in richness and diversity appear to be largely driven by the abundance of *Rickettsia* bacteria in the microbiome of the females, eggs, and larvae. Although little is known regarding the specific biology of the *Rickettsia* OTU's noted in this study, given that many of the OTU's we detected appear to be previously unidentified, their may be specific aspects of the female tick and egg biology that lend to an abundance of *Rickettsia* bacteria. Given the large blood meal that females take before producing and laying eggs, it is possible that *Rickettsia* thrive in blood rich environments, leading to dramatic increases in *Rickettsia* density and a subsequent exclusion of other bacterial species. Prior studies showed significant levels of *Rickettsia* in many of their adult ticks; however, these were not noted in *Ixodes scapularis*. Swei and Kwan (2017) found *Rickettsia* was dominant in adult *Ixodes pacificus* ticks while Van Treuren et al. found significant levels of *Rickettsia* in *Ixodes affinis* but found that an unknown species of *Enterobacteriacceae* dominated the microbiome of *Ixodes scapularis*, although this was only noted in North Carolina (Swei and Kwan, 2017; Van Treuren et al., 2015). Although Swei and Kwan (2017) found an abundance of *Rickettsia* in their samples, these ticks were *Ixodes pacificus* rather than *Ixodes scapularis* and their findings on richness and diversity differed slightly from our own, as previously discussed. Even more intriguing are the findings noted in Van Treuren et al. where their samples, which were *Ixodes scapularis*, where dominated by *Enterobacteriacceae* rather

than *Rickettsia* (Van treuren et al., 2015). However, they noted that *Rickettsia* was significantly more present in Virginia than *Enterobacteriacceae*, which is more present in North Carolina, which may explain why our study found such high levels of *Rickettsia* in our samples, given that they were taken from locations in Virginia (Van Treuren et al., 2015). Were we able to conduct our study across multiple states, as performed in Van Treuren et al., we may have found similar differences in diversity and prevalence of various OTU's of bacteria in the microbiome as noted in their study. These differences are striking and indicate that blood meals may not be the only factor in determining which species of bacteria dominate the microbiome of various species of ticks and their geographical locations.

In conclusion, it appears that the shape of the microbiome in *Ixodes scapularis* of Virginia appears to be largely influence by sex and feeding status, rather than location, and that this resulting microbiome is, at least partly, passed on to the resulting eggs. Perhaps most significant is the apparent dominance of *Rickettsia* bacterial species in female *I. scapularis* ticks and their resulting egg clutches. Given the profound potential impacts on public health that *Rickettsia* species may have in the near future, and the identification of new potential strains of this bacteria in this study, further research into their abundance, specific biology, and potential pathology should be investigated further. I plan to investigate this phenomenon further in the future in conjunction with colleagues at ODU to determine the specific strains of *Rickettsia* that were identified in this study and to begin analyzing their impacts on the microbiome and the potential impacts these findings may have on novel pathogen spread and emergence.

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