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Research Article

RESEARCH ARTICLE

Temporal variation of the microbiome is dependent on body region in a wild mammal (*Tamiasciurus hudsonicus*)

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One sentence summary: The buccal and gut microbial communities, or microbiomes, of North American red squirrels were sampled to investigate the impact of temporal variation on the characterization of microbiomes in a wild mammal.

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ABSTRACT

Microbial communities are increasingly being recognized as important to host health in wild mammals, but how these communities are characterized can have important consequences on the results of these studies. Previous research has explored temporal variation in microbial communities in humans and lab mammals, but few have investigated how microbiomes fluctuate in wild populations and none have examined the temporal dynamics of these fluctuations in different body regions on a wild mammal. Using Illumina MiSeq sequencing of the V3-V4 16S rRNA gene regions, we characterized the buccal and gut microbiomes of wild North American red squirrels, *Tamiasciurus hudsonicus*, to measure changes in these two microbiomes over short (<2 weeks), medium (2–4 weeks) and long (>1 month) term sampling periods. While we observed short and medium temporal stability in the buccal microbiome, the gut microbiome varied between medium and long-term sampling periods. There was no evidence of intra-individual correlations between buccal and gut microbiome change, suggesting that temporal stability is dependent on the body region and factors affecting microbial stability may be specific to body sites. From these findings, we urge researchers to be cautious in interpreting results from single temporal sampling periods when quantifying characteristic microbiomes in wild mammals.

Keywords: temporal variation; wild mammal; Illumina; microbiome

INTRODUCTION

Mammals are hosts to trillions of microbes and the structure of these microbial communities, or microbiomes, is important to host life history. Compositions of buccal and gut microbiomes have been linked to host health, such as the correlation between decreased oral microbiome diversity in humans and pediatric inflammatory bowel disease (Docktor *et al.* 2012) or the role of the gut microbiome in host immune development and physiology (Sommer and Bäckhed 2013). While emphasis has been placed on human and laboratory studies of host microbiomes, studies of natural populations are important when linking laboratory findings to ecologically relevant systems (Maurice *et al.* 2015). These may have noteworthy consequences for the conservation of Species at Risk, as demonstrated by the exploration of the relationship between dietary shifts due to habitat degradation,

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shifts in the gut microbiome and subsequent negative host health effects in endangered primates (Amato *et al.* 2013) or the re-introduction of captive individuals to the wild, with marked differences in microbiome composition between captive mammals and their wild counterparts (Cheng *et al.* 2015). However, first defining a 'typical' microbiome is crucial in evaluating the role of the microbiome on host fitness (Turnbaugh *et al.* 2007).

To date, most research in wild mammals has focused on characterizing microbiome composition from single collection periods (point samples), which serve as snapshots of the microbiome, or have provided evidence of seasonal changes in single microbiomes. For example, wild wood mice (*Apodemus sylvaticus*) and wild black howler monkeys (*Alouatta pigra*) show temporal changes of gut bacterial taxa that are linked with changes in host diet (Amato *et al.* 2015; Maurice *et al.* 2015). Variation in microbiome composition has also been linked to age; as demonstrated with changes in *Lactobacillus* species with age in dogs' intestinal microbiomes (*Canis lupus familiaris*; Masuoka *et al.* 2017); sex, as with differences in the gut microbiome of Siberian hamsters (*Phodopus sungorus*; Sylvia *et al.* 2017); social networking, as established by the correlation between gut microbiome variation and rates of interaction in wild savannah baboons (*Papio cynocephalus*; Tung *et al.* 2015); and stress, as demonstrated by an inverse relationship between fecal cortisol metabolite levels and microbiome diversity in North American red squirrels (*Tamiasciurus hudsonicus*; Stothart *et al.* 2016).

To our knowledge, there has been no research investigating how short and long-term temporal variation may influence buccal microbial characterization in wild mammals, or how multiple microbiomes may fluctuate over time in different body regions within a host. These patterns of variation in the microbiome may have important implications when attempting to link microbiome composition to fitness in wild mammals.

