



Sociality does not predict oral microbiome composition or diversity in free-living prairie voles

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ABSTRACT

The costs and benefits of group living and sociality have received much attention. Recent studies suggest that the acquisition of a more diverse microbiome may be another benefit of sociality which could be beneficial for host health and survival. We characterized the sociality of free-living prairie voles, *Microtus ochrogaster*, and their oral microbiome alpha diversity and dissimilarity (beta diversity) to assess associations between sociality and microbiome diversity. Voles were born in the laboratory and then released into seminatural enclosures, representing a shift in diet and changes in social interactions. Social interactions were monitored using an automated behavioural monitoring system from which we quantified degree (the number of social connections) and association index (the strength of the social connection between two individuals). Oral microbiome samples were collected throughout the field season and then the oral microbiome was characterized using 16S rRNA gene sequencing, representing the first ever characterization of the oral microbiome in this species. Oral microbiome alpha diversity increased when voles were moved to the field from the laboratory but did not change over time in the field. It was not related to the number of social connections (unweighted degree). Oral microbiome dissimilarity (beta diversity) between two voles was not related to the strength of their social association (association index) nor was it related to whether the pair produced offspring together. The oral microbiome became more dissimilar over the field season in sibling versus nonsibling pairs. Together, our results indicate a lack of association between social interactions and oral microbiome diversity and dissimilarity and provide an important contrast as other studies on this topic are conducted mostly in primate species and mostly in the gut microbiome. This highlights the importance of investigating other areas of the microbiome besides the gut and the relationship between these variables in additional species.

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Group living affords individuals a number of benefits such as reduced predation risk or increased access to a limited resource, but it can also expose individuals to increased risk of parasitism or disease or increased competition for resources (Krause & Ruxton, 2002). Similar costs and benefits apply to individual differences in sociality. Individuals within a given species, even those that are solitary, can and do differ in their number of interactions with conspecifics (Menz et al., 2021; Sabol et al., 2020), their level of

attention or responsiveness to cues in the social environment (Taborsky & Oliveira, 2012), or their apparent willingness to seek out interactions with other individuals (Rodríguez-Prieto et al., 2011; Cote et al., 2012). In turn, these individual differences in sociality may impact survival and reproduction (Schulke et al., 2010; Silk et al., 2010; Menz et al., 2021; Sabol et al., 2020).

Although the costs and benefits of individual differences in sociality have been documented across taxa, their effects on microbial diversity are less studied. This is surprising as the microbiome likely has close links to host health and survival in free-living animals (Antwis et al., 2019; Bates et al., 2018; Becker et al., 2009; Harris et al., 2009; Ruiz-Rodríguez et al., 2009; Hennersdorf et al., 2016).

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Alpha diversity in the microbiome can be beneficial from the perspective of resistance to pathogens. For instance, individuals with a more diverse gut microbiome (higher alpha diversity) exhibit increased resistance to infection from a pathogen (Cariveau et al., 2014; Gopalakrishnan et al., 2018; Knutie et al., 2017; Zhang & He, 2015) and the presence or absence of specific microbes can also play an important role in host health by altering immune responses (reviewed in Kamada & Núñez, 2014; Koch & Schmid-Hempel, 2011; Kamada et al., 2012). Consequently, a possible benefit of a higher degree of sociality is acquisition of a more diverse microbiome in general or specific beneficial microbes, although this could be offset by the risk of acquiring pathogenic bacteria from other individuals (Lombardo, 2008; Sarkar et al., 2020). It is possible that the beneficial microbes may counteract pathogenic bacteria or disease, but there is likely still a risk of acquiring potentially harmful bacteria or other diseases that cannot be or are not counteracted by acquiring beneficial microbes.

