

Linking fitness and the microbiota: exploring variation in microbial composition in  
the North American red squirrel (*Tamiasciurus hudsonicus*).

by

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## General Abstract

The importance of microbial communities, or microbiota, to host physiology and fitness is readily becoming apparent in laboratory animals and humans. Yet, little is known about the association between microbiota composition and fitness in wild animals, including the relation to host health and temporal or spatial variation. In this thesis, the promiscuous North American red squirrel, *Tamiasciurus hudsonicus*, was used to investigate if oral, gut, and genital microbiota differed with correlates of host fitness, sex, or capture period. These objectives were met using various sequencing techniques, including 454-Roche and Illumina MiSeq sequencing. Microbiota composition varied in all three body regions investigated, suggesting the impact of body region is influential on microbial composition in red squirrels. Evidence of differences in composition between sexes was only significant in the oral microbiota, which also displayed significant variation in beta diversity analysis between recaptures. As such, future research should be cautious in interpreting results from a single temporal sampling period when quantifying characteristic microbiota composition in wild mammals, and should account for potential sex bias within study designs. Body condition, a rudimentary measure of host fitness, was not correlated with composition in the microbiota of any body region. However, there were significant correlations between immune status and the oral microbiota composition, suggesting that some measures of fitness may be related to microbiota composition in a wild mammal. Together these results provide, for the first time, a comprehensive view of inter- and intra- individual factors contributing to microbiota variation across body regions and provide evidence for a correlation between microbial composition and fitness in a wild mammal.

Keywords: wild mammal; ecological immunity; next generation sequencing; microbiota

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## General Introduction

Bacteria are microscopic, single-celled organisms that inhabit almost every environment on Earth (Stumpf *et al.* 2013; Hogan 2014). Worldwide bacterial biomass is greater than that of all plants and animals on Earth and bacteria can be found in high densities in or on most living organisms (Hogan 2014). These bacterial communities, or microbiota, have complex relationships with their hosts, and the nature of these interactions depends on both the host and bacterial species. Most relationships can be split into three broad categories, which include mutualistic, commensal, and pathogen interactions (Stumpf *et al.* 2013). In mutualistic relationships, both the host and bacteria benefit from the presence of one another (Stumpf *et al.* 2013). For example, *Bacteroides thetaiotaomicron*, a normal component of the mammalian intestinal microbiota, modulates genes responsible for a variety of intestinal functions including nutrient absorption (Hooper *et al.* 2001). In commensal relationships, there is little to no cost to the host after the primary infection (Stumpf *et al.* 2013). Finally, pathogen relationships involve bacteria that are capable of causing disease in a host (Stumpf *et al.* 2013). *Yersinia pestis*, for example, is a bacterium responsible for several plagues throughout human history and has the potential to be pathogenic in other mammalian hosts (Perry and Fetherston 1997).

### Variation in mammalian microbiota

Mammalian microbiota tend to display large variation in inter- and intra-individual composition, driven by numerous factors. For example, anal pouch secretions in meerkats (*Suricata suricatta*) and vaginal microbiota composition in mice (*Peromyscus californicus* and *Peromyscus maniculatus*) may be largely influenced by the opportunity for bacterial transfer, driving similarity in microbiota composition in individuals who live in close-proximity and increasing diversity with promiscuous mating systems (MacManes 2011; Leclaire, Nielsen and

Drea 2014a; Yildirim *et al.* 2014). Similarly, spatial location (Linnenbrink *et al.* 2013), habitat degradation (Amato *et al.* 2013), sex (Leclaire, Nielsen and Drea 2014a) and diet (Phillips *et al.* 2012; Ooi *et al.* 2014) have also been shown to influence mammalian microbiota composition.

### Role of the microbiota in fitness

The immune system is vital to mammalian hosts' fitness and includes two subsystems: the innate and acquired immune system. The innate immune system generically recognizes and responds to pathogens and, while this subsystem provides immediate defense against infection, it does not confer long-lasting protective immunity to the host (Alberts *et al.*, 2002). The innate immune system is composed of several types of cells, including mast cells, macrophages, and certain white blood cells, such as basophils, eosinophils, and neutrophils (Stvrtinova, Jakubovsky and Hulin 1995; Janeway *et al.* 1999). The acquired immune system typically provides long-lasting protection to the host, and includes antibody responses and cell mediated immune responses (Alberts *et al.*, 2002). During an antibody response, B cells secrete large Y-shaped proteins known as antibodies, which neutralize specific pathogens (Janeway *et al.* 1999). Of the five types of mammalian antibodies, the most common found in the blood circulatory system is Immunoglobulin G (IgG) which binds to bacterial, viral and fungal pathogens (Junqueira and Carnerio 2003).

Most measures of the innate and acquired immune systems are expected to increase during bacterial infection in mammals (Janeway *et al.* 1999; Norris and Evans 2000; Weil, Martin and Nelson 2006). However, ecological immunity is a complex system and these increases are not always apparent during infection (Norris and Evans 2000). In some cases, an immune 'response' may instead signal 'readiness', in that an individual's resources are available to fight off infection or invading pathogens (Norris and Evans 2000). Similarly, a poor immune response could indicate

that an individual is in poor condition and is unable to mount a response to the infection (Norris and Evans 2000). It is therefore important to use multiple measures of the immune system to evaluate both the innate and acquired immune response effectively (Lee 2006).

Non-pathogen microbiota diversity is essential for proper immune function in mammals. In the gut microbiota, metabolic functions (such as the absorption of ions to help promote immune function throughout the body), trophic functions (such as the development of the postnatal immune system) and protection functions (the ‘barrier’ effect, in which bacterial diversity acts as a defensive layer against pathogens) all interact to ensure immune function in mammals (Guarner and Malagelada 2003). These roles are evident in studies which show that germ-free animals, such as chickens and mice, are at an increased risk of infection than animals with normal gut microbiota (Baba *et al.* 1991; Taguchi *et al.* 2002). More broadly, many studies have shown that gut microbiota diversity is positively correlated with health in humans, such as the discovery that *Helicobacter pylori* in the mammalian gut outcompetes other bacterial taxa, causing peptic ulcer disease in humans (reviewed in Suerbaum & Michetti, 2002). While increased bacterial diversity may help prevent the spread of pathogenic bacteria in the human gut (Dethlefsen *et al.* 2008), this is also true for other taxa such as mice, *Mus musculus* (He *et al.* 2014) and desert locusts, *Schistocerca gregaria* (Dillon *et al.* 2005), suggesting this relationship in the gut may be highly constrained across taxa.

Not all microbiota confer a healthy state in their host due shifts in bacterial diversity and potential for pathogens. The healthy human vaginal microbiota, for example, is dominated mainly by *Lactobacillus* species (Stumpf *et al.* 2013). A disequilibrium of this state, due to an increase in bacterial diversity, is known as Bacterial Vaginosis and can have negative fitness consequences by increasing susceptibility to sexually transmitted infections and premature birth or miscarriage

during pregnancy in humans (Taha *et al.* 1998; Ugwuada *et al.* 2003; Liu *et al.* 2013). The differences in ‘healthy’ states in the body region microbiota highlights the inability to describe concrete microbiota composition that confers health in all body regions, and brings into focus the complex relationship between microbiota composition and fitness in mammals.

### Thesis description

While emphasis has been placed on human and laboratory studies of the microbiota, studies of natural populations of animals are important in linking laboratory studies to ecologically relevant systems (Maurice *et al.* 2015). Our knowledge of microbial composition in natural systems is increasing, however studies of wild animals have previously focused on sampling a host once, representing only a ‘snapshot’ of the microbiota, or have focussed on seasonal changes in the microbiota of a single body region. Understanding how microbiota of different body regions in a single host are linked to host fitness correlates over long time periods is necessary to further our understanding of how microbiota composition may ultimately impact natural populations.

In this thesis, I aim to examine the microbiota composition of multiple body regions in a wild mammal, and link variation in composition to various intra- and inter-individual factors. Using the North American red squirrel (*Tamiasciurus hudsonicus*) as a model species, I will evaluate the composition of the genital, oral, and gut microbiota and explore the relationship between microbiota variation and the relative importance of sex, capture period, immune status, and body condition of the host.

The first chapter describes an exploratory study that involves evaluating the composition of the external genital microbiota in red squirrels throughout the summer season and investigating the relationship between microbiota variation, sex and body condition in these individuals.

The second chapter has two main objectives. The first is to quantitatively assess variation in microbial composition in a wild mammal, by investigating intra- and inter-individual variation in the microbiota of two body regions over a short recapture period. The second objective of this study is to investigate the factors driving microbiota variation within these individuals by exploring correlations between microbiota composition and immune status, sex, and body condition. As studies of humans and lab animals have shown a negative relationship between bacterial diversity in the gut and oral cavity and the propensity for hosts to be invaded by pathogens (reviewed by Brook, 1999), I expect to find similar trends in a wild mammal, in which individuals' with more diverse microbiota will also display measures of increased immune system functioning than individuals with less diverse microbiota.

### Study species

North American red squirrels (*Tamiasciurus hudsonicus*) are small, semi-arboreal rodents, who inhabit coniferous dominated forests in North America. They are a highly territorial and solitary species and, with the exception of a short mating season, there is little direct interaction between conspecifics (Smith 1968). Red squirrels are an ideal study species for this project for numerous reasons. First, their large populations and territorial nature allows for large sample sizes and relatively high recapture rates. Second, they are fairly closely related to mice and rats, species that have been a main focus in laboratory studies investigating variation in microbiota composition. This relatedness allows for comparisons between the results of laboratory studies and this thesis. To date, little research has been conducted on the microbiota composition in wild mammalian populations and this study is the first to explore the link between inter-capture variation, host fitness, and microbiota abundance and diversity in a wild mammal.

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Chapter 1: Evidence of broad similarities in genital microbiota composition in a wild mammal: relations to sex and fitness correlates

### 1.1 Abstract

While the study of the microbiota has flourished in recent literature, relatively little is known about the association between fitness, sex and microbiota composition in wild mammals. Here I use the promiscuous North American red squirrel, *Tamiasciurus hudsonicus*, to a) characterize the core external genital microbiota (EGMs) in a wild mammal and b) investigate if prevalent microbiota composition differs with host sex or body condition, a common fitness correlate. Evidence of a core microbiota existed within these individuals and, while some variation in bacteria taxa did exist, the prevalent EGM composition was similar between sexes and across individuals with varying body condition, suggesting that the genital microbiota of red squirrels is similar across conspecifics. Overall microbial abundance and diversity was not significantly correlated with body condition, highlighting the need to study the relationship between microbiota and fitness more robustly in wild mammals. This is the first study to characterize the red squirrel genital microbiota and provides evidence of a core microbiota in a wild mammal.

## 1.2 Introduction

Microbiota can directly impact individual health by influencing immunity (Hooper *et al.* 2001; Oliver *et al.* 2003; Macpherson and Harris 2004), and weight management or obesity (Turnbaugh *et al.* 2006, 2009; Bäckhed *et al.* 2007), among other factors. Genital microbiota composition, in particular, influences reproductive health and output and varies widely with age, sex and overall health in humans (Tamrakar *et al.* 2007; Nelson *et al.* 2010). In other mammals, individual anal pouch secretions and vaginal microbiota composition may be largely influenced by the opportunity for bacterial transfer, driving similarity in microbiota composition in individuals who live in close-proximity and increasing diversity with promiscuous mating systems (MacManes 2011; Leclaire, Nielsen and Drea 2014b; Yildirim *et al.* 2014). As a mammal's microbiota may be tightly linked to development and survival, exploring microbiota composition is important in order to fully understand a species' ecology and can have broad implications in understanding disease and health in both the study species and other related taxa.

The connection between fitness, sex and microbiota composition is particularly important in the genital microbiota, as the presence of pathogenic bacteria in the genitalia has been shown to potentially impact host fitness (Menard *et al.* 2010). However, relatively little is known about the connection between genital microbiota composition and fitness in wild mammals, which is important in order to connect laboratory based studies to ecologically relevant systems. Here I use the North American red squirrel, *Tamiasciurus hudsonicus*, to a) characterize the core microbiome of this wild mammal and b) investigate if the prevalent external genital microbiota (EGM) of red squirrels vary with sex or body condition, a common fitness correlate.

Red squirrels are a highly territorial and solitary species and, for the majority of the year, there is little direct interaction between conspecifics (Smith 1968). During the mating season, individuals participate in scramble mating competition, in which males leave their territories and

search for receptive estrous females (Lane *et al.* 2009). On their single day estrous period, females will mate with multiple males multiple times (Lane *et al.* 2008). This leads to a dichotomy in the opportunity for bacterial transfer between individuals, in which this opportunity is high during the short mating season and low throughout the rest of the year. If genital microbiota composition is strongly shaped by the opportunity for bacterial transfer, as demonstrated in other mammals, then it is likely that red squirrels will display large amounts of variation in microbiota composition. In contrast, if composition is strongly shaped by other factors, such as environmental influences or evolutionary selective pressures, then the microbial communities may display evidence of a core microbiota, common to all conspecifics.

### 1.3 Methods

#### 1.3.1 Study site and sample collection

16 red squirrel individuals (nine females and seven males) were live-trapped using tomahawk traps (Tomahawk Live Trap Co., Tomahawk, Wisconsin, USA) in Algonquin Provincial Park, Ontario, Canada ( $45^{\circ}35'N$ ,  $78^{\circ}31'W$ ) between May and August 2013. Upon first capture, each individual was marked with two numbered ear tags for future identification. Morphological measurements were taken at the first and all subsequent captures. These measurements included mass (Pesola scale  $\pm 1g$ ), right hind foot length (heel to tip of longest nail; ruler  $\pm 1mm$ ), skull length (distance from occipital crest to tip of nose; calipers  $\pm 0.1mm$ ), skull width (distance between parietal bones; calipers  $\pm 0.1mm$ ) and reproductive condition (females: non-reproductive, pregnant, lactating; males: scrotal, non-scrotal). Upon each individuals' capture, the external genitalia were swabbed using two dry sterile calcium alginate swabs (AMD Ritmed Inc., Quebec, Canada). Genitals were swabbed for 30 seconds, with light pressure applied in order to maximize DNA transfer. Swabs were placed into 1.5 mL microcentrifuge tubes, put on ice in the field, and stored in a  $-20^{\circ}C$  freezer within two hours. All individuals were sampled at least

twice throughout the field season to ensure adequate DNA collection for extraction and sequencing.

This study was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Laurentian University, protocol number 2013-03-01.

### 1.3.2 DNA extraction, 16S rRNA gene library preparation and sequencing

DNA samples were extracted from the swabs using the DNeasy Blood and Tissue DNA Extraction Kit (Qiagen, #69504), following the manufacturer's guidelines. Swabs collected over the field season were combined during DNA extractions and concentrated to increase DNA concentrations. DNA purity was quantified via spectrophotometry (Nanodrop, Thermo Fisher Scientific, Waltham, MA, USA).

Extracted DNA was processed at Mr. DNA Labs in Shallowater, Texas, United States. In a modified version of amplicon pyrosequencing (bTEFAP) process (Dowd *et al.* 2008), 16S rRNA gene universal primers 28F and 519R were used in a single-step 30 cycle PCR using HotStarTaq Plus Master Mix Kit (Qiagen, Valencia, CA, USA). This was conducted under the following conditions: 94°C for 3 minutes, followed by 28 cycles of 94°C for 30 seconds; 53°C for 40 seconds and 72°C for 1 minute. A final elongation step at 72°C for 5 minutes was performed. Following PCR, all amplicon products from different samples were mixed in equal concentrations and purified using Agencourt Ampure beads (Agencourt Bioscience Corporation, MA, USA). Samples were sequenced using Roche 454 FLX titanium instruments and reagents and following the manufacturer's guidelines.