In this present paper, we use free-living North American red squirrels to evaluate temporal variation in the buccal and gut microbiomes of a wild mammal. Red squirrels are small, semi-arboreal rodents that inhabit coniferous dominated forests in North America. They are a highly territorial, solitary species and, with the exception of a short mating season, there is little direct interaction between conspecifics (Smith 1968). Throughout the summer months, red squirrels show little dietary variation, mainly ingesting conifers (81%) and supplementing this food source with buds, flowers, fruits and sap from deciduous trees (13%), berries (3%) and mushrooms (2%) when available (Ferron, Ouellet and Lemay 1985). Red squirrels' well-studied ecology, territorial nature and high recapture rates make them an ideal study species when investigating microbiome temporal variation in wild mammals.

We examine the hypothesis that the buccal and gut microbiomes exhibit temporal variation by testing the prediction that relative abundance, alpha-diversity and beta-diversity fluctuate over short (>2 weeks), medium (2–4 weeks) and long (>1 month) time periods. We also investigate intra-individual microbial changes across both body regions and predict that, if factors influencing buccal and gut microbiomes are consistent, there will be a significant correlation between buccal and gut microbial changes within individuals.

MATERIALS AND METHODS

Study site and sample collection

In brief, 14 North American red squirrels (females: n = 8; males: n = 6; non-breeding) were trapped using tomahawk live traps (Tomahawk Live Trap Co., WI, USA) in a mixed-wood forest located in Algonquin Provincial Park, Ontario, Canada. All individuals were given tags with unique alphanumeric codes and captured three times (first capture: July 17–19, 2014; second capture: July 28–30, 2014; third capture: August 24–26, 2014). Further details on trapping protocols are reported in Stothart *et al.* (2016). This study was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Laurentian University, protocol number 2014–03-01.

Buccal microbiome samples were collected using sterile cotton-tipped swabs (Fisher Scientific, ON, Canada). The oral cavity was swabbed for a minimum of 10 s, with light pressure applied to the cheeks and tongue to maximize microbial transfer. All individuals were swabbed at least twice (<1 min apart) during each capture to ensure adequate microbial collection for extraction and sequencing. Both swabs from each individual were placed into a sterile 1.5 mL microcentrifuge tube and stored on ice in the field before being transferred to a –20°C freezer until further processing. Fecal samples were collected directly from the traps using sterilized tweezers within 2 h of capturing each individual. Fecal collection in this manner has been shown to be largely uncontaminated by environmental factors and is a good representation of the gut microbiome in small mammals (Kohl, Luong and Dearing 2015). Samples were placed into sterile 1.5 mL microcentrifuge tubes and stored on ice in the field before being transferred to a –20°C freezer until further processing.

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 the manufacture's protocol for purification of DNA from frozen animal feces. DNA purity was quantified via spectrophotometry (Nanodrop, Thermo Fisher Scientific, Waltham, MA, USA) and samples were stored at –20°C until sequencing.

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, on an Illumina MiSeq sequencing system (see Supplementary Methods section). Raw sequences have been deposited in NCBI under the accession SRA #SRP064395.

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 ('RDP Classifier') was re-trained using the GreenGenes database (version 13.5, DeSantis *et al.* 2006) to assign taxonomic classifications to each OTU. Sequences were aligned using the GreenGenes reference sequences and filtered. A midpoint rooted tree was produced using FastTree (Price, Dehal and Arkin 2010) in QIIME.

Composition statistical analyses

Statistical analyses on microbiome composition were 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), indispecies (DeCaceres and Legendre 2009) and RColorBrewer (Neuwirth 2014).

Average relative abundance values were calculated based on the average of all individuals' microbiomes for each capture. For each microbiome, the relative abundance of the three most abundant phyla and genera were calculated using the tax_glom function in the phyloseq package. To identify differences in abundance across phyla and genera between captures, abundance values were checked for normality and one-way ANOVA tests were run. If significant differences were found, Tukey's *post hoc* comparisons of multiple means (Tukey 1949) were conducted to identify where the significant changes between captures lay.