The relationship between sociality and microbiome diversity has been investigated in several species. Studies in group-living primate species have provided the most thorough examinations of this relationship because of the relative ease of observing social interactions compared to more secretive species (Amato et al., 2017; Moeller et al., 2016; Perofsky et al., 2017; Tung et al., 2015). For example, in yellow baboons, *Papio cynocephalus*, pairs of individuals that were more often observed grooming one another had more similar gut microbiomes (Tung et al., 2015). In chimpanzees, *Pan troglodytes*, two individuals that spent more time with one another also had more similar gut microbiomes (Moeller et al., 2016). In one nonprimate example, gut microbiomes were more similar among feral Welsh mountain ponies, *Equus ferus caballus*, from the same group and were related to particular social interactions within groups (Antwis et al., 2018). Studies about the association between sociality and microbiome diversity in nongroup-living species or nonprimate species are much rarer, often because of the difficulty of observing social interactions. Such studies in less social species may be particularly important given that group-living species share the same environment, which should also increase microbiome similarity. A recent study in free-living wild wood mice, *Apodemus sylvaticus*, which are not a group-living species, showed that two individual male mice (but not females) with a stronger social association had a more similar gut microbiome (Raulo et al., 2021). This relationship remained even after controlling for how close the individuals lived to one another and their relatedness (Raulo et al., 2021).

The links between sociality and the microbiome have been mostly studied by characterizing the gut microbiome (e.g. Antwis et al., 2018; Moeller et al., 2016; Raulo et al., 2021; Tung et al., 2015), but microbiota are present in many different parts of the body and may also be affected by social interactions. The human oral microbiome is thought to be the most diverse microbiota after the gut (Deo & Deshmukh, 2019; He et al., 2015). Like the gut microbiome, the function of the oral microbiome is largely viewed under the lens of human health. From this perspective, a 'healthy' oral microbiome is thought to play essential roles in the digestion of food as well as the proper functioning of oral mucosa, including the first line of defence against oral pathogens via the antimicrobial properties of the oral microbiome that can maintain oral health including protection against dental cavities (Deo & Deshmukh, 2019). There is also some suggestion of an association between a 'healthy' oral microbiome and general/systemic health (Deo & Deshmukh, 2019; Kilian et al., 2016; Sharma et al., 2018). Also similar to the gut microbiome, higher levels of diversity in the oral microbiome are thought to be favourable to the host (Scannapieco, 2013), such as a dysbiotic microbiome being characterized by low diversity and poorer oral health (Kilian et al., 2016; Sampaio-Maia

et al., 2016). However, much like in the gut microbiome, the direct links between oral microbiome diversity and health are largely unknown, as we are only starting to understand these links (Sampaio-Maia et al., 2016).

Past work has illustrated that close social interactions or a shared environment can increase oral microbiome similarity (Schaefer et al., 2010). Many species engage in behaviours, such as self- and allogrooming, that could facilitate the transfer of microbes from the oral cavity of one organism to another, making grooming a potentially important source of microbial transfer during social interactions. By grooming each other, individuals may directly acquire microbes from the other individual's skin or fur microbiome; if they are groomed and then groom themselves there is likely to be transfer of oral microbes through the saliva on the fur.

To better understand associations between the degree of sociality and the microbiome, more studies are needed of nonprimate species and of species that do not live in large groups. Additionally, because most previous studies focused on the correlation between sociality and the gut microbiome, it is not clear whether the same patterns exist for microbial communities that exist in other parts of the body. Finally, previous studies on this topic have been associative, where cause and effect are not clear as individual animals started with a nonstandardized environment and diet, which would also influence the microbiome. Ideally, individuals start out with a similar environment and diet, resulting in a more similar microbiome and then the specific effects of social interactions on microbiome similarity can be better quantified.

In this study, we were able to harness a powerful system of experimental enclosures to test the hypothesis that oral microbiome alpha diversity and dissimilarity (beta diversity) between two individuals are linked to their frequency of social interactions. We predicted that individuals with more social interactions with different individuals would have a more diverse microbiome and that pairs with a higher social association strength would have more similar (less dissimilar) microbiomes. To test these predictions, we reared prairie voles, *Microtus ochrogaster*, under standard laboratory conditions by controlling environment and diet to induce similar baseline microbiomes given their shared environment. We then released the voles into seminatural field enclosures where they could freely interact with each other. Prairie voles exhibit considerable variation in their social behaviour, with social monogamy and polygyny existing within the same population (Getz et al., 1993; Madrid et al., 2020). They can also vary in whether socially monogamous pairs are genetically monogamous or not (Solomon et al., 2009). Prairie voles sometimes live in groups of more than two adults due to philopatry, particularly at higher densities (Getz et al., 2005; Lucia et al., 2008). The number of social interactions between two individuals can vary substantially even when they inhabit the same population (Sabol et al., 2020). Further, prairie voles are known to engage in both self- and allogrooming, which could facilitate the transfer of oral microbes between social partners (Burkett et al., 2016).