### 1.3.3 Bioinformatic and statistical analyses

Sequences were processed using Quantitative Insights into Microbial Ecology (QIIME) (version 1.8.0) (Caporaso *et al.* 2010). V4 sequences were filtered to a length of 200-1000bp and all passed a minimum quality base score of 25. Filtering excluded ambiguous bases and primer

mismatches. Sequences were dereplicated, sorted by abundance, and singletons were removed. Operational Taxonomic Units (OTUs) were formed by grouping sequences with 97% similarity using UPARSE (Edgar 2013). Chimeras were filtered out using Usearch's 'Gold' database (version microbiomeutil-r20110519). Representative sequences from OTUs were aligned against the GreenGenes core reference set (version 13.5). Using a 97% confidence minimum, the Ribosomal Database Project Classifier assigned taxonomic classification to each representative OTU using the GreenGenes database (version 13.5). Alignments were then filtered and a midpoint rooted tree was produced using FastTree in QIIME. As the swabs used in DNA collection were found by the manufacturer to be potentially contaminated pre-packaging (Health Canada 2014), the identified potential contaminant genera (*Bacillus*, *Paenibacillus*, and *Brevibacillus*) were filtered before further downstream analysis was conducted.

Statistical analysis was completed in R (R Core Team 2014) using the base and stats packages, as well as the packages vegan (Oksanen *et al.* 2015), phyloseq (McMurdie and Holmes 2013), indispecies (De Caceres and Legendre 2009), ggplot2 (Hadley 2009), ggtthemes (Arnold 2015) and RColorBrewer (Neuwirth 2014).

As all individuals were caught more than once over the study period, mean morphological dimensions were calculated. The first principal component from a principal component analysis (PCA) was used as a proxy for body size from individuals' hind foot length, skull width and skull length measurements. Body condition was then estimated through the regression of body mass and body size (Schulte-Hostedde *et al.* 2005).

Shannon diversity was calculated based on the raw dataset, and compared statistically using the non-parametric Wilcoxon signed-rank test. OTUs representing  $\geq 1\%$  abundance in at least one individual were used as a proxy for 'prevalent' bacteria, and OTUs that were present in all

individuals were used as a proxy for the ‘core’ microbiome. As encouraged by McMurdie and Holmes (2014), in order to negate large differences in library sizes in the dataset, count data of all OTUs were normalized by applying variance stabilizing transformations (VST) with the DESeq2 package (Love, Huber and Anders 2014). Normalized count data were then used to calculate weighted UniFrac distances. Adonis, a permutational MANOVA, was used to calculate statistical differences in weighted UniFrac distance matrices. Each test was run with 9999 permutations and pseudo F, R<sup>2</sup> and p values are reported. Principal Coordinate Analysis (PCoA) ordinations using weighted UniFrac distances were performed to visualize intersexual differences in abundance and diversity in overall microbial composition. Additionally, a non-parametric indicator species analysis was run on the prevalent microbiota dataset to identify OTUs that best described differences between the sexes. To identify significant differences in taxa abundance in the prevalent microbiota, type-3 linear models were run using VST normalized read counts, sex and body condition. If significant sex-body condition interactions were present, the sexes were divided and run separately using regression analyses. Non-significant interactions remained in the linear models. Statistics for taxa differences are reported only for the lowest taxa resolution, which were the drivers of the significant differences within individuals.

## 1.4 Results

### 1.4.1 Body condition analysis

The first component (eigenvalue 3.36) of the principal component analysis (PCA) using the three morphological measures explained 52.6% of the variance in the data and was used as a proxy for body size in the analysis. The first component loaded positively with skull length (0.913), hind foot length (0.297) and skull width (0.279); There were no intersexual differences in body size ( $W=49$ ,  $p=0.07$ ) or body condition ( $W=24$ ,  $p=0.47$ ).

#### 1.4.2 Characterization of red squirrel abundant and core microbiota

For the 16 individuals sampled, a total of 82 286 sequences were detected in the dataset, with a mean sequencing depth of  $5\ 142.9 \pm 975.26$  sequences per sample. After OTU binning and quality filtering, the ‘prevalent’ microbiota was established by subsetting the raw microbiota to include OTUs that were composed of >1% relative abundance in the dataset. In total, 1 198 OTUs and 57 906 sequences remained in the dataset, with an average of  $3\ 619.13 \pm 697.13$  sequences per individual. Of the 20 unique phyla detected in the prevalent microbiota, Firmicutes, Proteobacteria and Bacteroides were the three most abundant, representing 36.8%, 30.8%, and 11.2% respectively of the microbiota in all individuals (Figure 1-1). Notably, *Clostridium colinum* and *Staphylococcus aureus* were found to be present in multiple individuals ( $n=3$ ;  $n=11$  respectively).

The core microbiota included all OTUs that were present in every individual and was composed of seven OTUs and 10 527 sequences, with an average of  $657.94 \pm 266.44$  sequences per individual. The core microbiota accounted for 18.2% of the prevalent microbiota. The seven OTUs included taxa that could reliably be identified as Bacillales, Pasteurellaceae and Bradyrhizobium, among others (Figure 1-2).

#### 1.4.3 Composition in the prevalent microbiota similar across conspecifics’ sex and body condition

Among sexes, 638 OTUs were present in both males and female microbiota, while 289 and 271 unique OTUs were present in only males and females respectively (Figure 1-3). The most abundant of these shared OTUs included those taxonomically classified as Bacillales, *Staphylococcus* and *Flexispira* (Figure 1-3; Figure 1-4). Of the 638 shared OTUs, males were significantly more likely to have a higher abundance of any given OTU compared to females ( $X^2=73.39$ ,  $p<0.0001$ ).

There were no differences between sexes in alpha diversity metrics (Shannon's diversity index: W=32, p=0.99), nor could differences in abundance be explained by host sex (VST normalized sequence counts: W= 27, p= 0.68). Indicator species analysis revealed that OTU\_405 (genus *Mycobacterium*) was associated with males and OTU\_125 (family Isosphaeraceae) was associated with females in this dataset (Table 1-1).

Beta diversity metrics, through the calculation of Weighted UniFrac distances of VST normalized sequence counts, also did not show any significance between sexes (adonis:  $F_{1,14}=0.96$ ,  $R^2=0.07$ ,  $p=0.38$ , Figure 1-5), although female variation was confined within male variation. Body condition was not significantly correlated with weighted UniFrac distances (adonis:  $F_{1,14}=0.53$ ,  $R^2=0.04$ ,  $p=0.72$ ).

Linear models revealed that, in males, body condition was negatively correlated with Acidobacteriaceae ( $F_{1,5}=8.5$ ,  $R^2=0.63$ ,  $p=0.03$ ) and *Sphingomonas wittichii* abundance ( $F_{1,5}=5.3$ ,  $R^2=0.46$ ,  $p=0.04$ ), while females did not show significant correlations between body condition and any classified taxa (Figure 1-6).

## 1.5 Discussion

I have sampled the EGMs of 16 North American red squirrels, explored the composition of these microbiota, and investigated the relationship between microbiota composition and variation in both host sex and a fitness correlate. Firmicutes was the dominant phylum in individuals' microbiota, consistent with findings in studies examining genital and fecal microbiota of other members of the order Rodentia (MacManes 2011; Ericsson *et al.* 2015). While MacManes (2011) found that two mice species' (*Peromyscus californicus* and *Peromyscus maniculatus*) vaginal microbiota were also dominated by Actinobacteria, this was not the case in the females in this study. This difference in major phyla abundance can potentially be explained by either

interspecific differences or interspatial microbiota sampling methods, which have been noted to influence microbiota composition (reviewed in Stumpf *et al.*, 2013).

There is evidence of broad similarities in microbiota composition between sexes, suggesting that a prevalent microbiota exists in this species. Previous mammalian microbiota studies have shown that inter-individual homogeneity in anal pouch secretions and genital microbiota composition is influenced by the opportunity for bacterial transfer, such as the opportunity for transmission through close proximity in meerkats (Leclaire, Nielsen and Drea 2014b). However, North American red squirrels are highly territorial for the majority of the year (Smith 1968), leading to a low probability of bacterial transfer. In this study, I have found evidence of similar composition between conspecifics. This may be explained by the short mating season at the beginning of the sampling season, suggesting that mating behaviours may have large, lasting influences on microbiota composition in red squirrels. Alternatively, genital microbiota composition may perhaps be less driven by the opportunity for bacterial transfer than predicted in this species, and is instead driven by other external variables, such as environmental or evolutionary factors.

An indicator species analysis revealed two OTUs that best described differences between the sexes. While the genus *Mycobacterium* was present in both males and females, it was relatively more abundant in the males of our study. Similarly, the family Isosphaeraceae was found in greater abundance in females than males in this dataset. As these OTUs were only able to be broadly taxonomically classified, it is difficult to discern why these sex differences may exist. However, there are only a few OTUs that are statistically different between the sexes, and there are a relatively high number of shared OTUs in this dataset. These lines of evidence provide support for evidence of the persistence of a prevalent microbiota within this species.

The abundance of several bacterial taxa showed significant interactions between sex and/or body condition in this study. Males showed a significant negative correlation between body condition and abundance in Acidobacteriaceae and *Sphingomonas wittichii*. As these taxa have so far been isolated from environmental and marine samples (Kuske, Barns and Busch 1997; Barns, Takala and Kuske 1999; Yabuuchi *et al.* 2001; Becking 2006), and little is known about the influence of these taxa on mammalian fitness, future studies should directly investigate why these significant correlations may exist within male red squirrels.

Two notable putative pathogens were detected in this dataset. First, *Staphylococcus aureus* was found to be present in 11 individuals in this study. This species acts as both a commensal and pathogenic species in humans and other animal hosts (Gordon and Lowy 2008). Colonies can be found in up to 30% of human hosts and have the potential to act as reservoirs for later infection when hosts' immune systems and defensive barriers are compromised (Gordon and Lowy 2008). *S. aureus* was present in 68% of the EGMs in this study, but abundance of this bacterial species was not significantly correlated with either host sex or body condition. This finding suggests that the presence of *S. aureus* does not directly decrease host body condition, but that the bacteria may act as a commensal organism or opportunistic pathogen in red squirrels. Future research is encouraged in this area to increase the knowledge of the impact of *S. aureus* presence and potential ability for infection in red squirrel hosts.

The presence of another putative pathogen species, *Clostridium colinum*, was detected in the EGMs of three individuals. *C. colinum* is a causative bacteria of ulcerative enteritis ('quail disease') in numerous avian species (Berkhoff, Campbell and Naylor 1974; Berkhoff 1985). This infection is highly virulent and is transmitted via the feces of infected individuals in natural environments (Wages 2008). As *C. colinum* has been found to have no pathogenic effect on

inoculated guinea pigs (Berkhoff 1985), was found in low abundance in the individuals in this study, and had no correlation with body condition in red squirrel hosts, it may be unlikely that *C. colinum* has a direct impact on red squirrels' fitness. The potential for red squirrels to act as a reservoir for *C. colinum* bacteria, however, cannot be discounted and further research is recommended to explore this possibility.

The results of this study suggest that red squirrels exhibit a high degree of interindividual similarity across EGMs, spanning variation in both host sex or body condition. I have used body condition to demonstrate that microbial composition at various taxa levels is only rarely significantly correlated with a basic measure of fitness in wild mammals, highlighting the need to study the relationship between the microbiota and fitness more robustly in wild mammals.

Broadly, future studies should improve our understanding of the relationship between microbiota and fitness, which can be accomplished by increasing sample sizes and implementing longer-term studies effective in directly evaluating host fitness. With these future directions in mind, I hope this paper serves as a founding step in disentangling the relationship between microbiota composition and fitness in wild mammals.

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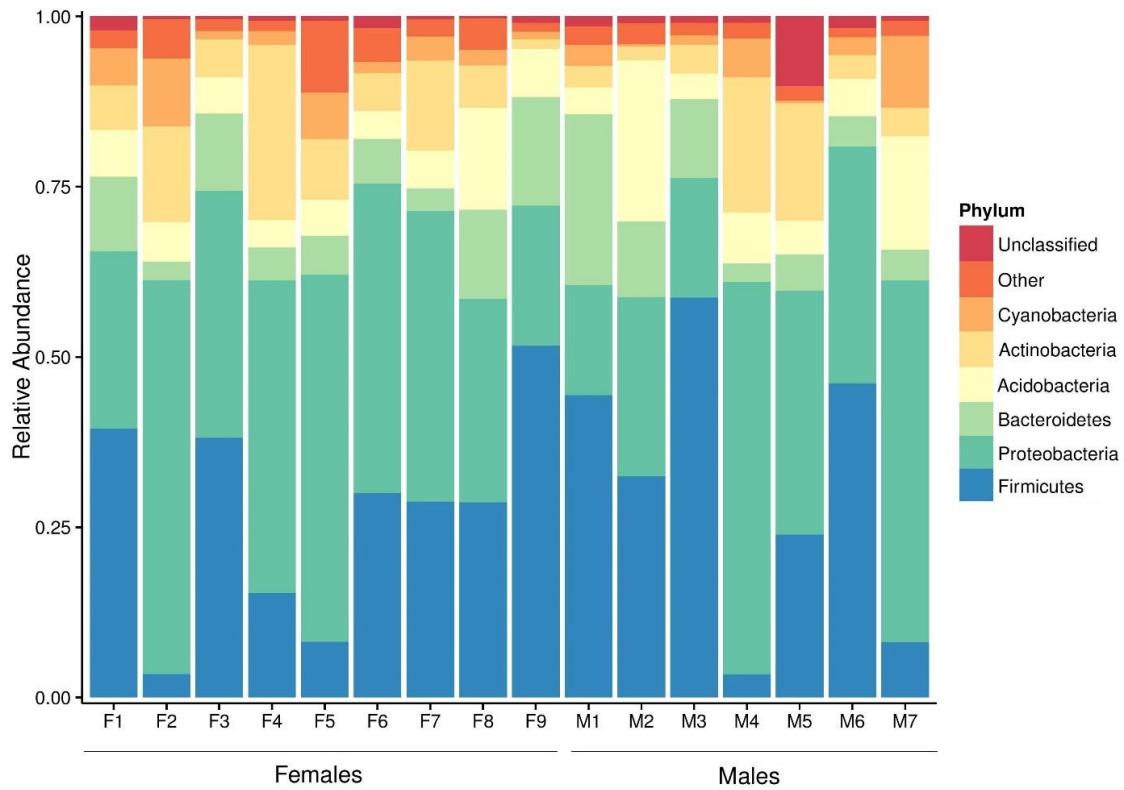
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*Table 1-1:* Indicator Species Analysis characterizing differences in genital microbiota composition between male and female red squirrels. P-value is calculated based on Monte-Carlo test with 999 permutations.

<b>Sex</b>	<b>Indicator OTU (lowest classified taxa)</b>	<b>Indicator value</b>	<b>p-value</b>
<b>Male</b>	OTU_405 ( <i>Mycobacterium</i> )	75.6	0.016
<b>Female</b>	OTU_125 (Isosphaeraceae)	74.6	0.022



*Figure 1-1:* Relative abundance of the top six most abundant phyla in the prevalent genital microbiota in 16 red squirrels. Phyla are stacked in decreasing overall abundance. Firmicutes, Proteobacteria, and Bacteroidetes were the three most abundant phyla in the microbiota, representing (respectively) 36.8%, 30.8% and 11.2% of overall bacterial abundance.