To negate large differences in library sizes, and to be able to reliably identify interindividual changes in abundance in the gut and buccal microbiomes, count data of all OTUs were normalized by applying variance stabilizing transformations (VST) with the DESeq2 package (see Supplementary Materials; Love, Huber and Anders 2014; McMurdie and Holmes 2014). To evaluate α-diversity, Shannon's diversity index measures (Shannon 1948) were calculated based on the raw datasets following the methods in the study by Stothart *et al*. (2016). Diversity metrics were log-transformed to conform to normality, if necessary.

Changes in the microbiomes were defined as short term (between the first and second capture; <2 weeks), medium term (between the second and third capture; 2–4 weeks) and long term (between the first and third capture; >1 month).

The relationships between α-diversity in each microbiome and capture period were examined using general linear mixed-effect models (GLMM), including sex and individual as random effects. Similarly, these models were run using normalized VST abundance values to determine the effect of capture period on bacterial abundance in both the buccal and gut microbiomes. Pairwise Tukey's tests for all significant models were made with the general linear hypothesis test (glht) function from the multicomp package (Hothorn, Bretz and Westfall 2008), correcting p-values for multiple comparisons.

Normalized count data were used to calculate weighted UniFrac distances (Lozupone and Knight 2005), a measure of β-diversity. To ensure that the UniFrac distances were Euclidian and appropriate for downstream analysis, distances were checked with the is.euclid function from the ade4 package (version: 1.7–4, Dray and Dufour 2007) prior to conducting a principal coordinates analysis (PCoA). As both datasets were Euclidian, no changes were made to the datasets. Within R, using the package vegan, Adonis tests (equivalent to permutational MANOVA) were used to calculate statistical differences in weighted UniFrac distance matrices between captures, including sex as a fixed effect and individual a random effect. Each test was run with 9999 permutations and the seed for each test was set at 123. Non-parametric indicator species analyses were run to identify the important OTUs driving differences between captures in each microbiome. OTUs with an indicator value >0.3 and a Bonferroni-adjusted p-value <0.05 were selected for analysis. For each capture, the three OTUs with the highest IV values in each microbiome were identified (as in Yildirim *et al*. 2014). Finally, intra-individual changes in abundance and diversity in the buccal and gut microbiomes were evaluated to investigate whether changes in the buccal microbiome could be directly related to changes in the gut microbiome within individual hosts. To statistically test this, and to account for potential non-linearity in the data, linear regressions (Zar 2007) were conducted using individuals' change in buccal and gut microbiome composition.

RESULTS

Temporal variation in buccal microbiome

The buccal microbiome contained 1174 OTUs and 5493 499 (mean: 130 797.6, min: 46 577, max: 337 210) sequences. A total of 444 OTUs were present in all three captures. The third capture had the most unique OTUs, followed by captures 2 and 1 (respectively: 252, 152 and 90 unique OTUs; Fig. 1A). The top three most abundant phyla (Proteobacteria, Firmicutes and Bacteroidetes) remained constant across captures (ANOVA: $P = 0.58$, $P = 0.25$, $P = 0.19$, respectively; Fig. S1A, Supporting Information).The three most abundant genera included *Streptococcus*, *Ralstonia and Coprococcus* (Fig. 2A). The relative abundance of *Streptococcus* was significantly higher in the first than second and third captures (ANOVA Tukey's: 1–2: $P = 0.012$; 1–3: $P = 0.006$), and the relative abundance of *Coprococcus* was higher in the second than first and third captures (ANOVA Tukey's: 2–1: 0.016; 2–3: $P = 0.011$). The relative abundance of *Ralstonia* was not significantly different across captures (ANOVA: $P = 0.877$). While normalized abundance was significantly different between the first and third captures (GLMM Tukey's: $z = 3.34$, $P = 0.002$, Fig. 3A), α-diversity did not significantly change across capture periods (Fig. 3C). A significant difference existed in β-diversity metrics between captures (adonis: $F_{2,39} = 1.044$, $R^2 = 0.15$, $P = 0.05$, Fig. 4A), but not sexes