We characterized the sociality of individual voles while they were in the field enclosures using an automated behavioural monitoring system that used a network of radiofrequency identification (RFID) antennas distributed within the field enclosure to track spatiotemporal co-occurrence of voles that all had unique passive integrated technology (PIT) tags (Sabol et al., 2018, 2020). We then used these data to construct both weighted and unweighted social networks to quantify the number of social connections each individual vole had (unweighted degree) and the strength of their social association with different voles in their enclosure (association index). We used DNA parentage analyses to identify which individuals produced offspring with one another; genetic relatedness for adults released into the enclosures was

known as they had been bred in the laboratory. We have already used this system of social network analysis to document individual differences in sociality in terms of the number of different voles each individual interacted with (Sabol et al., 2020). We obtained regular oral swabs from all individuals and then used 16S rRNA sequencing to describe the prairie vole oral microbiome as well as quantify oral microbiome diversity and dissimilarity between samples.

We predicted that (1) oral microbiome alpha diversity would increase after the voles were moved from the laboratory to the field, (2) voles with a higher unweighted degree (more social connections) would have a higher mean oral microbiome alpha diversity, (3) voles would exhibit a lower oral microbiome dissimilarity index (beta diversity, and therefore be more similar) if they had a higher association index (due to more time in close proximity and possibly the same burrow, possible food sharing or grooming) or if they produced offspring with each other, and (4) that siblings would have more similar microbiomes overall.

METHODS

Fieldwork was conducted at the Miami University Ecology Research Center in Oxford, OH, U.S.A. from May 2017 to August 2017. Voles were bred in the laboratory in plastic cages (24 × 45 cm and 21 cm high) containing straw and Tekfresh paper bedding (Harlan Laboratories, Indianapolis, IN, U.S.A.). Around 21 days of age, they were separated from their parents and housed with littermates in cages of the same size with the same substrates. Voles were maintained on a 14:10 h light:dark cycle (lights on at 0600 hours), fed Purina Autoclavable laboratory Diet 5010 (Nestle Purina PetCare Company, St Louis, MO, U.S.A.), and provided with water ad libitum. Their diets were supplemented with Purina Hi Fiber Rabbit Chow 5326 twice each week. These housing conditions helped keep the experiences of all founding voles as similar as possible prior to their release into the enclosures. Founder voles released into enclosures were at least 40 days of age, old enough to breed (Solomon, 1991). We ensured that no parent–offspring or opposite-sex siblings were released into the same enclosures to prevent inbreeding.

The two outdoor enclosures the voles were released into were 0.1 ha (ca. 32 × 32 m) surrounded by a sheet metal fence (75 cm above and 45 cm below ground) that prevented voles from moving in or out of the enclosures. An electrified wire was run across the top of the fence and turned on whenever researchers were not in the enclosure; this functioned to keep out most terrestrial predators. No supplemental food was provided to voles in the enclosures except for small amounts of cracked corn used to bait traps; this bait is a low-quality food source (Desy & Batzli, 1989). Therefore, the bait likely made up a portion of the diet, but voles would not be able to subsist solely on the bait. The enclosures contained a mix of local vegetation including goldenrod, *Solidago* spp., bluegrass, *Poa pratensis*, clover, *Trifolium* spp., fescue, *Festuca* spp., timothy, *Phleum* spp., and ryegrass, *Elymus* spp., that provided natural habitat and food for the voles. One enclosure initially had 48 individuals (a density of 480 voles/ha, enclosure 1) while the other enclosure had 24 individuals (a density of 240 voles/ha, enclosure 2) for another experiment, although these densities changed across the study period (Sabol et al., 2020).