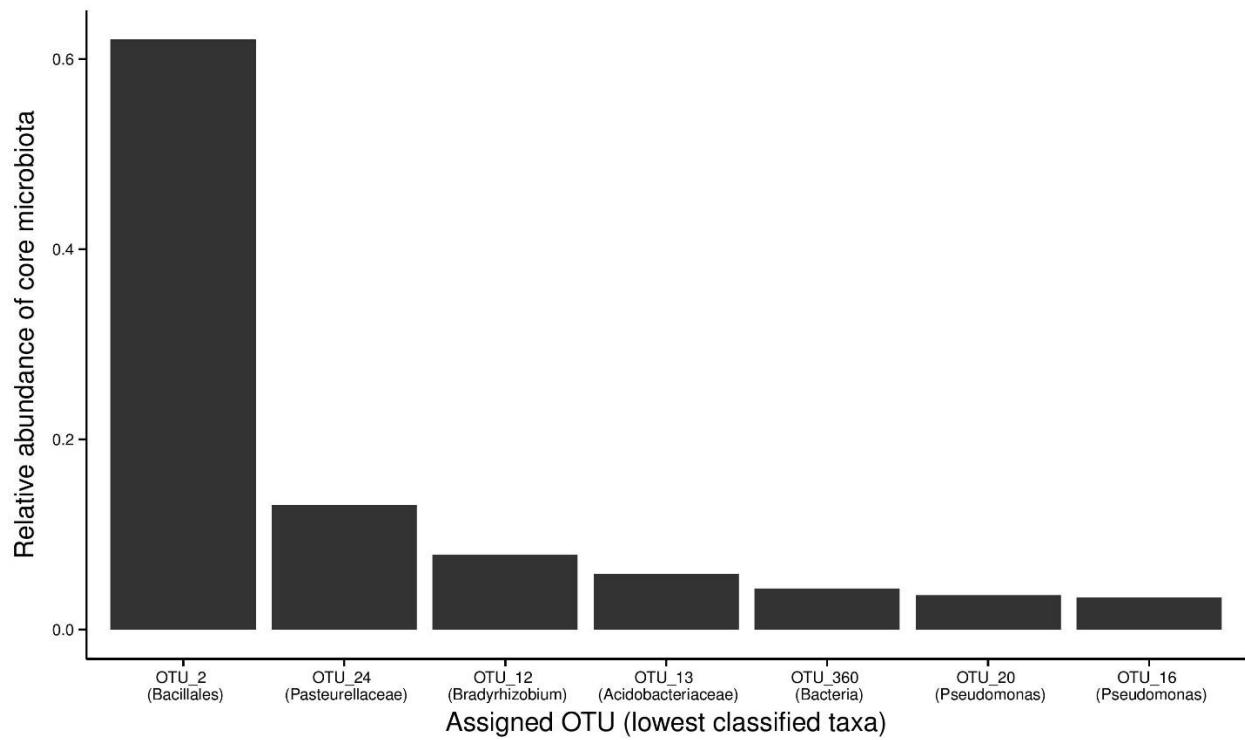
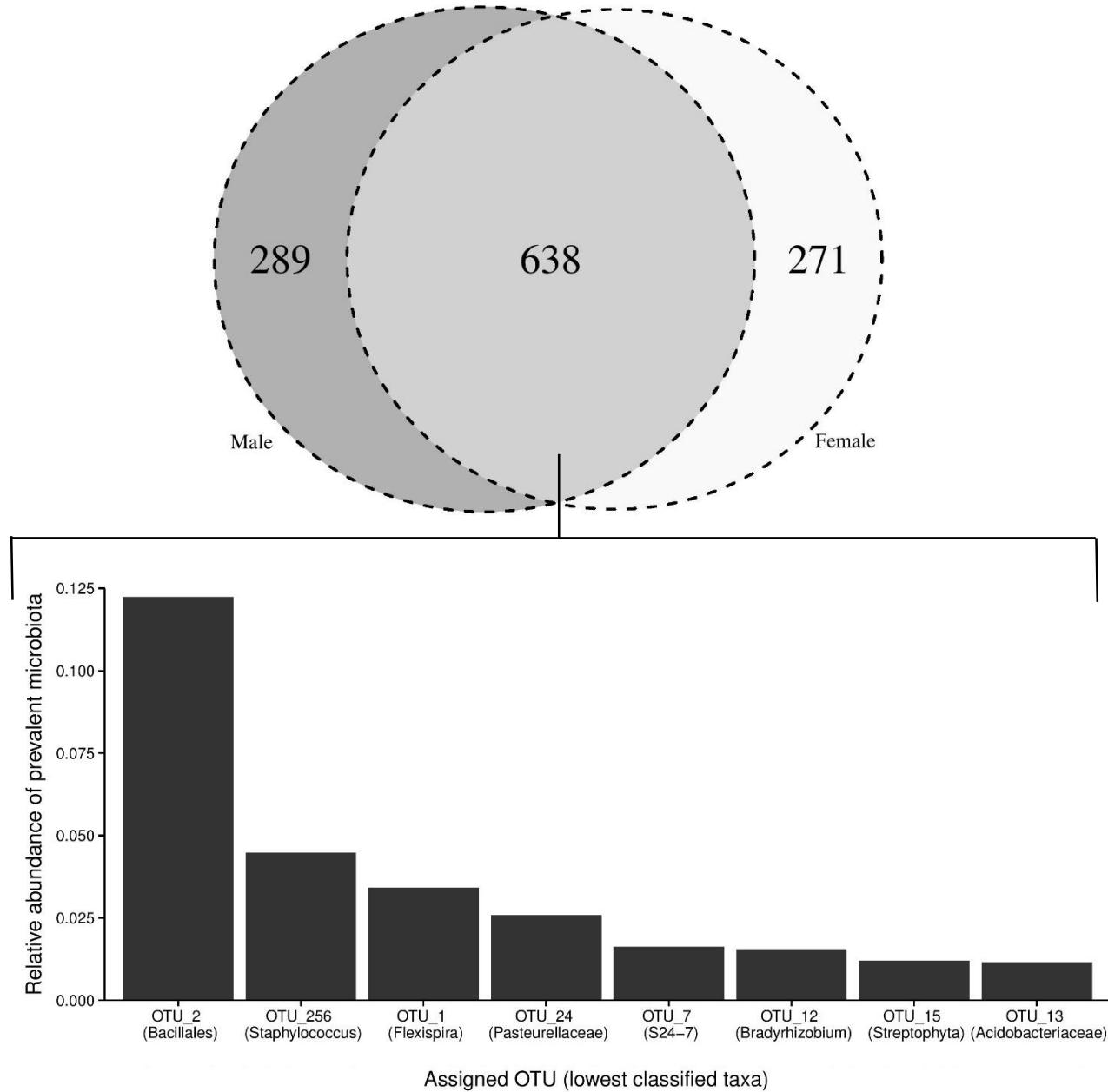
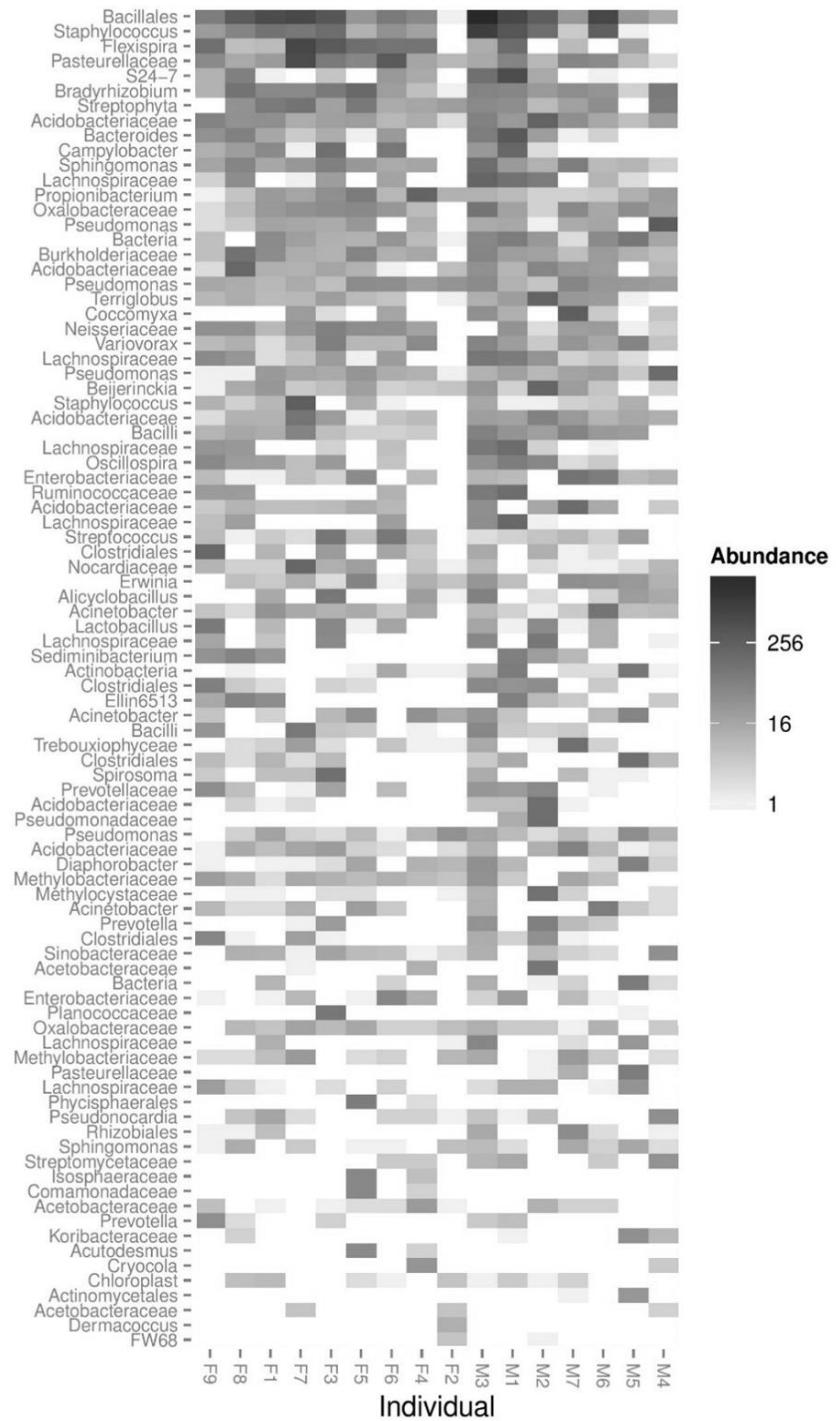


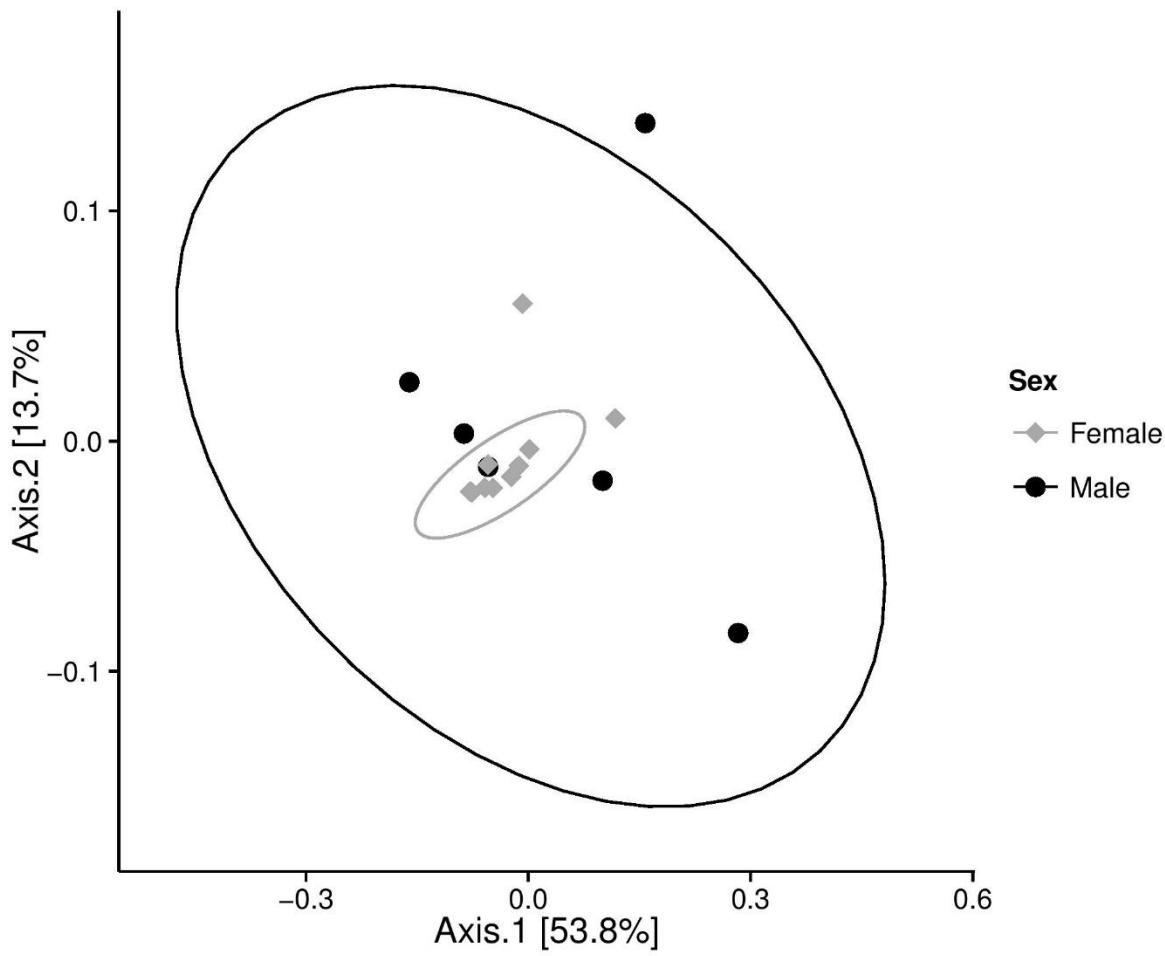
Figure 1-2: Relative abundance of the seven OTUs representing the core microbiota of red squirrel genitalia. Each OTU was present in all individuals in the study. Lowest classified taxa corresponding to each OTU are reported.



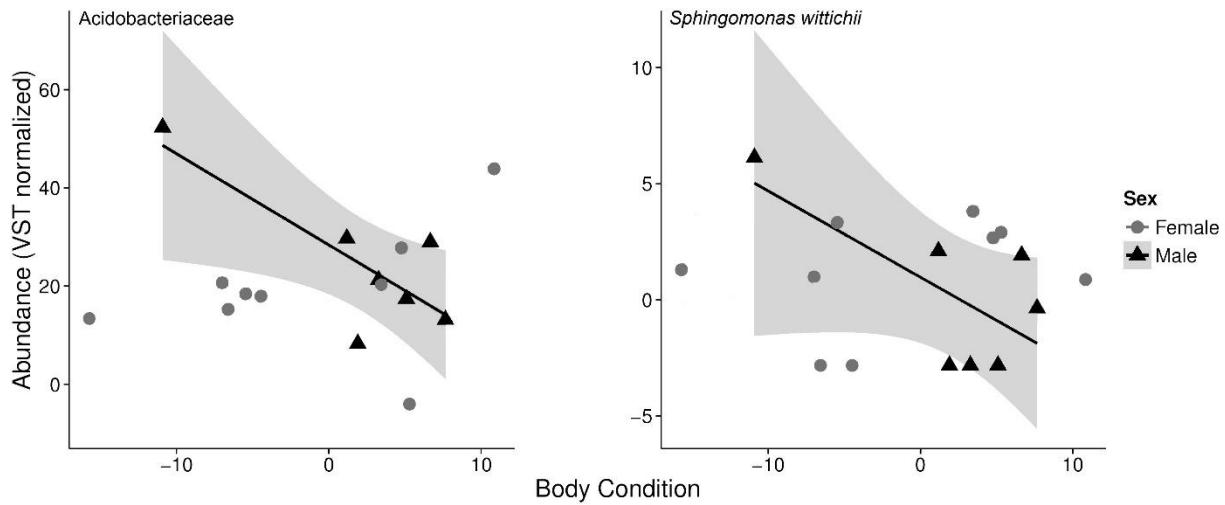
**Figure 1-3:** Operational Taxonomic Unit (OTU) relationships between male and female prevalent microbiota. Of the 638 shared OTUs between the sexes, eight OTUs had >1% relative abundance in the prevalent microbiota, including OTUs assigned to the taxonomic classifications Bacillales and Staphylococcus.