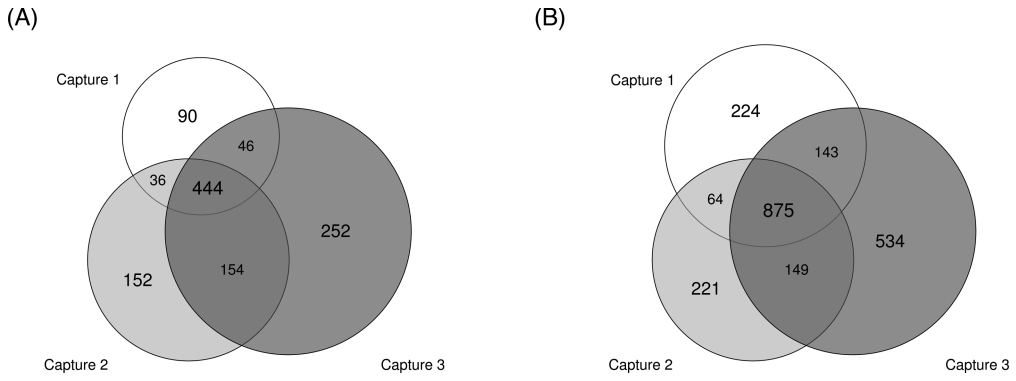

Figure 1. Shared and unique OTUs between all three captures in (**A**) the buccal and (**B**) the gut microbiomes of 14 North American red squirrels (*Tamiasciurus hudsonicus*).

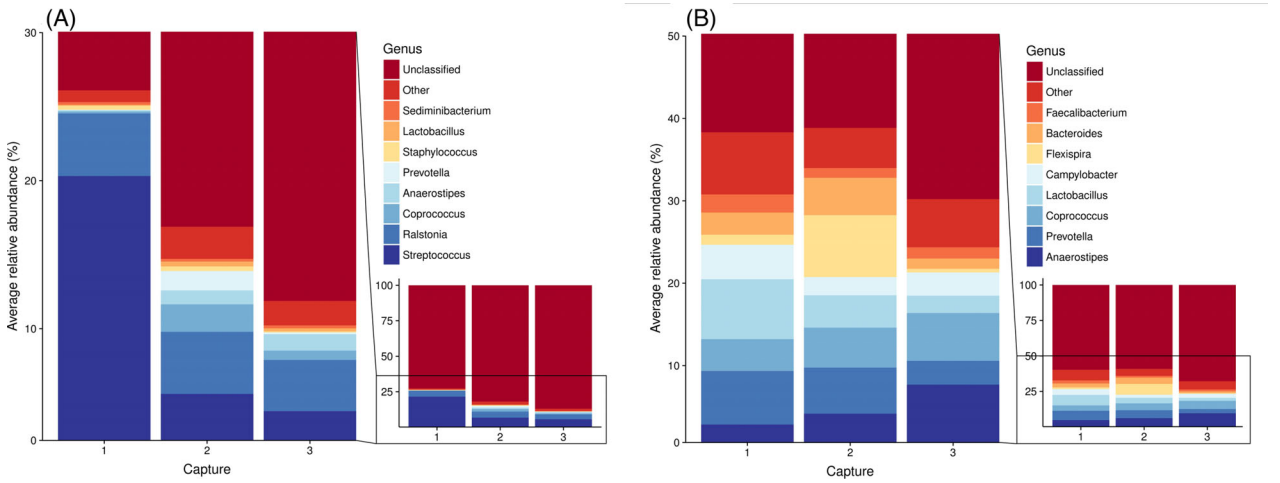

Figure 2. Average relative abundance of the top eight most abundant genera in the (**A**) buccal and (**B**) gut microbiomes of 14 North American red squirrels (*T. hudsonicus*) across three captures. Taxa are stacked by decreasing relative abundance.