RFID Monitoring System

All voles were implanted with a PIT tag (12 mm HPT tags; Bio-mark; Boise, ID, U.S.A.) before being released into the enclosures and offspring produced within the enclosures were PIT tagged when they were large enough (>25 g). This allowed us to

distinguish individuals and for their movements and interactions to be recorded by the RFID system. Twelve RFID antennas were placed around the enclosures in two different arrays. Arrays were changed every 3 days and moved between enclosures every 6 days (Sabol et al., 2018). These antennas recorded the PIT tag number of any vole within 15 cm of the antenna once a second, for the entire duration the animal was within range (Sabol et al., 2018). We have previously shown that this system can accurately track the social relationships of free-living prairie voles (Sabol et al., 2018).

Oral Swab Collection

To obtain oral swabs and to PIT-tag offspring produced within the enclosures, we conducted regular live trapping using Ugglan multiple capture traps (Grahnbab, Gnosjö, Sweden) that were covered with aluminium shields (to protect voles from sun or unexpected rain). We placed two live traps at each stake in a 5 m × 5 m grid and then placed two live traps at each nest or burrow as we found them. The trapping schedule changed throughout the field season (Sabol et al., 2018) but traps were set approximately every other day for a few hours in the morning and evening, so corn was available during this time. Oral microbiome swabs were collected and voles were weighed once per week for individuals that were live-trapped that week. To collect samples from captured voles, a sterile, nylon-flocked tip swab (product B220529, Fisher Scientific, Waltham, MA, U.S.A.) was moved thoroughly around inside both cheeks for approximately 3–5 s. Swabs were then stored individually in sterile containers in a –20 °C freezer immediately afterwards, transferred to a –80 °C freezer on the day of collection and kept there until analysis.

We chose to analyse swabs for individuals that had a high number of samples over the course of the study to test the change in the oral microbiome over time (although we made sure to not only include individuals that survived to the end of the field season). Specifically, we chose one to two samples from individuals while they were in the laboratory, the first one or two samples after release into the enclosures (to investigate how quickly the microbiome changed once released) and the last sample collected for that vole. We then added any samples from 5 June, as this was the day with the highest sampling of the populations overall (Appendix Fig. A1), 6 June, to include any additional samples at a similar time point to 5 June, and any samples collected in August (to ensure we included some samples that represented the longest amount of time in the enclosures, as this was the last month of the experiment) that were not already included (see Appendix Fig. A1 for the number of oral swabs collected per day throughout the study).

Microbiome Analysis Methods

We prepared 16S rRNA gene sequencing libraries from 367 individual oral swabs as previously described (Kozich et al., 2013; Schloss et al., 2009). Briefly, microbiome communities were released from swabs using bead agitation and DNeasy PowerSoil HTP 96 kits (Qiagen, Hilden, Germany) were used to extract DNA on an epMotion 5075 automated liquid handler (Eppendorf, Hamburg, Germany). The V4 region of the 16S rRNA gene was amplified with Accuprime Pfx Supermix (Invitrogen, Waltham, MA, U.S.A.) and custom barcoded primers. The ZymoBIOMICS microbial community standard (Zymo Research, Irvine, CA, U.S.A.) was used as a positive amplification control and water was used as a negative control. Libraries were then pooled using SequalPrep normalization plates (Applied Biosystems, Foster City, CA, U.S.A.) and quantified with KAPA library quantification kits (Kapa Biosystems, Wilmington, MA, U.S.A.). Sequencing was performed on a MiSeq system (Illumina, San Diego, CA, U.S.A.) to produce 250 bp paired-end reads.

We analysed the 16S rRNA gene sequences using mothur (v1.42.3, <https://mothur.org>) following the standard workflow. Reads were deduplicated, aligned to the V4 region of the 16S rRNA gene of the SILVA reference alignment (v132; Quast et al., 2013), and chimeras were removed using VSEARCH (v2.13.3; Rognes et al., 2016). Resulting sequences were clustered into 4774 bacterial operational taxonomic units (OTUs) via the OptiClust algorithm and a 97% similarity threshold (Westcott & Schloss, 2017). We assigned OTU taxonomies using the Ribosomal Database Project (v16) with an 80% confidence threshold (Cole et al., 2014).