*Figure 1-4:* Heatmap of red squirrel individuals' prevalent bacteria. Operational Taxonomic Units (OTUs) which represent  $\geq 1\%$  abundance in at least one individual are shown. OTUs are labelled with their lowest taxonomic classification and sorted by highest (top) to lowest (bottom) abundance. Individuals are grouped by sex and ordered based on weighted UniFrac distances with Principle Coordinate Analysis.



*Figure 1-5:* Principal Coordinate Analysis using weighted UniFrac distances of microbiota composition. Axis 1 explained 53.8% of the variation in the dataset, Axis 2 explained 13.7%. Confidence ellipses represent 95% confidence around distance centroids for each sex. While males varied more widely across the axes than females, there were no significant differences in distances between males and females (adonis:  $F_{1,14}=0.96$ ,  $R^2=0.07$ ,  $p=0.38$ ).



**Figure 1-6:** Evidence of significant interactions in red squirrel males between body condition and abundance of two bacterial taxa. Males exhibited a significant negative correlation between abundance and body condition in Acidobacteriaceae ( $F_{1,5} = 8.5$ ,  $R^2=0.63$ ,  $p=0.03$ ) and *Sphingomonas wittichii* ( $F_{1,5}= 5.3$ ,  $R^2=0.46$ ,  $p=0.04$ ). Shaded areas represent 95% confidence intervals around male data, abundance values represent normalized read counts.

Chapter 2: Linking temporal variation, fitness, and the microbiota in a wild  
mammal (*Tamiasciurus hudsonicus*)

## 2.1 Abstract

The importance of microbial communities, or microbiota, to host physiology and health is readily becoming apparent. While our knowledge of the interaction between fitness and microbiota composition is increasing, few studies have investigated this relationship in wild mammals. Here I use the North American red squirrel (*Tamiasciurus hudsonicus*) to explore variation in composition of buccal and gut microbiota across short time periods and link this variation to measures of fitness through the exploration of individual immune status. To meet these objectives, I used 16s ribosomal RNA gene sequencing-based surveys of the buccal and gut microbiota in 24 individuals captured two weeks apart and measured individuals' immune function at each capture. Beta diversity analysis revealed that the buccal, but not gut, microbiota showed significant variation between capture periods, suggesting that temporal variation is dependent on the location of the microbiota, but that wild mammal microbiota have the ability to display large amounts of variation over short time scales. Measures of innate immune system negatively correlated with microbiota diversity in the buccal microbiota, while the presence of antibodies, used as a measure of immune preparedness, positively correlated with microbiota diversity in the buccal microbiota. Together our results provide, for the first time, a comprehensive view of inter- and intra-individual microbiota variation over a short time scale and provide evidence for a correlation between microbial composition and fitness in a wild mammal.

## 2.2 Introduction

Mammals are hosts to trillions of microbes and variation in these microbial communities, the microbiota, are rapidly being recognized as important to host physiology. Variation in microbiota has been linked to spatial location (Linnenbrink *et al.* 2013), habitat degradation (Amato *et al.*, 2013), sex (Leclaire, Nielsen and Drea 2014b) and diet (Phillips *et al.* 2012; Ooi *et al.* 2014).

While emphasis has been placed on human and laboratory studies of host microbiota, studies of natural populations are important in linking laboratory studies to ecologically relevant systems (Maurice *et al.* 2015). Our knowledge of microbial composition in natural wildlife populations is increasing, however, studies of wild mammals have previously focused on single time scales, which serve as a ‘snapshot’ of the microbiota or have focussed on seasonal changes in single microbiota. Understanding how multiple microbiota in a single host are linked to correlates of host fitness over multiple time periods is necessary to further our understanding of how microbiota composition may ultimately impact natural populations.

The first objective of this chapter is to investigate intra- and inter-individual variation over a short recapture period and investigate how this variation differs between two separate regions on the body of a wild mammal. While seasonal variation has been found in wild mice (Maurice *et al.* 2015), lab studies have also shown that variation in microbiota is dependent on both the body region and time (Caporaso *et al.* 2011). As a result, I hypothesize that there will be spatially dependent variations in microbiota composition and predict that the buccal microbiota will shift in composition at a faster rate than the gut microbiota.

The second objective in this study is to investigate the factors driving this microbiota variation within individuals. Colonization resistance, or bacterial interference, suggests a negative relationship between bacterial diversity and the propensity for hosts to be invaded by pathogens

(review: Brook, 1999). This has been demonstrated across numerous other mammals, such as mice, and humans, where individuals in diseased and non-diseased states differ in microbiota composition (He *et al.*, 2014; review: Kinross *et al.*, 2011), and individuals with more diverse microbiota are less likely to be invaded by pathogens (Dillon *et al.* 2005). The link between wildlife immunology and microbiota composition has largely remained unexplored in the rapidly expanding field of microbial ecology, despite the fact that the ability for a host to fight off infection or invading pathogens ('immunocompetence'; Norris & Evans, 2000) is an important factor in fitness in wild mammals. I hypothesize that the microbiota and immune system is tightly correlated with one another in this wild population, and predict that I will find evidence of colonization resistance, in which individuals with more diverse microbiota will also display higher fitness correlates than individuals with less diverse microbiota.

## 2.3 Methods

### 2.3.1 Study site and sample collection

Red squirrels were sampled from a 0.1 km<sup>2</sup> grid of 80 tomahawk live traps (Tomahawk Live Trap Co., Tomahawk, Wisconsin, USA) in Algonquin Provincial Park, Ontario, Canada (45°54'N, 78°26'W). Traps were spaced every 20 m, set 0600-1900 h, baited with an oatmeal-peanut butter mixture (~10 g) and checked every ≤2 h. A total of 24 squirrels (12 males; 12 females) were captured twice for repeated sampling (sample 1: July 17-19, 2014; sample 2: July 28-30, 2014). Upon first capture, each individual was marked with two uniquely numbered ear tags. Sex, reproductive condition (females: non-reproductive, pregnant, lactating; males: scrotal, non-scrotal), and mass (Pesola scale ±1 g) were recorded for all individuals at all captures. Only 14 individuals were assessed for complete body condition measurements, taken at a third capture. This was done to reduce handling time and subsequent stress during the first two captures. Morphological measurements included right hind foot length (heel to tip of longest nail; ruler ±1 mm), skull length (distance from occipital crest to tip of nose; calipers ±0.1 mm), and skull width (distance between parietal bones; calipers ±0.1 mm).

Oral microbiota samples were collected using sterile cotton-tipped swabs (Fisher Scientific, Ontario, Canada). The oral cavity was swabbed for a minimum of 30 s, with light pressure applied to the cheeks and tongue in order to maximize DNA transfer. All individuals were swabbed at least twice during each capture to ensure adequate DNA collection for extraction and sequencing.

Fecal samples were collected from the traps, placed into 1.5mL labelled microcentrifuge tubes and were stored on ice in the field before being transferred to a -20°C freezer until further processing. Fecal collection in this manner has been shown to be largely uncontaminated by

environmental factors, and is a good representation of the gut microbiota in small mammals (Kohl, Luong and Dearing 2015).

### 2.3.2 Fitness analyses

Blood samples were taken from a toenail on a hind foot of each individual. Nails were clipped to the quick with sterilized toenail clippers. Blood was collected in a standard heparinized capillary tube (Ram Scientific, Yonkers, NY). Styptic powder (Kwik Stop, Mississauga, Ontario) was applied to stop any excessive bleeding. Upon returning to the lab, a collected tube was centrifuged at 3,000 RPM for 5 min. Hematocrit parameters, through analyses of packed cell volume, were measured, and plasma was extracted and stored at -20°C until immunoglobulin tests were performed. Blood smears were made to assess leukocyte counts and set in the field using Methyl Alcohol. Smears were then stained using the Wright's Staining method (Kwik<sup>TM</sup> –Diff stain kit; Thermo Electron Corporation), and leukocytes were counted per 10,000 red blood cells. Specific leukocyte types were tallied to give total counts based on 100 white blood cells.

Immunoglobulin antibody tests were conducted to measure the amount of total immunoglobulin G (IgG) antibody in plasma. A preliminary dilution test was conducted to determine the appropriate species-specific anti-body/plasma volumes. The optimal concentration was found to be 1:32,000, which lays at the center of the linear change in the negative sigmoidal relationship between absorbance and plasma IgG concentration. Once this was optimized, an enzyme-linked immunosorbent assay (ELISA) technique based on Martínez *et al.* (2003) and adapted by Gooderham (2010) was conducted using a commercial anti-guinea pig anti-body (Sigma A5545). In brief, 100 µL of the diluted samples (first dilution: 2 µL plasma/1000 µL dilution solution; second dilution from the optimized concentration), were placed in a 96-well, flat bottom sterile ELISA plate (Sigma CLS 3370). The plate was incubated for 1 h at 37°C and then

overnight at 4°C. The plate was then emptied and rinsed with 200 µL of PBS-Tween solution. 100 µL of Carnation non-fat dry milk-PBS Tween solution was added to each well and incubated for 1 hour at 37°C. The plate was then rinsed with PBS-Tween solution and filled with 100 µL of guinea pig anti-body-PBS Tween solution. The plate was incubated for 2 h at 37°C. 100 µL of PBS-Tween solution was rinsed in each well three times and 100 µL of revealing solution (ABTS solution with hydrogen peroxide) was added to each well. The plate was incubated for 1 h at 37°C. Using a 405 nm wavelength, the plate was read in a plate reader (Max Plus 384 MAIR05340, Molecular Devices, Sunnyvale, CA, USA), values retrieved were averaged for each individual and were used as a direct measure of immunoglobulin circulating in plasma. Intra-assay sample variation was 1.09% based on average relative variation of duplicate samples.

### 2.3.3 DNA extraction, 16S rRNA gene library preparation and sequencing

Bacterial DNA extraction from swabs was performed using the QIAamp DNA Mini kit (Qiagen, Mississauga, ON, Canada) following the manufacturer's Buccal Swab Extraction Protocol. Fecal DNA extraction from fecal samples was performed using the DNeasy Blood and Tissue Kit (Qiagen, Mississauga, ON, Canada) following a user-developed protocol for purification of DNA from frozen animal stool (Qiagen 2006). DNA purity was quantified via spectrophotometry (Nanodrop, Thermo Fisher Scientific, Waltham, MA) and samples were stored at -20°C until sequenced.

A volume of 20 µL of the extracted DNA solution was sent to Metagenome Bio Inc. (Toronto, Canada) for V3-V5 sequencing of the 16S rRNA gene using the 341F and 806R universal primers, using an Illumina MiSeq sequencing system. The primers contained Illumina adaptor sequences and priming sites for sequencing. Indexing sequences (6 bp) were also incorporated into both primers for pooling multiple samples in one run. The 25uL PCR reaction

contained 5 $\mu$ L of standard OneTaq buffer (5x), 0.25  $\mu$ L of 25 mM dNTP, 0.5  $\mu$ L of forward and reverse primers (10 uM each), 1  $\mu$ L BSA (12 mg/mL), 0.125  $\mu$ L of OneTaq DNA polymerase (NEB), 1-10 ng DNA, and water up to 25  $\mu$ L. PCR was run under the following conditions: 94°C for 5 min, 30 cycles at 94°C for 30 s, 45°C for 45 s, 68°C for 1 min, and finally at 68°C for 10 min. Triplicate PCR reactions were performed for each DNA sample in order to minimize bias. Each PCR product was checked on 2% agarose gels. Following pooling three PCR products of each sample, the amount of 16S rRNA gene amplicon was quantified on 2% agarose gels. All PCR products were combined in equal amount of the 16s rDNA and loaded on an agarose gel with SYBR Green (Invitrogen). The DNA band was excised with a Qiagen MinElute gel extraction kit. The purified library DNA was quantified using Qubit dsDNA HS assay kit (Life Technologies). Library pool (8 pM) was spiked with 5% phiX control (V3, Illumina) to improve bae imbalance. Paired-end sequencing with read lengths of 251 bp was performed using MiSeq Regent Kit V2 (2x250 cycles) on an Illumina MiSeq sequencing system. Raw sequences have been deposited in NCBI under the accession number SRA #SRP064395.

#### 2.3.4 Bioinformatic analysis

Sequences were processed using PANDAseq (Masella *et al.* 2012) and Quantitative Insights into Microbial Ecology (QIIME; version 1.8.0; Caporaso *et al.*, 2010). Sequences were filtered to a length of 390-590 bp. Additional filtering thresholds included an alignment penalty of 0.01, default parameters of 2 k-mers and a matching primer threshold of 0.6. Sequences were dereplicated and sorted by abundance. Operational Taxonomic Units (OTUs) were clustered using UPARSE (Edgar 2013) and chimeras were filtered out using USEarch's 'Gold' database. Using a minimum of 97% confidence cut-off, the Ribosomal Database Project classification tool assigned taxonomic classifications to each OTU using the GreenGenes database (version 13.5). Sequences

were aligned using the GreenGenes reference sequences and filtered. A midpoint rooted tree was produced using FastTree in QIIME.

### 2.3.5 Composition statistical analyses

Statistical analysis on microbiota composition was completed in R using the base and stats packages (R Core Team, 2014) as well as the packages vegan (Oksanen *et al.* 2015), phyloseq (McMurdie and Holmes 2013), ggplot2 (Hadley 2009), ggthemes (Arnold 2015) and RColorBrewer (Neuwirth 2014).

To assess body size, the first principal component from a principal component analysis (PCA) was used as a proxy for body size from 14 individuals' hind foot lengths, skull widths and skull length measurements. All variables were log transformed to conform to normality. Body condition was estimated through the regression of body mass and body size (Schulte-Hostedde *et al.* 2005).

Shannon's diversity index was calculated based on a dataset pruned to remove doubletons and singletons. While literature commonly recommends calculating alpha diversity indices from raw, complete datasets, Shannon's diversity index is sensitive to the inclusion of singletons and doubletons (Hill *et al.* 2003) and therefore I excluded these reads to limit false conclusions based on rare bacteria.

The pruned dataset was used to calculate weighted UniFrac distances for both gut and buccal microbiota. A principal coordinate analysis (PCoA) using weighted UniFrac distances including both microbiota was used as a visualization technique to explore differences between the two microbiota for all individuals.

To investigate differences between capture and across sexes, weighted UniFrac distances were calculated and PCoAs were conducted for each separate microbiota. Adonis tests (equivalent

to permutational MANOVA) were used to calculate statistical differences in weighted UniFrac distance matrices. Each test was run with 9999 permutations and the seed for each test was set at 123. Tests investigated differences in distances in buccal and gut microbiota and included capture period, sex, and microbiota as fixed effects. Individual was also included in a strata to control for repeated measures across individuals.

### 2.3.6 Fitness parameter statistical analyses

Principal component analyses (PCA) were run to reduce fitness correlates for overall general linear models. The first PCA included log-transformed white blood cell (WBC) counts, packed cell volume (PCV), log-transformed neutrophil, log-transformed lymphocyte, and monocyte measurements for individuals across both captures. As no eosinophil or basophil were identified in the smears, they were not included in these analyses. The first component of the PCA (PCA1) had an eigenvalue of 320.41 and explained 90.99% of the variance in the data. PCA1 contained two high loading variables: neutrophils (0.70) and lymphocytes (-0.71). A second PCA was conducted to explore the changes in the above blood parameters across captures. The first component of the second PCA explained 92.90% of the variation in the data (eigenvalue: 463.11), and the change in neutrophils (loading: 0.61) and change in lymphocytes (-0.71) loaded in this component. In both models, WBC, PCV, and monocytes did not load on the first component, and therefore the first components, representing neutrophil and lymphocyte counts of both models was used as a proxy for fitness correlates in these 24 individuals. PCA1 components, hereafter referred to as ‘WBC metrics’, from both ordinations were log-transformed to conform to normality for use as fixed variables in the overall general linear models.