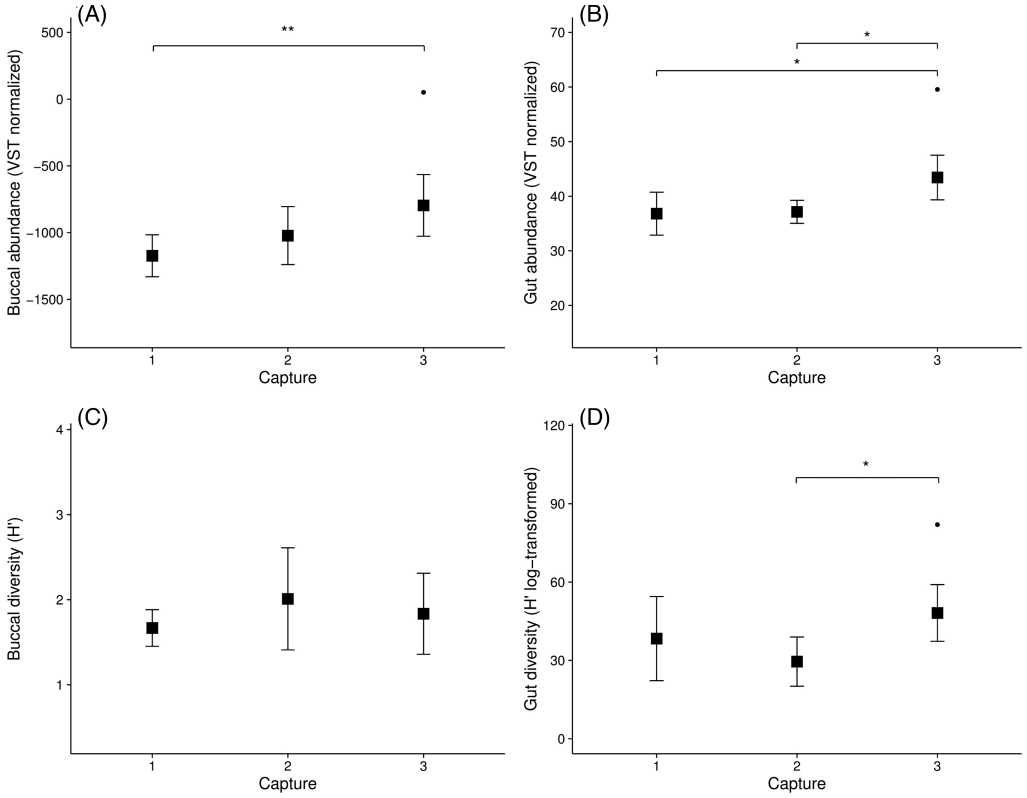

Figure 3. Change in average abundance measures and average diversity values across captures in North American red squirrels' (*Tamiasciurus hudsonicus*) buccal and gut microbiomes. Boxes and intervals represent the mean and 95% confidence limits respectively.

($P = 0.28$). Indicator species analysis revealed multiple specific OTUs that were significantly associated with each capture, including *Alicyclobacillus* in the first capture, *Coprococcus* in the second and Coriobacteriaceae in the third (see Table S1, Supporting Information).

Temporal variation in gut microbiome

The gut microbiome consisted of 2229 OTUs and 3714 125 (mean: 88 431.55, min: 48 413, max: 177 124) sequences. A total of 874 OTUs were present in all three captures. Capture 3 had the most unique OTUs, followed by capture 1 and capture 2 (respectively: 534, 244 and 221 unique OTUs; Fig. 1B). The three most abundant phyla in the gut microbiome, Firmicutes, Proteobacteria and Bacteroidetes, did not change significantly between capture periods (ANOVA: $P = 0.96$, $P = 0.61$ and $P = 0.63$, Fig. S1B, Supporting Information). While two of the most abundant genera, *Prevotella* and *Coprococcus*, remained stable across captures (ANOVA: $P = 0.17$ and $P = 0.43$, respectively; Fig. 2B). Normalized abundance values were significantly different between the first and third captures (GLMM Tukey's: $z = 2.89$, $P = 0.01$; Fig. 3B) as well as between the second and third captures (GLMM Tukey's:

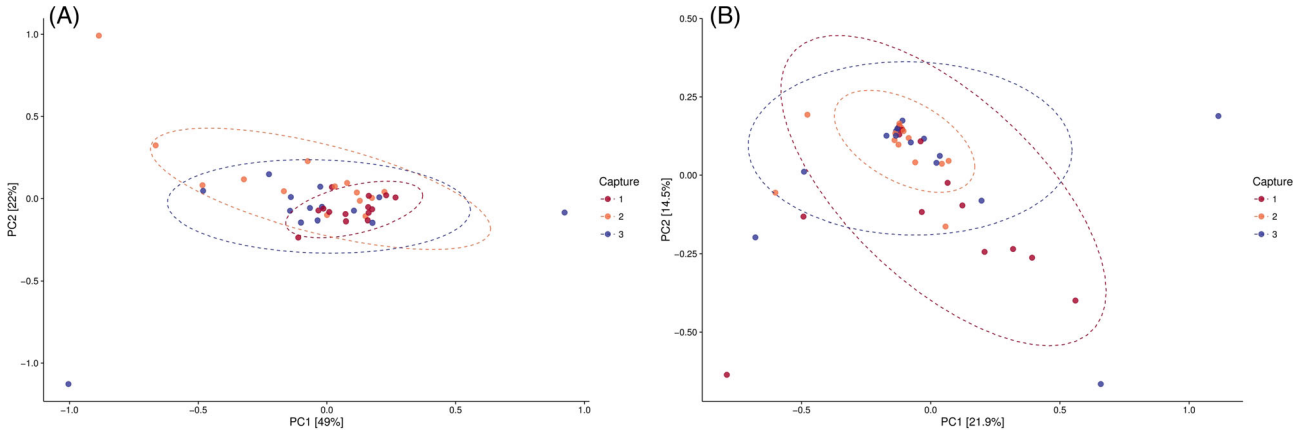

Figure 4. PCoA of weighted UniFrac distances in the (**A**) buccal and (**B**) gut microbiomes of North American red squirrels (*T. hudsonicus*) across three capture periods. Ellipses represent 95% confidence limits around the mean of each capture.

$z = 2.75$, $P = 0.02$; Fig. 3B). α-Diversity significantly differed between the second and third captures (GLMM Tukey's: $z = 2.69$, $P = 0.02$; Fig. 3D), and there was a significant difference in β-diversity metrics between captures (adonis: $F_{2,39} = 2.904$, $R^2 = 0.22$, $P = 0.01$; Fig. 4B) but not sexes ($P = 0.98$). Indicator species analysis revealed that several OTUs were specifically associated with each capture, including *Ruminococcus* in the first, *Lactobacillus* in the second and *Coprococcus* in the third (Table S1, Supporting Information).

Intra-individual temporal variation

Intra-individual change in buccal abundance across captures was not correlated with change in gut abundance in the first and second captures (regression: $R^2 = 0.01$, $F_{1,12} = 1.97$, $P = 0.30$), first and third captures (regression: $R^2 = 0.18$, $F_{1,12} = 3.914$, $P = 0.07$), or second and third captures (regression: $R^2 = 0.08$, $F_{1,12} = 2.214$, $P = 0.16$). These relationships were also not significant when investigating differences in α-diversity across captures (1–2: $R^2 = -0.08$, $F_{1,12} = 0.0001$, $P = 0.99$; 1–3: $R^2 = 0.04$, $F_{1,12} = 1.57$, $P = 0.23$; 2–3: $R^2 = 0.04$, $F_{1,12} = 0.04$, $P = 0.84$).

DISCUSSION

We sampled the buccal and gut microbiomes of North American red squirrels to investigate the impact of temporal variation on the characterization of multiple microbiomes in a wild mammal. We defined the changes between the first and second (<2 weeks) captures as a short-term change, the changes between the second and third (2–4 weeks) as medium-term change and the changes between the first and third (>1 month) as long-term change.