To account for variation in sequencing depth, samples were rarefied to 1202 reads. Alpha and beta diversity metrics were calculated by mothur using the Shannon diversity index and Bray–Curtis dissimilarity index, respectively. We included both alpha and beta diversity metrics as we were interested in both how diverse the microbiome samples were overall (alpha) in relation to number of social connections and how similar (or dissimilar) the microbiome samples were (beta) between voles depending on the strength of their social interactions.

Owing to the complexity of social interactions, the Shannon diversity index was chosen to measure alpha diversity because it reflects the functional diversity of the microbial community as well as the relative abundance of those traits within the population. The Bray–Curtis dissimilarity index was also chosen over other beta diversity metrics for its ability to account for similar properties of community membership and distribution within the sample populations.

Parentage Analysis

Before releasing voles into the enclosure, we collected a small tissue sample from the ear pinnae of adults and stored the sample in alcohol in a -80°C freezer. When offspring were trapped for the first time, we collected a toe clip that served as both a unique identifier until offspring were large enough to be PIT tagged and a sample for parentage analyses. DNA was extracted using Qiagen DNeasy kits.

We used PCR to genotype all founding voles and their offspring captured in the enclosures at six microsatellite loci previously used for parentage analysis in prairie voles (for details see Keane et al., 2007; Solomon et al., 2009). The resultant PCR products were diluted, combined with an internal size standard (LIZ GS500, Applied Biosystems) and detected using an ABI 3730 DNA sequencer (Applied Biosystems). Base-pair lengths of the fluorescently labelled DNA fragments were analysed using PeakScanner (version 2.0) fragment analysis software (Applied Biosystems) and microsatellite alleles were compiled into discrete size classes using FlexiBin (Amos et al., 2006).

Parentage was assigned using the parent-pair analysis option in Cervus 3.0, which uses a simulation to calculate the statistical confidence of parentage assignments (Kalinowski et al., 2007). We conducted separate parentage analyses for each enclosure and all simulations were performed for 10 000 cycles with a genotyping error rate of 0.02. This error rate was based on empirical estimates of two potential sources of error: mutation and mis-scoring of alleles (Solomon et al., 2009). All founders within an enclosure were considered as candidate parents for each offspring. We accepted a parentage assignment only when the confidence level among a male–female–juvenile trio was $\geq 95\%$.

Ethical Note

All work involving live animals was approved by the Institution of Animal Care and Use Committee at Miami University (protocol number 979) because this was where all work with live animals

occurred. When deciding how to mark animals and collect tissue samples as well as to address IACUC's requirement of refinement, we considered alternative methods. For marking animals, hair clipping has been used, but these identifying marks are temporary because the fur is shed or regrows (Johnson 2001; Sikes et al., 2016). Also, these techniques are not appropriate for marking altricial neonates, like voles, that are hairless or juveniles that will moult their early pelage. Nontoxic dyes were deemed inappropriate due to the lack of information on the short- and long-term consequences in small mammals. Therefore, we decided to PIT-tag adult voles (>25 g) for individual identification by injecting a transponder underneath the skin between the shoulder blades of the voles. This technique is commonly used in small mammals for individual identification (Gibbons and Andrews 2004). We were concerned that injecting a PIT tag into a juvenile prairie vole would be risky due to the gauge of the needle needed to inject the tag. There is no published information that indicates how large an animal must be to safely inject a PIT tag; therefore, we used toe clipping for individual identification in juveniles that seemed too small (<25 g) to be PIT tagged. According to the guidelines of the American Society of Mammalogists (ASM), toe clipping is a permanent method of marking that 'might be especially appropriate in studies where tissues samples also are required' (Sikes et al., 2016, p. 677), which was the case in this study.