GLMs were run to investigate differences in diversity and abundance in both microbiota, across WBC metrics, normalized IgG values, sex, and capture periods. Individuals were included

as random effects in the models. I also explored intra-individual change across capture periods in both microbiota. These models included transformed change in WBC metrics, change in IgG values and sex as fixed effects. Tukey's pairwise comparisons were used as a post-hoc analysis to determine, if necessary, the relationship between significant variables.

In order to also test for differences in composition due to body condition, these tests were then repeated with a parsed dataset which only included the 14 individuals' body condition. Body condition was added as a fixed effect to each of the models. All continuous fixed effects were transformed, if necessary, to conform to normality.

A separate study was performed, in which I attempted to disrupt the squirrel bacterial microbiota with a broad spectrum skin antibiotic (n=12; 0.3 mL/kg cefovecin sodium; Zoetis; Kirkland, Quebec). I also injected a control group (n=12) with 0.3 mL/kg sterile saline solution. In subsequent analysis, no effect of the antibiotic was observed in these individuals. Treatment was added as a fixed effect in all GLMs for this chapter, but did not change significance levels within each model. Due to the lack of evidence showing differences between these groups, all individuals have been pooled from the control and treatment groups to increase statistical power in analyses for this chapter. In-depth methodology for this experiment has been provided in Appendix 1.

## 2.4 Results

### 2.4.1 Microbiota Analyses

#### *Buccal composition analysis*

A total of 6,825,774 sequences were detected in the buccal dataset, with a mean sequencing depth of  $136,720 \pm 8,732$  sequences per sample. After OTU binning and quality filtering, 1,830 OTUs and 6,425,735 sequences remained in the dataset, with an average of  $133,860 \pm 8,352$  sequences per sample.

An abundant buccal microbiota was parsed from the complete dataset in order to visualize and statistically test the most relevant taxa differences between individuals. This subset microbiota discarded singletons and doubletons, and included 1,531 OTUs and 6,425,323 sequences. Of the 21 unique phyla detected in this dataset, the most abundant across both captures were Proteobacteria, Firmicutes and Bacteroidetes, representing a relative abundance of 75.0%, 20.2%, and 0.7% respectively in the first and 71.5%, 21.0%, and 2.9% of the second capture (Figure 2-1). Within each capture, variation in relative abundance of each phyla could not be attributed to differences in sex (Table 2-1). Overall abundance (number of sequences) was not correlated with capture period ( $V=155$ ,  $p=0.90$ ). There were 127 unique OTUs in the first capture and 481 in the second, while 923 OTUs were present in both captures (Figure 2-2).

There were no significant difference in Shannon's diversity (hereafter: diversity) across captures or between sexes ( $F_{3,44}=0.80$ ,  $p=0.51$ , Figure 2-3). However, there was a broader range in diversity in the second capture than the first (first range: 1.16-2.46; second range: 0.37-3.90). Whether individual's had decreased or increased microbial diversity across captures was not linked to sex; 13 individuals (six males, seven females) increased diversity between captures periods, while 11 individuals (six males, five females) showed a decrease in diversity across captures.

PCoA analysis revealed overlaps in confidence intervals across sexes, and the first and second components explained a noticeable amount of variation in the data (Axis 1: 19.5%, Axis 2: 7.4%; Figure 2-4). There were no significant difference across sexes or within individuals using weighted UniFrac distances for this microbiota (adonis: sex: p=0.55; individual: p=0.40), however, these distances were significantly different across captures (adonis: p=0.008).

#### *Gut Composition analysis*

A total of 5,604,024 sequences were identified in the gut dataset, with a mean sequencing depth of  $87,530 \pm 6,323$  sequences per sample. After OTU binning and quality filtering, 2,442 OTUs and 4,570,052 sequences remained in the dataset, with an average of  $87,512 \pm 4,472$  sequences per sample.

The abundant gut microbiota included 1,920 OTUs and 4,200,590 sequences. Inter-capture and intra-capture variation existed in the prevalent microbiota (Figure 2-1; Figure 2-5). Of the 20 unique phyla detected in this dataset, the most abundant across both captures were Firmicutes, Proteobacteria, and Bacteroidetes, representing a relative abundance of 49.5%, 27.7%, and 20.5% respectively of the first and 51.3%, 25.6%, and 20.5% of the second capture. While these remained the top most abundant phyla, Tenericutes and Elusimicrobia increased in relative abundance across captures and Cyanobacteria and Actinobacteria decreased in relative abundance. Within the first capture, Actinobacteria relative abundance was significantly higher in males than females ( $W=31$ ,  $p=0.02$ ) – this was the only phyla to show significant inter-sexual differences upon first capture (Table 2-2). In the second capture, Proteobacteria was significantly higher in males than females ( $W=36$ ,  $p=0.04$ ; Table 2-2). Firmicutes and Tenericutes were significantly lower in males than females (respectively:  $W=109$ ,  $p=0.03$ ;  $W=124$ ,  $p= <0.01$ ; Table 2-2). There were 489 unique

OTUs in the first capture, 265 unique OTUs in the second, and 1166 OTUs were present in both captures (Figure 2-2). Overall abundance was not correlated with capture ( $V=148$ ,  $p=0.97$ ).

The microbiota of several males in capture one and two were predominately composed of Proteobacteria (Figure 2-1). Despite broad similarities in higher taxa, there were large variations in lower taxa among these individuals between and within captures. In the first capture, the majority of the Proteobacteria abundance could be attributed to three OTUs, which were taxonomically assigned Neisseriaceae and Pasteurellaceae (two OTUs) (Figure 2-5, Table 2-3). A fourth OTU, assigned to *Campylobacter*, was responsible for the majority of one male's Proteobacteria abundance (M8, Table 2-3). In the second capture, Proteobacteria dominance could be attributed to Neisseriaceae, Pasteurellaceae, and *Flexispira* (Table 2-3). In the remaining individuals in the dataset, other taxa such as *Lactobacillus reuteri* and Lachnospiraceae were abundant across the first and second captures (Figure 2-5).

Overall, diversity was significantly different between groups ( $F_{3,44}=4.02$ ,  $p=0.02$ , Figure 2-6). Tukey's pairwise comparisons revealed that females had significantly higher gut microbial diversity than males in the second capture ( $p=0.01$ ), but intersexual variation was not significant in the first capture ( $p=0.82$ ) and there were no significant intra-sexual differences in diversity between captures (females:  $p=1.00$ ; males:  $p=0.15$ ). Fourteen individuals (nine males, five females) showed a decrease in diversity from the first capture to the second, while ten (three males, seven females) showed an increase in diversity across captures.

The first and second components of a PCoA using weighted UniFrac distances explained a large amount of variation in the data (Axis 1: 64.2%, Axis 2: 8.6%; Figure 2-4b), and revealed that there were large overlaps in confidence intervals between captures and across sexes. This was

confirmed statistically, as distances were not significantly different between sexes (adonis:  $F_{1,43}=9.177$ ,  $R^2=0.16$ ,  $p=0.60$ ) or across captures ( $p=0.65$ ).

#### *Comparisons between buccal and gut microbiota composition*

The abundant datasets for each microbiota contained 953 shared OTUs, and 967 and 578 unique OTUs for the fecal and buccal microbiota, respectively (Figure 2-2).

Visually, a PCoA revealed large differences in weighted UniFrac distances between the two microbiota (Figure 2-7). Statistical analysis confirmed that, when controlling for individuals, there were significant differences in the weighted UniFrac distances between the two microbiota (adonis:  $F_{1,95}=60.22$ ,  $R^2=0.39$ ,  $p=0.0001$ ). Capture and the interaction between microbiota and capture were not significant (adonis, respectively:  $p=0.15$ ,  $p=0.33$ ).

#### 2.4.2 Relationships between fitness measures and microbiota composition

##### *Body condition analysis*

The first component (eigenvalue 2.32) of a Principal Component Analysis using the three morphological measures was used as a proxy for body size in subsequent body condition analysis. This component explained 71.5% of the variance in the data and loaded skull length (-0.342), skull width (-0.280), and hind foot length (-0.897). For ease in interpretation, all body size values were negatively transformed. There were no intersexual differences in body size ( $W=100$ ,  $p=0.87$ ), and body size was significantly and positively correlated with body mass ( $R^2=0.15$ ,  $p=0.04$ ). Body condition values were not significantly different between sexes ( $W=70$ ,  $p=0.24$ ).

Body condition did not show a significant correlation with either gut or buccal microbiota abundance or diversity in either capture. Across captures, a change in body condition was also not significantly correlated with either change in abundance or diversity in either the buccal or gut microbiota datasets.

### *Immune function and buccal microbiota composition*

In the buccal microbiota, diversity was negatively and significantly correlated with WBC metrics across captures ( $t=2.16$ ,  $p=0.03$ ), driven by a negative correlation in the second capture ( $p=0.0009$ , Figure 2-8a). Abundance of the buccal microbiota was not significantly correlated with WBC metrics ( $t=0.65$ ,  $p=0.52$ ; Figure 2-8b). Change in WBC metrics between captures was significantly and negatively correlated with change in diversity ( $t=-3.18$ ,  $p=0.005$ ; Figure 2-8c) and significantly and positively correlated with change in abundance ( $t=2.50$ ,  $p=0.008$ ; Figure 2-8d).

IgG values were positively correlated with diversity ( $t=2.40$ ,  $p=0.021$ ; Figure 2-9a), driven by a significant positive correlation in the second capture ( $t=2.91$ ,  $p=0.008$ ). IgG values were not significantly correlated with microbiota abundance ( $t=0.68$ ,  $p=0.50$ ; Figure 2-9b). Similarly, change in these values were also not significantly correlated with either change in microbiota diversity ( $t=-0.16$ ,  $p=0.87$ , Figure 2-9c) or change in abundance ( $t=-1.19$ ,  $p=0.25$ ; Figure 2-9d).

### *Immune function and gut microbiota composition*

Diversity and abundance were not significantly correlated with immune variables in the gut microbiota, however a negative trend between diversity and WBC metrics existed ( $t=-1.687$ ,  $p=0.1$ ), as did a positive trend between diversity and IgG values ( $t=1.909$ ,  $p=0.063$ ). There were no significant correlation between any fitness variable and either change in diversity or abundance across captures in the gut microbiota.

## 2.5 Discussion

### 2.5.1 Microbiota composition

The red squirrel buccal microbiota showed a dominance of Proteobacteria (75.0%) and Firmicutes (20%), which is a different composition from other described mammalian buccal microbiota. Domestic dogs, for example, display dominance of Firmicutes and Bacteroidetes, while the rat buccal microbiota is dominated by Actinobacteria and Firmicutes (Dewhirst *et al.* 2012; Manrique *et al.* 2013). These differences in sampling may be in part due to micro-habitat sampling differences (Mager *et al.* 2003; Aas *et al.* 2005) and highlights the inability to make broad inferences in buccal microbiota composition across mammals. In contrast to the buccal microbiota, red squirrel gut microbiota composition is broadly consistent with the composition of other mammalian gut microbiota, such as mice and red pandas (Kong *et al.* 2014; Zhang *et al.* 2014). The similarity in the three main phyla, Firmicutes (49.0%), Proteobacteria (27.7%), and Bacteroidetes (20.5%), suggests highly constrained bacterial taxa composition in the gut microbiota among mammals, as has been previously shown in other taxa (Ley *et al.* 2008).

*Lactobacillus reuteri* and Lachnospiraceae were found in high abundance in the gut microbiota of some individuals in this study, while other individuals showed a dominance of Neisseriaceae and Pasteurellaceae. *L. reuteri* is a potential probiotic which has been shown to limit pathogenic infection of *E. coli* in germ-free mice (Eaton *et al.* 2011). Lachnospiraceae is a family of Clostridia that are abundant in mice and human gut microbiota (Meehan and Beiko 2014). Members of the family play key roles in the human gut microbiota and mice pre-colonized with Lachnospiraceae isolates partially restore colonization resistance against *C. difficile* infections (Reeves *et al.* 2012). The Neisseriaceae family includes commensal and opportunistic pathogenic species, with the majority of species commonly found on mammalian mucosal and dental surfaces (Knapp 1988; Bennett *et al.* 2014). Species belonging to the family Pasteurellaceae are frequently

isolated from mucosal surfaces and genital tracts of mammals and in most cases act as commensal flora, although some genera can be opportunistic pathogens (Christensen and Bisgaard 2006; Pritchett-Corning, Cosentino and Clifford 2009).

### 2.5.2 Variation in microbiota composition: body habitat, sex, and capture

#### *Body habitat*

The buccal microbiota of red squirrels was overall more abundant than the gut microbiota, however the gut microbiota was more diverse. In the buccal microbiota, Proteobacteria was the dominant phylum, (average relative abundance between both captures: 73.03%) but was the second most abundant in the gut microbiota (average: 26.65%). Firmicutes was the dominant phylum in the gut microbiota (average: 50.4%) but was the second most abundant in the buccal microbiota (average: 20.6%). Bacteroidetes was the third most abundant phylum in both microbiota, but had a higher average relative abundance across individuals in the gut microbiota than the buccal microbiota (average: 20.5% and 1.8% respectively).

While bacterial taxa showed significant variation in body habitat, >40% of all detected OTUs were constrained in both microbiota datasets at both times of capture. Additionally, a few individuals displayed large similarities between microbiota within the same capture periods and several OTUs in the prevalent microbiota were identical across microbiota in the same sampling period. This suggests that a) I have found evidence for a prevalent opportunity of transmission of bacteria via the orofecal route in these individuals and b) there is strong evidence for a temporal core microbiota in red squirrels. As the fecal-oral route has been implicated in pathogen transmission in mammals (*Helicobacter mustelae* in ferrets: Fox *et al.*, 1991; widespread paratuberculosis caused by *Mycobacterium avium* subsp. *paratuberculosis* in wildlife: Beard *et*

*al.*, 2001), this finding has important implications for future studies studying pathogen infection in red squirrels.

#### *Sex*

In the buccal microbiota, no bacterial phyla variation could be explained by differences in sex. However, in the gut microbiota there was evidence of variation in bacterial taxa between sexes. In the first capture, Actinobacteria was the only phyla present in the gut microbiota to show significant differences between the sexes, in which females had a higher relative abundance than males. Speculatively, this change could be driven by the large (though non-significant) differences in Proteobacteria in five males. In the second capture, abundance of Proteobacteria, composed largely of the families Neisseriaceae and Pasteurellaceae, was higher in male gut microbiota than females, driven by a large difference in six males. Both Neisseriaceae and Pasteurellaceae have been isolated from the genitalia of North American red squirrels, although abundance for both families was higher in females than males (see Chapter 1) suggesting that sex differences in these families may be highly dependent on body region.

When investigating broad differences in diversity and abundance in gut microbiota composition in these individuals, I found evidence to support significant intersexual variation in diversity in the second capture, but not the first, which could be due to the smaller amount of variation displayed between individuals in the first capture. Abundance was not correlated with capture, however, nor was a beta diversity analysis, accounting for both abundance and diversity.

#### *Capture*

In the buccal microbiota, alpha diversity analysis showed no significant differences between capture, while beta diversity analysis displayed significant inter-capture variation. This disparity can be explained by the inclusion of both diversity and abundance in the beta diversity

metric (Lozupone *et al.* 2007), suggesting that there is a marked difference in abundance between captures. In the gut microbiota, there was no evidence for significant variation in diversity between captures, regardless of which diversity analysis was used to measure variation.