The buccal microbiome was dominated by Proteobacteria, Firmicutes and Bacteroidetes in all three captures. The dominance of these phyla is similar to other animals, such as salivary microbiomes of Komodo dragons (*Varanus komodoensis*; Hyde *et al.* 2016) and *Pan* species (Li *et al.* 2013). There was no evidence of change in the order of the most abundant phyla between captures and no significant differences in α-diversity measures. However, the most abundant genera shifted significantly between captures and long-term changes in normalized abundance and subsequent β-diversity analysis were evident. These conflicting results between diversity metrics of the microbiome point towards the importance of a robust analysis when characterizing a typical microbiome of a species, including applying distinct approaches to analyze the microbiome at unique taxonomic levels. Despite the uncertainty in long-term analysis, the buccal microbiome in this population showed stability in all microbiome measures over short and medium time periods, mirroring studies investigating the human salivary microbiome (Lazarevic *et al.* 2010; Belstrøm *et al.* 2016).

The gut microbiome was dominated by Firmicutes, Proteobacteria and Bacteroidetes, similar to humans (Eckburg *et al.* 2005) and mice (Maurice *et al.* 2015). This microbiome displayed more temporal variation than the buccal microbiome, consistent with findings in humans (Caporaso *et al.* 2011). Overall, there was evidence of near-significant change in the top three most abundant genera, medium and long-term changes in normalized abundance values, medium-term change in α-diversity, and significant differences in β-diversity metrics between captures. Evidence of long-term change has also been found in other mammals, such as field mice, who exhibit seasonal variation in the gut microbiome, likely due to changes in diet (Maurice *et al.* 2015). Our findings, however, also suggest that the gut microbiome of this population has the potential for relatively short (2–4 weeks) temporal variation, which ultimately may influence downstream analysis when characterizing a typical microbiome.

Our final goal of this study was to determine whether factors driving temporal variation in the buccal and gut microbiomes were synchronous (e.g. individuals with increasing buccal diversity may also show an increase in gut diversity). We found no evidence to support this claim, suggesting that body site variation in microbial composition may be driven independently by external factors.

While not the main focus of this study, the influence of various factors driving the variation in both microbiomes is an interesting point of conjecture. Previous research has found variation in gut microbiomes of mammals to be impacted by numerous factors (including sex, age, social interaction, stress and diet) and these factors may help explain the variation seen here. As sex was not significant in our statistical models, this likely did not play a large factor in driving variation seen in this study. Conspecifics in this study were all of a similar age class and, as the study was conducted outside of the breeding season, the likelihood of social interactions influencing microbial variation is

low. As all squirrels were sampled within 2 h of capture, it is unlikely that short-term stress influenced the noted variation (Bosson, Islam and Boonstra 2012). However, as short-term cortisol changes have been linked to buccal microbial composition (Stothart *et al*. 2016), it is possible this may also be evident in the long term and across several body sites. Slight dietary changes throughout the summer months could also be influencing this variation, and future research should focus on increasing our knowledge of these interactions within both the buccal and gut microbiomes over short and long time periods.

Overall, the buccal microbiomes in this population of red squirrels displayed temporal stability over short and medium capture periods, while the gut microbiome showed temporal variation across medium and long-term capture periods. Ultimately, we have demonstrated that the site of the microbiome studied, the length of study and type of analysis (including diversity and abundance metrics) may influence the characterization of a typical microbiome in a population of wild mammals. We have also found evidence that variation in microbiome composition behaves independently in two body sites, suggesting that body sites' microbiomes may be influenced by different factors. As more research is conducted to determine the impact of microbiome composition on the health and fitness of study species, we urge researchers to be cautious when characterizing typical microbiomes from point samples and to consider incorporating measures to eliminate this bias when evaluating microbiome composition in wild mammals.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSEC* online.

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Conflict of interest. None declared.

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