Previous studies suggest that there are only moderate effects known to occur from toe clipping small mammals. For example, in house mice, *Mus musculus*, individuals that were toe-clipped versus not toe-clipped did not differ in corticosterone levels (Schaefer et al., 2010) and toe clipping in juvenile mice did not induce deficits in locomotor behaviour or climbing (Castelhano-Carlos et al., 2010) or in somatosensory and nociceptive thresholds in adulthood (Frezel et al., 2019). This is also consistent with our previous observations (e.g. Lucia & Keane, 2012; Solomon et al., 2009) and those from a previous field study in prairie voles (Wood & Slade, 1990) where toe clipping did not induce any observable adverse effects on the animals. We toe-clipped juvenile voles when they were first captured. Based on data on growth rates of prairie voles from Nadeau (1985), we estimate that 83% (34/41) of the animals first trapped as juveniles in our study were less than 17 days of age when first captured. We note that this is an estimate because there is likely to be considerable variation in the size at which a vole would reach 17 days of age, since growth rates in prairie voles are known to be affected by numerous factors (Solomon, 1991, 1994; Sauer and Slade, 1986; Keane et al., 2007). In conclusion, we think that toe clipping the animals, as was permitted by ASM and our IACUC protocol, was the most feasible method of permanently marking the juvenile prairie voles and at the same time providing tissue for DNA extraction for parentage analysis.

Toe clipping of voles was performed in the field using a sharp, clean pair of scissors without the use of anaesthetics and analgesics. PIT tagging was also done without the use of anaesthetics and analgesics. The ASM guidelines do not recommend using anaesthetics and analgesics because 'the prolonged restraint of small mammals to apply these substances and the potential consumption of the analgesic substances (e.g. creams) via licking likely cause more stress and harm than conducting the procedure without their use' (Sikes et al., 2016, p. 678). Further, Paluch et al. (2014) found that there were no negative effects of toe clipping without anaesthesia in house mouse pups at 17 days of age. No vole had more than one toe clipped per foot as recommended by ASM guidelines (Sikes et al., 2016). Because we could only generate 16 unique marks by clipping one toe per animal, we found it necessary to clip more than one toe in 25 juveniles. Individuals who performed toe clips were trained by an experienced individual and had taken an IACUC training course. This training included how to use the

scissors to remove a digit for toe clipping as well as how to use toe clipping for unique marking of individuals.

To obtain tissue for parentage analysis from adult voles, which were not toe-clipped, we decided to take a small piece of one ear pinna following guidelines from the American Society of Mammalogists (Sikes et al., 2016). This method is considered one of the USDA-approved tissue collection procedures that involve no or only momentary or slight pain (Sikes et al., 2016). The same procedures as described for toe clipping juveniles were used for obtaining a sliver of ear pinna and students were trained as described previously.

Traps were covered with an aluminium shield to protect animals in them from sun and rain. They were baited with cracked corn, which is a low-quality food and therefore would not have been strongly desirable to voles compared to the vegetation in the enclosures (Desy & Batzli, 1989). Voles were free to enter traps as they wished. In addition, traps were only set for 2–3 h in the morning and 2–3 h in the evening before being checked, so that is the maximum amount of time animals would have been in the traps each day. The rest of the time the traps were left in the enclosures but not set. Often voles were caught in traps during multiple trapping sessions in a row, suggesting that trapping is not stressful for them. We have seen this in previous studies in our enclosed populations as well as in the natural populations (Lambert et al., 2021; Solomon et al., 2009; Streatfeild et al., 2011).

Swabbing the oral cavity may have caused some temporary discomfort to the voles. Therefore, we used the minimum number of rotations of the swab in each cheek to obtain an adequate microbiome sample (approximately three rotations or 3–5 s).

Regarding IACUC's requirement of replacement, if possible, to study behaviour we had to use live animals. Models or simulations of animal social behaviour are not yet advanced enough or have enough background information to be used in place of live animals. To address IACUC's requirement of reduction we only used two enclosures, one with high density (48 voles per 0.1 ha) and one with lower density (24 voles per 0.1 ha) at the beginning of the study and we used the minimum number of individuals in each enclosure to have enough statistical power for the density experiment. We stocked each enclosure with the number of voles that was representative of low and high density based on field studies from natural populations of voles (Getz et al., 1993). We also reduced the number of trapping sessions compared to previous studies of prairie voles in these enclosures (Castelli et al., 2011; Lambert et al., 2021; Lichter et al., 2020) so voles were handled less frequently.

Statistical Analysis

All statistical analyses were run in R version 4.0.3 (R Core Team, 2020). For all linear mixed-effects models (LMMs) we used the lmerTest package (version 3.1–3, Kuznetsova et al., 2017) and for all linear models and LMMs we visually checked residuals for normality, homogeneity of variance and the lack of high leverage data points. For LMMs, we either included a random effect for vole identity (when we had repeated samples per individual), enclosure identity (when we had repeated samples from the same enclosure), or both. We originally included litter as a random effect but had to remove it due to model convergence issues (models would not converge when it was included).