Human studies sampling temporal variation in body habitats have shown that within-subject variation was greater in gut communities than oral communities on short (daily) and longer (two month) time scales (Costello *et al.* 2009; Caporaso *et al.* 2011). These conclusions are inconsistent with our findings and suggest that not all mammals display similar patterns in temporal variation in microbial composition. As such, future research should be cautious when interpreting single time-point samples to quantify ‘core’ microbiota composition in wild mammals.

### 2.5.3 Variation in microbiota composition: immune status and body condition

#### *Immune status*

Colonization resistance predicts a negative correlation between diversity and immune function, wherein hosts are more susceptible to invading pathogens with decreasing microbiota diversity. This has been demonstrated in numerous taxa, such as lab mice (He *et al.* 2014) and desert locusts (Dillon *et al.* 2005). Correlations between immune status and buccal microbiota composition were observed in the current study, suggesting that colonization resistance may explain how fitness and buccal microbiota composition are linked in wild mammals. Specifically, there is a significant negative correlation between diversity in the second capture and WBC metrics (representing low loadings of lymphocytes and high loadings of neutrophils). This finding suggests that, when diversity is widely variable within a population, there is a negative correlation between bacterial diversity and the activation of the immune system. This negative correlation was also significant when investigating the intra-individual change over capture periods. Change in abundance across the two captures was positively correlated with microbiota abundance (overall

sequences, corrected for initial sequence count). These results, taken together, suggest that individuals with low diversity and high abundance may have lower fitness correlates, although I am unable to determine the directionality of this correlation.

In other mammalian studies, IgG levels are commonly found to rise during bacterial infection. However, IgG can also be interpreted as a measure of ‘immunocompetence’, or a preparedness to fight off bacterial infection in the absence of a present infection (through high IgG values; Norris & Evans, 2000). Indeed, immune preparedness, in which individuals with high energetic reserves devote energetic resources to costly circulating immune defences independent of infection, is a concept that has been shown in numerous taxa (Read and Allen 2000; Bordes and Morand 2009). If this is true, then individuals with high IgG values may also display high microbial diversity. This is indicative of our findings, as IgG was positively correlated with diversity in the buccal microbiota, suggesting that individuals who are less prone to invasion of pathogens through an increased diversity, also may have higher immunocompetence.

There were no significant correlations with immune status in the fecal microbiota, although trends mirrored the significant relationship between the buccal microbiota and immune function (negative trend between diversity and WBC metrics, positive trend between diversity and IgG). This is consistent with the findings of Rautava *et al.* (2015), who reported that changes in the saliva microbiota were greater than in the gut microbiota following colonic inflammation in lab mice. The buccal microbiota may be more sensitive to change than the gut microbiota for numerous factors. For example, the saliva mucosal surface is thinner than the gut, and saliva is continuously renewed in mammals (Rautava *et al.* 2015).

### *Body Condition*

Finally, I did not find any significant correlations between microbial composition and body condition in these individuals. This suggests that in red squirrels, while fitness measures such as IgG and hematological parameters may be able to explain subtle variation in composition, coarse measures, such as the measurement of body condition through size-corrected mass, cannot.

#### 2.5.4 Variation in microbiota composition: stress

The hypothalamic-pituitary-adrenal (HPA) axis is an important factor in the mammalian stress response and directly interacts with immune function, wherein an increase in fecal glucocorticoid metabolites can be correlated with the activation of the innate immune system and simultaneously diminishing adaptive immunity (Bailey *et al.* 2009).

Similarly, activation of the HPA axis has been linked to bacterial microbial composition (the ‘brain-gut’ axis) in captive animals (Bailey *et al.*, 2010; reviews: Cryan & O’Mahony, 2011; Grenham *et al.*, 2011). In wild mammals, a study of fecal glucocorticoids in red squirrels has shown a proximate link between the oral microbiota and the HPA axis (Stothart *et al.* 2016). Evidence for a positive correlation between fecal glucocorticoid metabolites (FGM) concentrations and phylum abundance exists, and a negative correlation between FGM concentrations and phylum diversity. When coupled with our findings, these results provide evidence for a complex relationship between stress, immune function, and microbiota composition in wild red squirrels. As such, I suggest that future studies be aware of the close-knit relationship between these variables and control for the confounding effect of stress when studying the relationship between microbiota composition and immunity in wild populations.

### 2.5.5 Conclusions and future directions

Microbial ecology is an important and rapidly expanding field, although few studies have investigated the dynamics of multiple microbiota within individuals across multiple capture periods. There has also been little investigation into the interaction between fitness and microbiota composition in wild mammals. I have found evidence for rapid body-habitat dependent changes in microbiota composition, suggesting that, due to these dynamic shifts in composition, it is difficult to obtain accurate ‘core’ microbiota composition analysis from a single capture. Additionally, a proximate link between fitness correlates and microbiota composition exists in the buccal microbiota, providing an initial view into the interaction between fitness and microbiota composition in a wild mammal.

Future studies should place emphasis on experimental designs, ideally aimed to a) tease apart causality from correlation in the interaction between fitness and microbiota and b) determine a direct link between fitness and the microbiota. Together these studies will help further our understanding of the importance of microbiota composition in wild mammals.

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## 2.7 Tables and Figures

*Table 2-1:* No sex differences existed in the relative abundance of the buccal microbiota most abundant phyla. Phyla are ranked by most abundant to least abundant, based on mean relative abundance in the dataset.

<b>Capture</b>	<b>Phyla</b>	<b>Test statistic (W)</b>	<b>p-value</b>
<b>One</b>	Proteobacteria	50	0.2189
	Firmicutes	96	0.1782
	Bacteroidetes	80	0.6707
	Actinobacteria	72	1
	Tenericutes	68	0.8428
	TM7	59	0.4776
<b>Two</b>	Proteobacteria	69	0.8874
	Firmicutes	83	0.5512
	Bacteroidetes	77	0.7987
	Actinobacteria	77	0.7987
	Tenericutes	49	0.1778
	Cyanobacteria	81	0.6297

*Table 2-2:* Sex differences existed in the relative abundance of the gut microbiota most abundant phyla. Phyla are ranked by most abundant to least abundant, based on mean relative abundance in the dataset.

Capture	Phyla	Test statistic (W)	p-value	Nature of relationship
<b>One</b>	Proteobacteria	55	0.347	
	Firmicutes	95	0.197	
	Bacteroidetes	96	0.1782	
	Cyanobacteria	90	0.3185	
	Tenericutes	72	1	
	Actinobacteria	31	<b>0.017</b>	<b>Females &gt; Males</b>
<b>Two</b>	Proteobacteria	36	<b>0.03872</b>	<b>Males &gt; Females</b>
	Firmicutes	109	<b>0.03324</b>	<b>Females &gt; Males</b>
	Bacteroidetes	103	0.07802	
	Elusimicrobia	80	0.648	
	Tenericutes	124	<b>0.00183</b>	<b>Females &gt; Males</b>
	Cyanobacteria	93	0.2415	

*Table 2-3:* Taxonomic assignments of abundant Proteobacteria in six male *T. hudsonicus* gut microbiota that appeared in two capture periods.

<b>Capture</b>	<b>ID</b>	<b>Abundant Proteobacteria (Lowest Assigned Taxa)</b>	<b>Assigned OTU</b>	<b>Amount of Overall Proteobacteria Abundance Explained (Rel. Abundance of Taxa/Rel. Abundance Proteobacteria, %)</b>
1	M4	Neisseriaceae	2	50.3
		Pasteurellaceae	1	25.3
			23	10.5
	M5	Pasteurellaceae	1	42.6
		Neisseriaceae	2	29.9
	M6	Pasteurellaceae	1	67.4
	M7	Pasteurellaceae	1	48.9
		Neisseriaceae	2	43.6
	M8	Campylobacter	5	100
	M9	Pasteurellaceae	1	14.2
2			23	21.5
		Neisseriaceae	2	61.2
	M4	Pasteurellaceae	1	50.1
			23	19.4
		Neisseriaceae	2	25.2
	M5	Pasteurellaceae	23	63.8
			1	11.6
	M6	Pasteurellaceae	1	44.0
		Neisseriaceae	8	27.4
	M7	Pasteurellaceae	1	66.4
		Neisseriaceae	2	24.8
	M8	Pasteurellaceae	1	85.8
			23	11.9
	M9	Flexispira	6	100

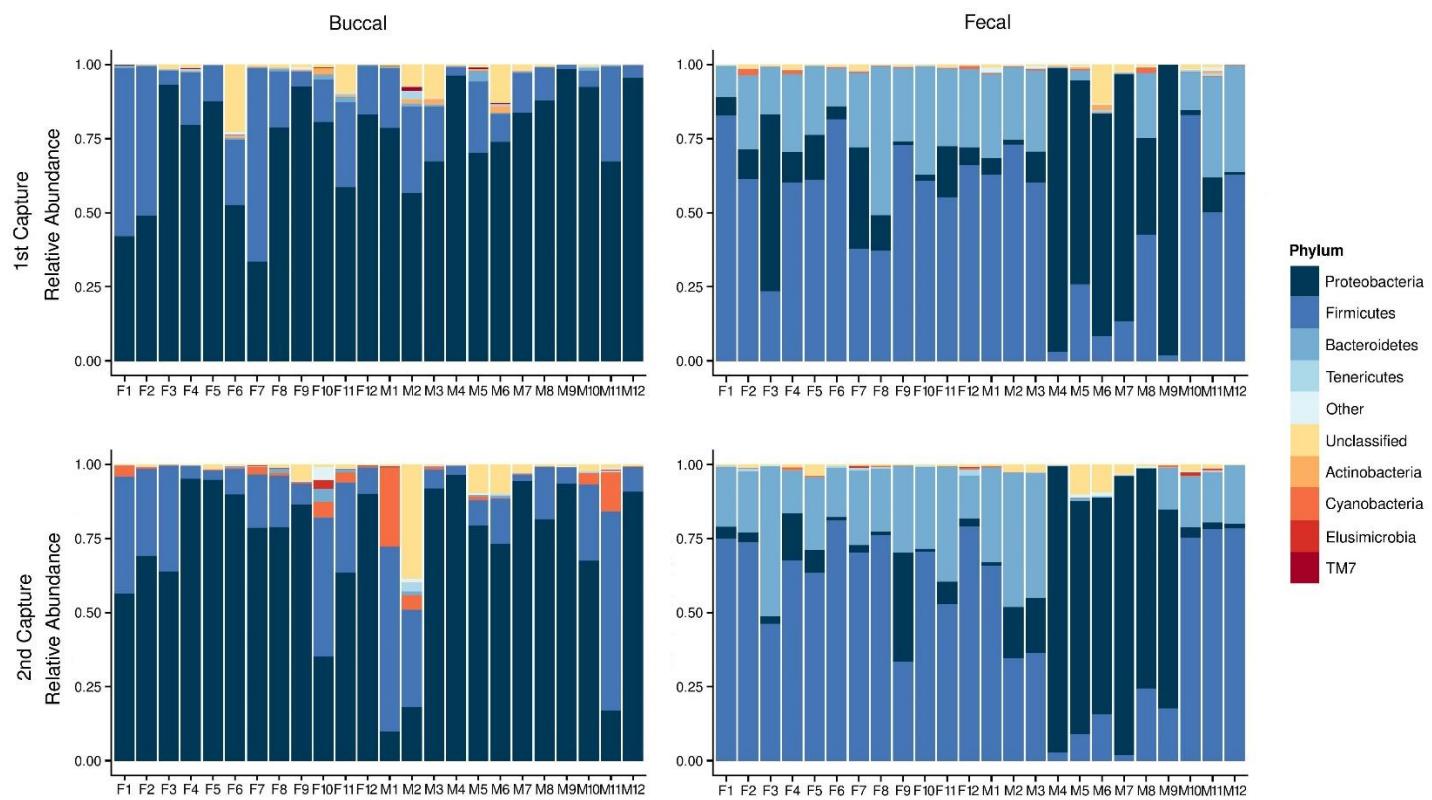


Figure 2-1: Relative abundance of the top most abundant phyla for each microbiota, across both capture periods.

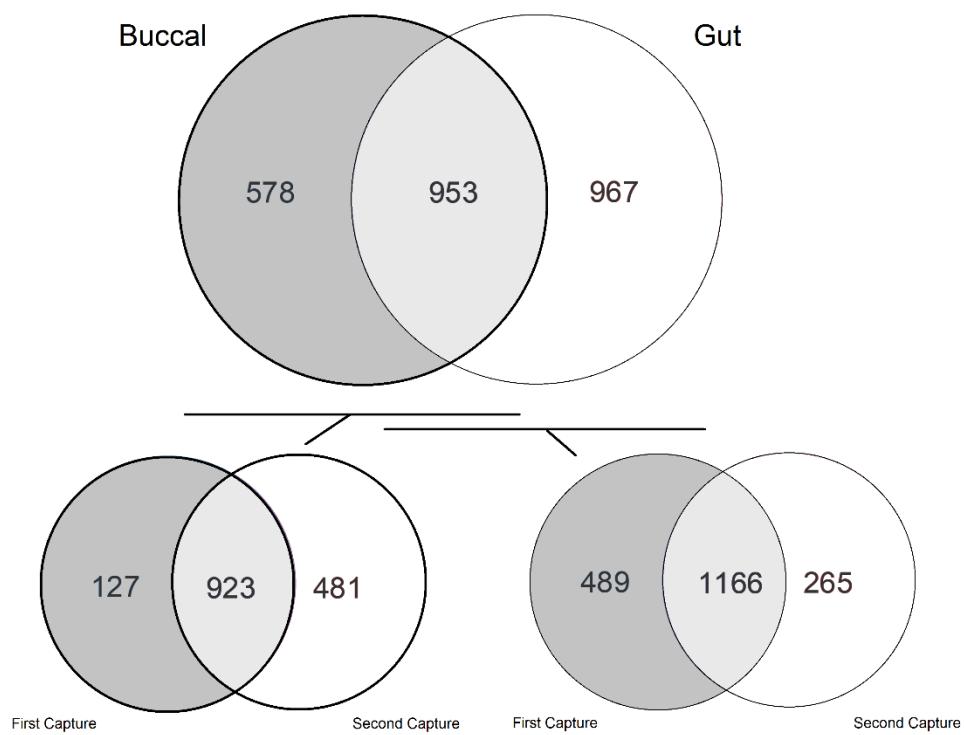
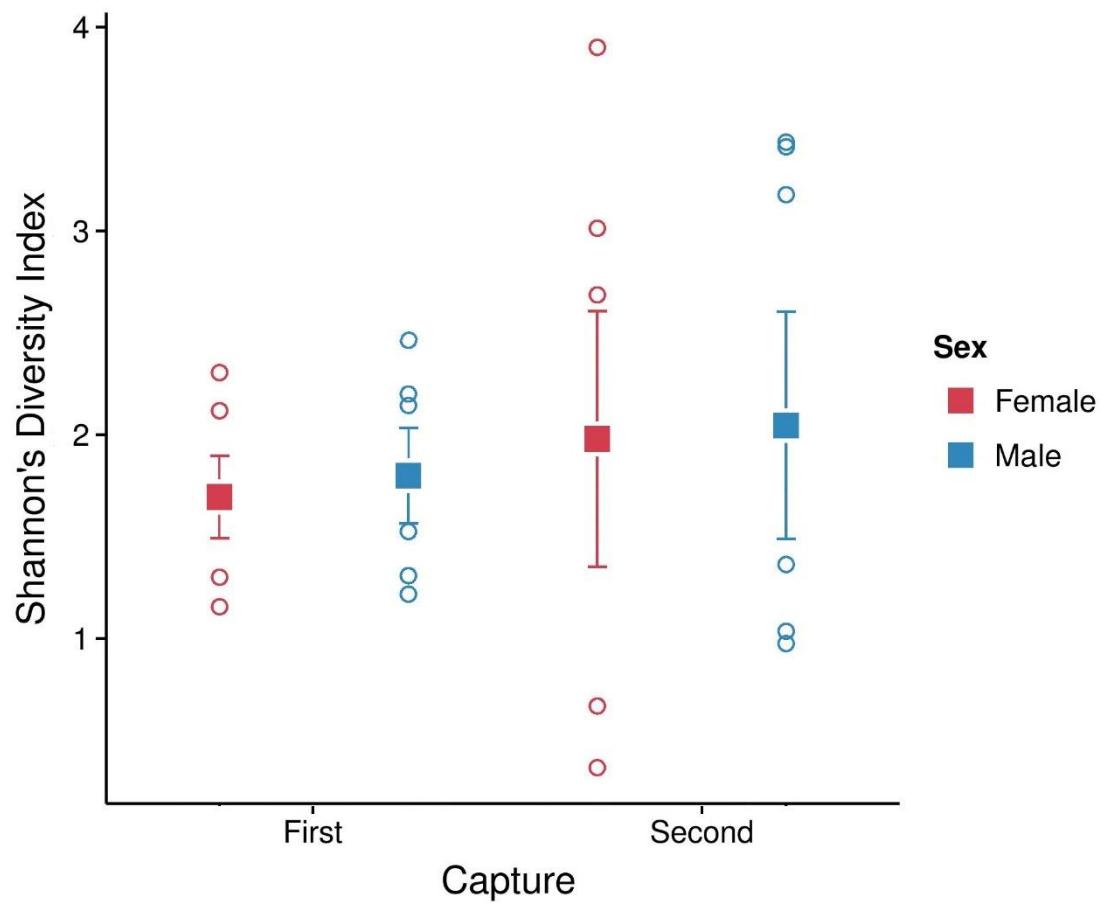
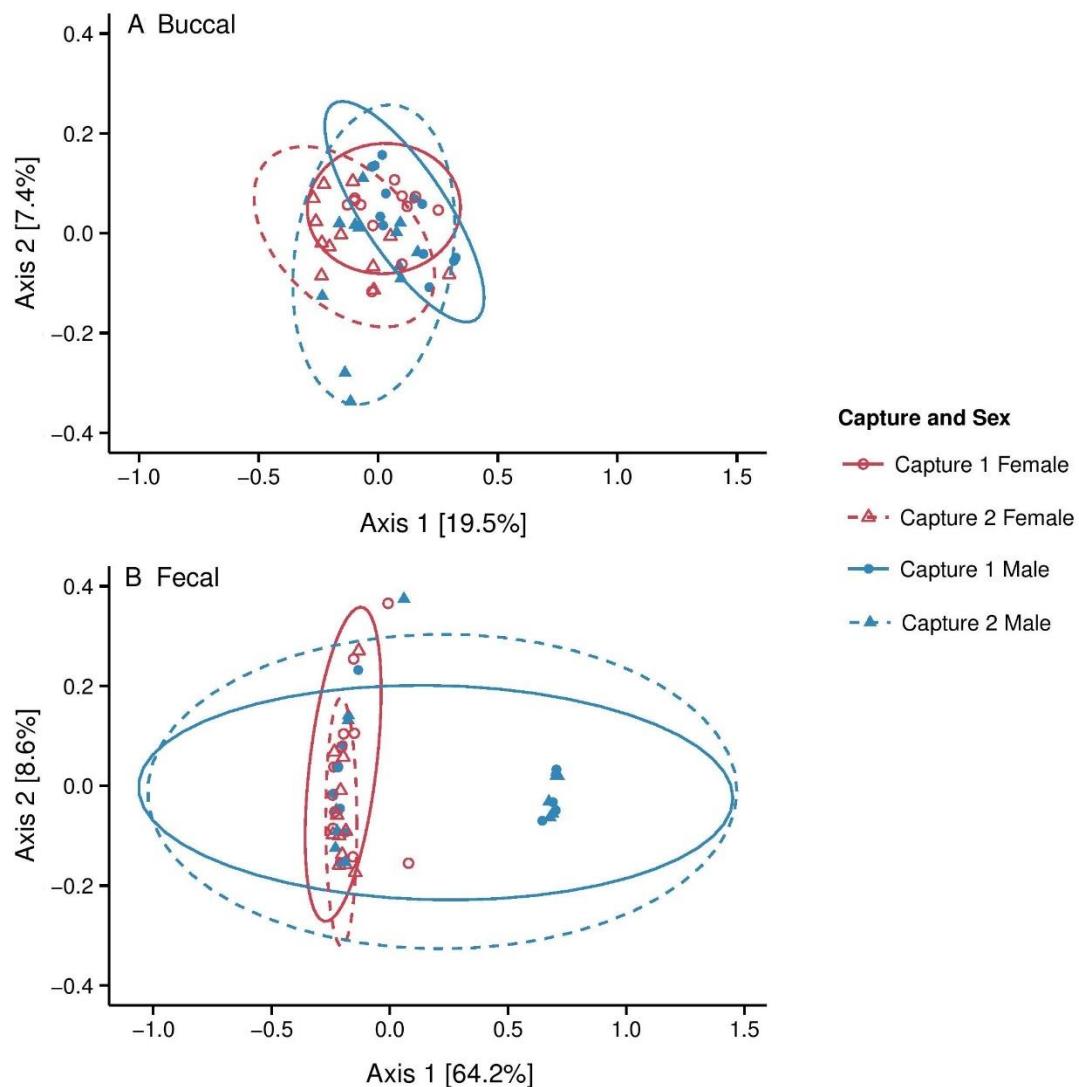


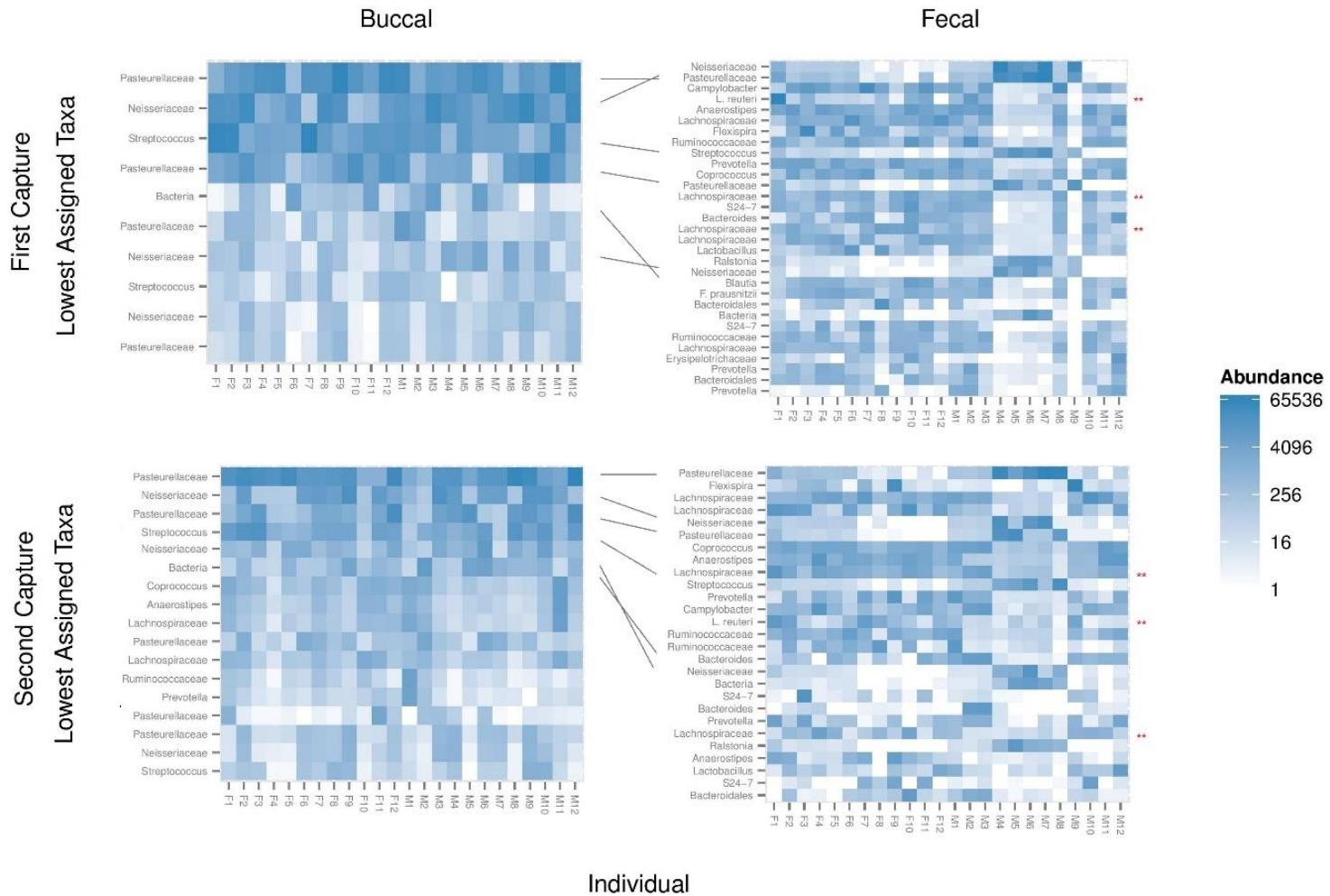
Figure 2-2: Shared and unique OTUs for abundant buccal and gut microbiota. Shared and unique OTUs across captures within each microbiota are also displayed.



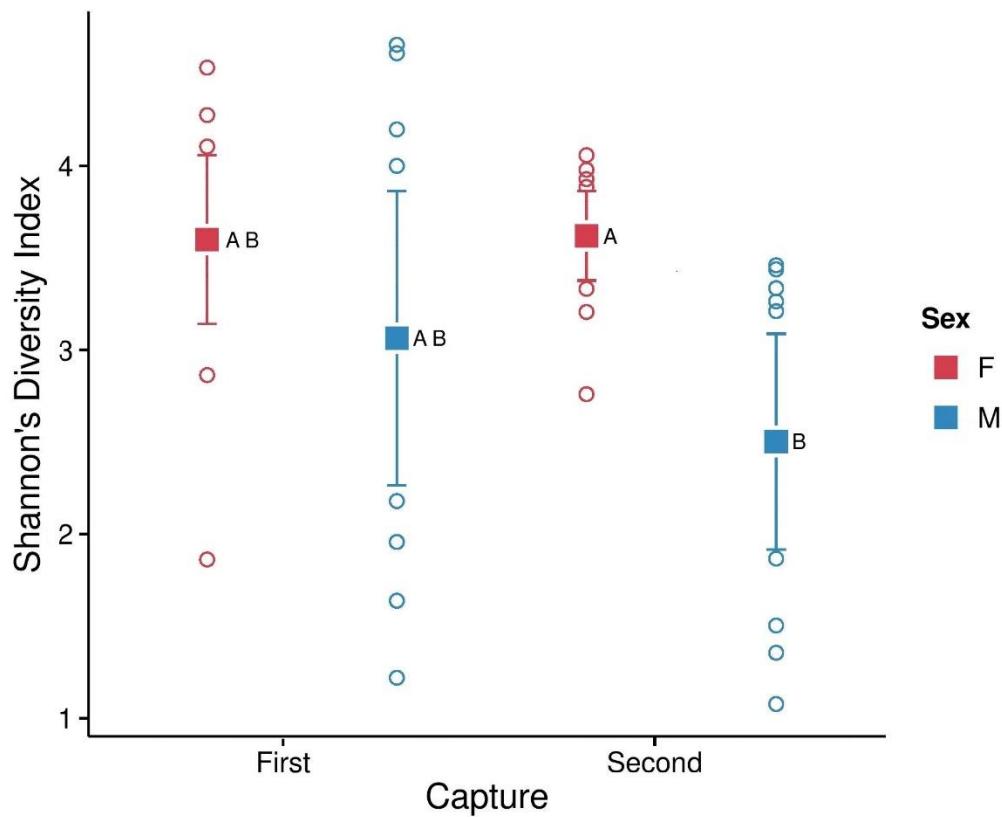
*Figure 2-3:* Changes in Shannon's diversity in the buccal microbiota across two capture periods of 16 red squirrels. There were no significant differences in diversity across capture periods, nor did diversity significantly change between sexes. Means and 95% confidence intervals are reported.



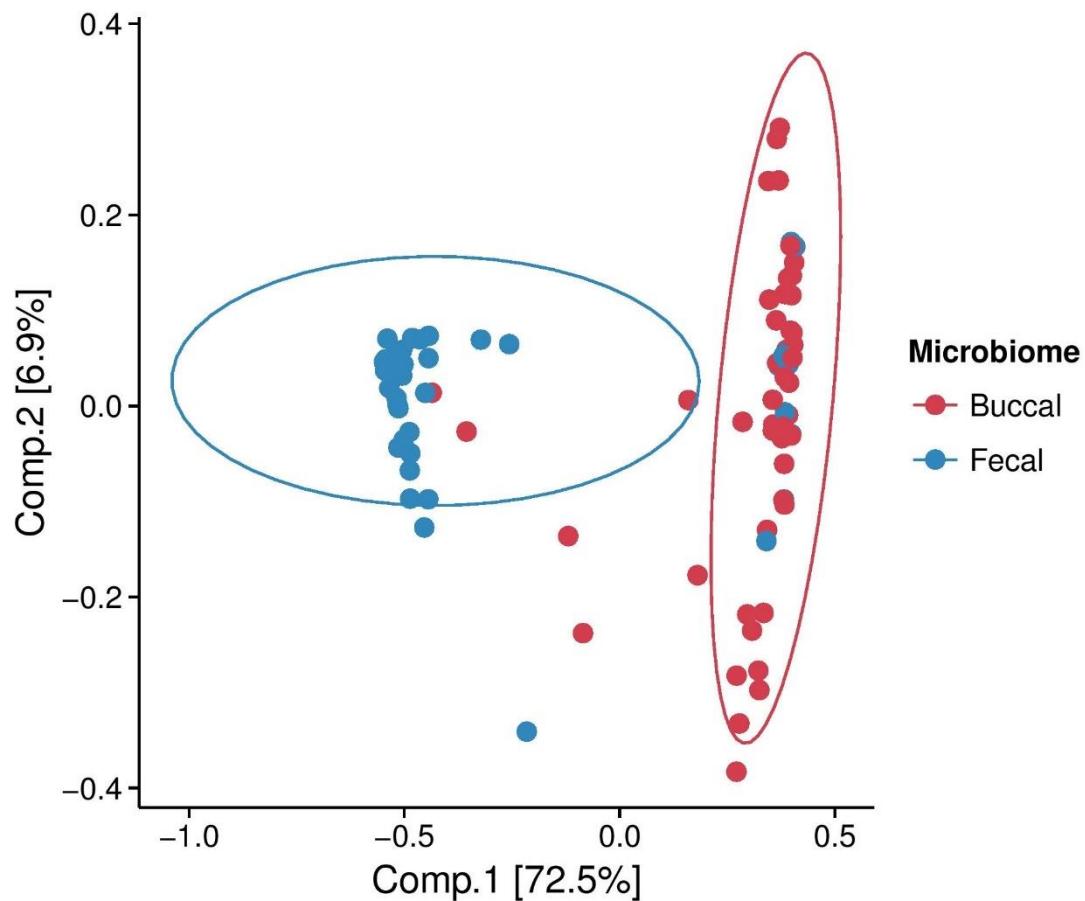
*Figure 2-4:* PCoA analysis for buccal and gut microbiota for 24 individuals across two capture periods. A) In the buccal dataset, there were no significant differences in sex, but captures were significantly different (adonis:  $p=0.008$ ). B) There were no significant differences in sex or capture in the gut dataset. Colours are designated based on sex (red, open symbols = female; blue, closed symbols = male); Line type and symbol are designated by capture (circle, solid line = capture 1; triangle, dotted line = capture 2). Ellipses represent 95% confidence intervals around distance centroids.