We identified the bacterial families whose relative abundances in the vole oral microbiome changed significantly with the transition from laboratory to field using a series of negative binomial mixed-effects models and the NBZIMM package (Zhang & Yi, 2020). Negative binomial models are better equipped to handle the zero-inflation and sparsity common to microbiome count data than

other differential abundance methods (Zhang & Yi, 2020). We filtered bacterial taxa to those with relative abundances >5% before testing to capture changes in the most abundant taxa present. Negative binomial models included the rarefied read count of each bacterial taxon as the dependent variable, whether the sample was collected from the laboratory or field and the sex of the individual as fixed factors, and animal enclosure and individual identity as random factors. We adjusted *P* values for multiple hypothesis testing using a Benjamini–Hochberg FDR correction to all *P* values. Adjusted *P* values < 0.05 were considered statistically significant.

To understand how microbiome diversity changed from the laboratory to the field, we ran an LMM with microbiome alpha diversity as the response variable and fixed effects of whether the sample was taken in the field or laboratory, vole sex and the interaction between these two terms (the interaction was included because, as the field season went on, females were likely to get pregnant and remain in the nest potentially limiting interactions with other individuals, while males would continue to roam the enclosure, potentially interacting with other individuals). The random effects were which enclosure the vole was in and the individual vole identity. Next, we ran an LMM to determine whether microbiome alpha diversity changed during the field study, with microbiome diversity as the response variable and the fixed effects of the number of days since the voles were released into the field for the day that particular sample was taken, vole sex, the interaction between these two terms (the interaction was included because, as the field season went on, females were likely to get pregnant and remain in the nest potentially limiting interactions with other individuals, while males would continue to roam the enclosure, potentially interacting with other individuals) and the body mass of the vole on the day the sample was taken as a potential indicator of body quality. The random effects were which enclosure the vole was in and the individual vole identity.

We used the R package *asnipe* (version 1.1.13, Farine, 2013) for all social network analyses. We used co-occurrence information from the RFID antennas to build the social networks by running the PIT tag readings on the RFID antennas through a Gaussian mixture model (Psorakis et al., 2012) with each day run separately. This model creates associations based on clusters of PIT tag readings throughout time. Therefore, groups of social associations are not based on a uniform time frame but are instead created based on the clustering of the data. These groups ranged from 0 s difference (direct co-occurrence at the same antenna at the same time) to 66 161 s, with a mean \pm SD of 655.2 \pm 3352.8 s. This model creates an individual by group matrix where voles are associated if they are placed in the same spatiotemporal group based on the Gaussian mixture model (for more details, see Sabol et al., 2018).

To measure the number of social connections of each individual we used a binary, unweighted measure of degree, so if two voles were associated this was marked as a '1' and if two voles were not associated this was marked as a '0'. Unweighted degree is the sum of total connections for each individual. To compare the number of social connections to microbiome diversity, we calculated the mean diversity for all of the individual's microbiome samples from the enclosures (excluding laboratory samples) and used this as the response variable in a linear model with the main effects of the individual's unweighted degree (total number of the individual's social connections), vole sex and the interaction of these two terms (the interaction was included because, as the field season went on, females were likely to get pregnant and remain in the nest potentially limiting interactions with other individuals, while males would continue to roam the enclosure, potentially interacting with other individuals). Because social network data are inherently nonindependent, we used the network permutation method in *asnipe* (Farine, 2013). This allows us to compare the

observed relationship to the relationships from randomly generated networks with the same structure as our social network. The network permutation method swaps an individual from the group by individual matrix with another individual from the same enclosure for each randomization (10 000 randomizations in total, Farine, 2013). We also limited these swaps to only include voles recorded at the antennas that same day to control for differences in how long each vole survived. This method then compares the regression coefficient from the observed model to the corresponding regression coefficients from the randomized models and calculates a new *P* value based on how many randomized models produced a regression coefficient that had an absolute value higher than the observed coefficient. We ran the network permutations three times to ensure that the relationship was either consistently significant or consistently not significant and present all three *P* values (Sabol et al., 2020).