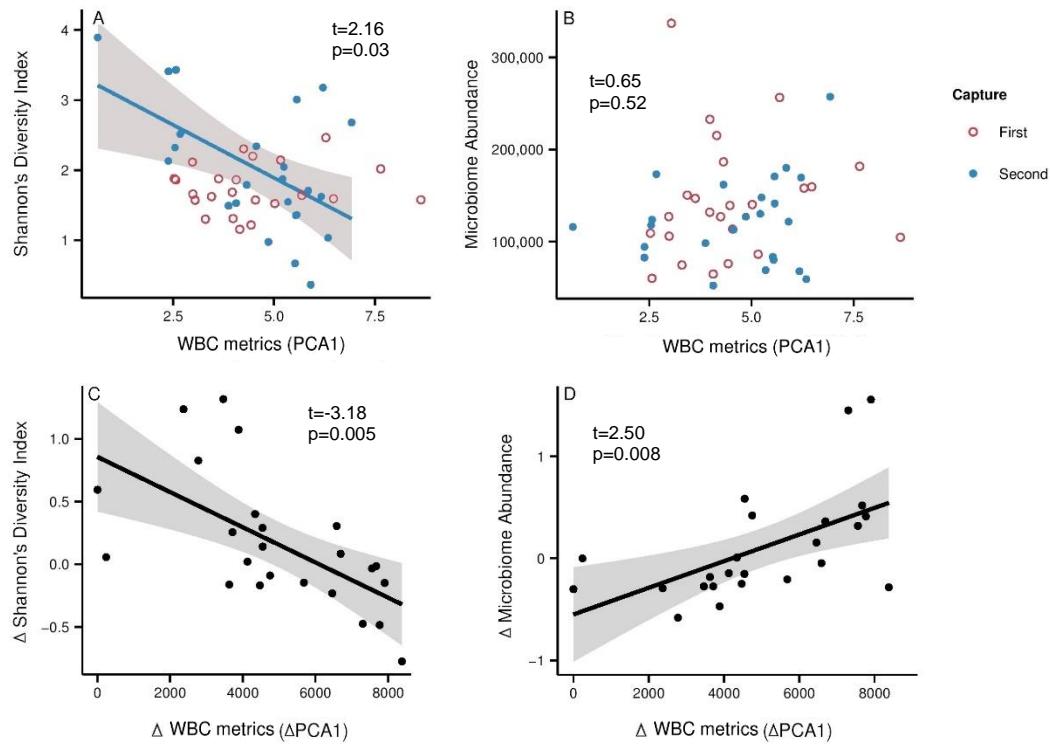
*Figure 2-5:* Heatmaps of the abundant buccal and gut microbiota, across two capture periods. Prevalent microbiota are composed of the top 10% most abundant sequences in each microbiota within each capture period. Individuals are labelled based on their sex (F= Female; M= Male), and taxa are ordered from highest to lowest relative abundance. Stars indicate taxa discussed in detail in text and connecting lines match identical OTUs in both microbiota within captures.



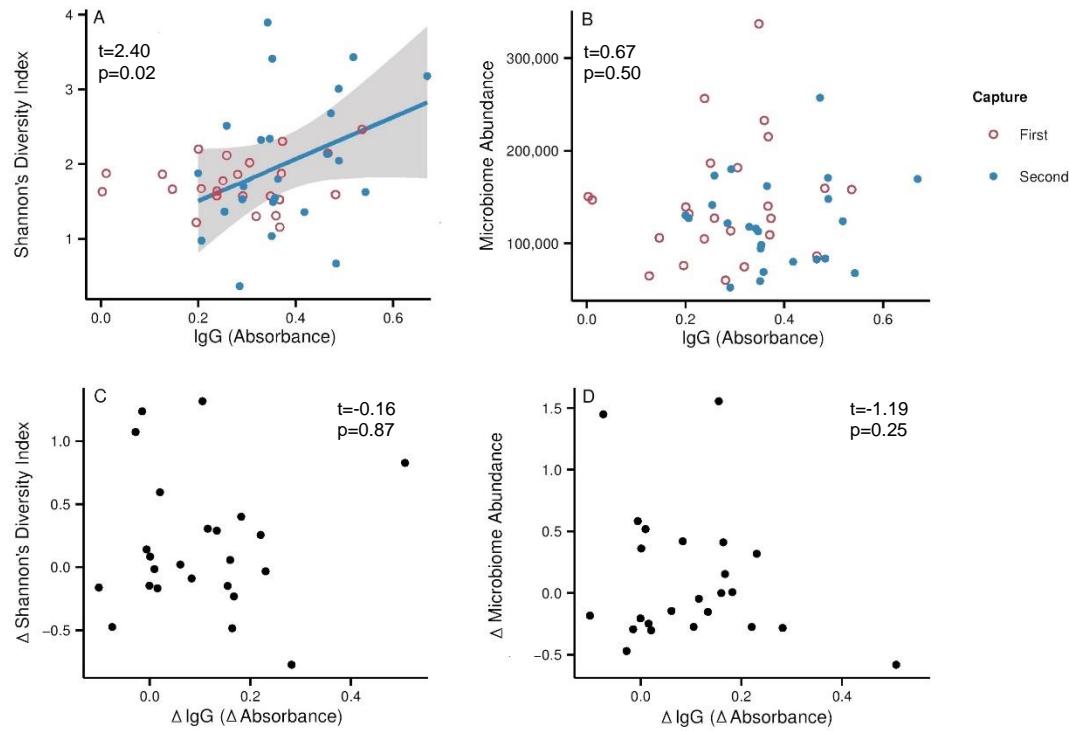
*Figure 2-6:* Changes in diversity in gut microbiota across two capture periods. Shannon diversity was significantly different between groups ( $F_{3,44}=4.02$ ,  $p=0.02$ ). Tukey's pairwise comparisons revealed that females had significantly higher diversity than males in the second capture ( $p=0.01$ ), and there were no other significant changes between groups. Means and 95% confidence intervals are reported.



*Figure 2-7:* PCoA analysis for buccal and gut microbiota for 24 individuals across two capture periods. There were significant differences between buccal and gut microbiota (adonis:  $F_{1,95}=60.22$ ,  $R^2=0.39$ ,  $p=0.0001$ ), but no significant differences within microbiota across captures. Ellipses represent 95% confidence around the distance centroids.



*Figure 2-8: A)* Shannon's diversity was negatively and significantly correlated with WBC metrics across captures, driven by significance in the second capture ( $p=0.0009$ ). *B)* Abundance of the buccal microbiota was not significantly correlated to WBC metrics. *C)* Change in WBC metrics was significantly and negatively correlated with change in diversity. *D)* Change in WBC metrics was significantly and positively correlated with change in abundance.



*Figure 2-9:* A) Shannon's diversity was negatively and significantly correlated with IgG values across captures, driven by significance in the second capture. B) Abundance of the buccal microbiota was not significantly correlated with IgG values. Change in IgG values across captures were not significantly correlated to C) change in diversity or D) change in microbiota abundance.

## Appendix: Antibiotic Experiment

In a secondary experiment, I attempted to disrupt bacterial communities in both the buccal and gut microbiota of North American red squirrels. Individuals were randomly categorized into a control ( $n=12$ ) or treatment group ( $n=12$ ) upon first capture. After body measurements and samples (including buccal swabs, fecal samples, and blood) were collected, individuals in the treatment group were given a subcutaneous injection of the broad spectrum antibiotic cefovecin (Convenia<sup>TM</sup>, Zoetis, Florham Park, NJ) at a dosage of  $2.3e^{-4}$ mL/g, as per the manufacturer's instruction. Control individuals were given an equivalent dose of saline subcutaneously.

Individuals were recaptured within 10-14 days of initial injection to coincide with the highest circulating level of antibiotic within the individual. Mass, buccal swabs, fecal samples and blood were collected at this time. Finally, a third capture period occurred 1.5 months after initial injection, when the antibiotic was expected to be completely clear circulatory system of each individual. This was to allow for a comparison of fitness correlates and bacteria community assemblage before, during, and after antibiotic treatment.

However, there was no evidence of an effect of antibiotic treatment on individuals' bacterial composition during the 2<sup>nd</sup> and 3<sup>rd</sup> capture periods. Antibiotic treatment was included as a fixed effect in all models in Chapter 2, but did not explain variation in bacterial diversity in both buccal ( $p=0.4$ ) and fecal ( $p=0.8$ ) microbiota, nor could it explain variation in bacterial abundance in both buccal ( $p=0.5$ ) and fecal ( $p=0.4$ ) microbiota. Thus, to increase sample size for fitness and temporal variation analyses, all individuals from treatment and control groups were pooled.

Cefovin was originally chosen as the antibiotic in this study due to its single dose application. As recapturing within short time frames can potentially be challenging in wild studies, it was determined that an antibiotic with a one-time injection would be ideal for this study design.

However, cefovin is a broad-spectrum antibiotic typically used to treat skin infections in dogs and cats. When administered subcutaneously in red squirrels, therefore, it is likely that the antibiotic was not effective in altering the buccal and gut microbiota as it may not have reached the microbiota in question.

Antibiotic experiments have the potential to answer many questions about the causality of microbiota composition in wild mammalian fitness. Future studies interested in this question, therefore, should carefully focus on ensuring that the antibiotic is delivered directly to the microbiota, perhaps through a dietary supplement or through water dosed with the antibiotic (J. Schertzer, personal communication).

*Appendix 2-1:* Control and Treatment individuals in the exploratory antibiotic study. Seven females and five males were given a saline subcutaneous injection (control) and five females and seven males were given a subcutaneous injection of cefovin (treatment).

Control or Treatment Group	EarTag ID	Sex
Control	2266 2267	Female
	2270 2271	Male
	2276 2280	Male
	2284 2286	Female
	2293 2294	Male
	2317 2318	Male
	2322 2323	Female
	2332 2338	Female
	2341 2342	Female
	2362 2363	Male
Treatment	2364 2365	Female
	2368 2369	Female
	2264 2265	Female
	2272 2273	Male
	2278 2287	Female
	2281 2324	Female
	2283 2301	Female
	2285 2321	Male
	2289 2290	Male
	2297 2298	Male

## General Discussion

Microbial communities are present in almost every environment on Earth and have a measurable impact on the environments in which they are found, including within other living organisms. Consequently, recent research has begun to recognize the large role the microbiota plays in shaping host health. While our understanding of the relationship between microbiota and health is increasing, studies have focussed primarily on humans and laboratory animals. This thesis aimed to a) provide an in-depth description of microbiota from multiple body regions in a wild mammal and b) link variation in microbiota composition to intra- and inter-individual factors, such as sex, temporal variation, and multiple measures of fitness correlates.

### Composition of genital, oral, and gut microbiota

While the top three most abundant microbial phyla were similar in all three body regions, the relative abundance of each phylum differed among each microbiota, suggesting that body region is influential on phylum composition in red squirrels. In the genital, oral and gut microbiota, the three most prevalent phyla were Firmicutes, Proteobacteria and Bacteroidetes, however relative abundance differed between body regions. As an important caveat, the genital microbiota was sampled from different individuals and within different years. However, these broad differences in phylum-level relative abundance among body regions are consistent with previous mammalian studies. For example, humans exhibit large variation in composition between body regions, such as gut, vaginal, oral and skin (Ding and Schloss 2014). This has been shown in other taxa as well, including non-human primates (Stumpf *et al.* 2013), laboratory mice (Rautava *et al.* 2015) and koalas (Alfano *et al.*, 2015), suggesting that many mammalian species display variation in body-region microbial composition.

### Sexual and temporal variation linked to microbiota composition

There are numerous inter- and intra- individual factors that influence microbiota composition. In this thesis, there was evidence of broad similarities between conspecifics of both sexes in the external genital microbiota composition and the oral microbiota. The gut microbiota displayed significant sex differences in bacterial taxa, this variation was dependent on the capture period, as intersexual variation in diversity was significant in the second capture but not the first. While previous studies have shown that microbiota composition is influenced by the opportunity for bacterial transfer, such as close proximity to conspecifics (Leclaire, Nielsen and Drea 2014a), red squirrels are generally a solitary species (Smith 1968), suggesting that composition should be less influenced by proximity than other species.

In the second chapter, the oral and gut microbiota were sampled twice, two weeks apart. The oral, but not gut, microbiota displayed significant differences in beta diversity between captures, consistent with oral and fecal microbiota comparisons in laboratory mice over a similar time period (Rautava *et al.* 2015). The authors suggested that, as salivary film on gut mucosal surfaces is thicker than the oral cavity (Collins and Dawes 1987; Atuma *et al.* 2001), the thinner film could provide an explanation for rapid changes observed in the oral, but not gut microbiota. While the gut microbiota may not display rapid changes, the gut microbiota of wild mice, *Apodemus sylvaticus*, showed seasonal variation (Maurice *et al.* 2015) suggesting that temporal variation is dependent on both temporal scale and body habitat. As such, future research should be cautious when interpreting results from a single sampling period in order to characterize typical microbiota composition in wild mammals.

### Fitness correlates and microbiota composition

Colonization resistance predicts a negative correlation between microbial diversity and immune function, in which hosts are less susceptible to invasion by pathogens when they are hosts to a more diverse microbiota (Dillon *et al.*, 2005; He *et al.*, 2014). It was thus predicted that red squirrels with highly functioning immune systems (showing little signs of infection) would have more diverse microbiota than conspecifics with lower functioning immune systems (or those who showed signs of infection). In the two sites sampled for this thesis, body condition was estimated through the regression of body mass and body size (Schulte-Hostedde *et al.* 2005) and used as a basic measure of host fitness. In the oral, genital, and gut microbiota, there was no significant correlation between overall microbiota composition and body condition, regardless of sampling year or site differences.

In the second chapter, individuals were also sampled for more robust measures of fitness correlates, including measures of both the innate and acquired immune systems. There were significant correlations between immune status and oral microbiota composition, following the predictions made by the colonization resistance hypothesis. In the second capture, when microbial diversity was highly variant in the population, there was a significant negative correlation between microbiota diversity and measures of the innate immune system, indicative of the activation of the immune system during a bacterial infection. There were also significant and positive correlations between microbiota and measures of the innate immune system. Further, immunoglobulin levels were used as a measure of the acquired immune system, and were positively correlated with diversity in the oral microbiota. This is contrary to my original prediction, but may be indicative of a measure of ‘immunocompetence’, in which hosts who are prepared to fight off bacterial infection (through high immunoglobulin levels) (Norris and Evans 2000). Together, these results

suggest that, while rudimentary fitness analyses were unable to detect a correlation between fitness and microbiota composition in this species, robust measures are able to do so at a higher resolution when measuring host fitness correlates.

### Future directions

As microbial ecology is a rapidly expanding field, there are many questions that remain unanswered regarding the link between the microbiota and mammalian host physiology. For example, while this thesis identifies a relationship between host fitness and the microbiota in a wild mammal, the causation of the patterns observed remain unknown. Experimental studies that allow for manipulation of the microbiota will be needed in order to tease apart this important relationship. Other variables, such as sampling measures of stress, should be incorporated into future study design to ensure confounding factors are also explored.

Finally, future research should also focus on evaluating the role of the microbiota in host survival and fitness, particularly in light of conservation in Species at Risk. To do this, long-term ecological studies, which can properly assess survival and fitness are needed, as well as a standardized method of sampling microbiota composition. This research direction is particularly important, as it will help enhance our knowledge of the role microbiota may play in wild mammalian fitness and survival.

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