To determine the strength of each social association, we used a simple ratio index which is calculated as the number of times a particular pair were recorded as associated together divided by the total number of times they were recorded either separately or together based on the RFID antennas for the entire field season (Cairns & Schwager, 1987). We then compared these association indices to the response variable of the measure of the pair's microbiome dissimilarity using a multiple regression quantitative assignment procedure (MRQAP, Krackhardt, 1988) in the package *asnipe* with 10 000 randomizations per model. To test whether voles that had higher association indices became less dissimilar over the course of the field season, we ran three separate models per enclosure: one comparing each pair's association index to the pair's microbiome dissimilarity for samples taken in the laboratory (as a control as the voles in the laboratory would have no social interactions besides the siblings with which they were housed), one comparing association index to the pair's microbiome dissimilarity for each vole's first sample taken after being released to the field enclosures, and one comparing association index to the pair's microbiome dissimilarity for the last sample taken during the field season for each vole (either the end of the experiment or the last time the vole was recorded during the experiment). We compared the association index to the sample dissimilarity taken at different times to see whether they would be associated by different points of the field season, so comparing the association index to the laboratory dissimilarity index was run as a control to compare with the results of the later sample dissimilarity models.

To test whether voles that produced offspring together (mates) had more similar oral microbiome communities, we ran a general linear model with microbiome dissimilarity as the response variable and the main effects of whether the pair were mates or not (defined as successfully producing offspring together), whether the samples for the pair were taken in the laboratory or were the last samples collected during the study (and therefore while the voles lived in the field enclosures), and the interaction of these two variables. Because enclosure 1 had very few offspring born ($N = 5$) we only ran this model on data for enclosure 2 ($N = 36$).

Finally, to test whether the oral microbiomes of siblings were more similar than those of nonsibling pairs, we ran a general linear model with microbiome dissimilarity as the response variable and the main effects of whether the pair were siblings or not, whether the samples for the pair were taken in the laboratory or were the last samples while the voles lived in the field enclosures, and the interaction of these two variables. Because enclosure 2 had very few sibling pairs ($N = 6$) we only ran this model on data for enclosure 1 ($N = 33$). Since we did not include any opposite-sex sibling pairs in the same enclosures to prevent inbreeding, all the sibling pairs tested were therefore same-sex siblings. Owing to problems with accessing all of the previous laboratory records, we

only had information about direct parent–offspring and sibling relationships, so sibling relationships were the extent of relatedness we could test (since we did not have any parent–offspring pairs in the voles released into the enclosure to prevent inbreeding).

RESULTS

The Vole Oral Microbiome

Following rarefaction, high-quality reads were sorted into 4774 operational taxonomic units (OTUs) using a similarity threshold of 97%. Taxonomic assignments revealed that the vole oral microbiome was highly diverse and comprised many common rodent gut microbes. Across 367 oral swab samples ($N = 302$ field samples and 65 laboratory samples), the vole microbiome included OTUs from nine phyla and 188 families. Bacteria assigned to the phylum Firmicutes were the most common in both field and laboratory samples (field: mean \pm SD = 49.78 \pm 10.6%; laboratory: 40 \pm 12.7%), followed by Proteobacteria (field: mean \pm SD = 23.0 \pm 11.0%; laboratory: 28.2 \pm 13.2%), Actinobacteria (field: mean \pm SD = 7.4 \pm 6.8%; laboratory: 17.1 \pm 15.3%), Bacteroidetes (field: mean \pm SD = 15.8 \pm 7.9%; laboratory: 12.1 \pm 8.8%) and Fusobacteria (field: mean \pm SD = 8.5 \pm 4.3%; laboratory: 8.3 \pm 4.1%). Bacteria assigned to the phylum Verrucomicrobia made up less than 6% of the relative abundance of the oral microbiome in both laboratory and field samples, and unclassified bacteria made up less than 5%. Notably, OTUs assigned to the phylum Tenericutes were absent in laboratory samples and only present (although at low relative abundance) in field samples.

Of the 188 detected bacterial families, 14 were found in more than 90% of all samples (field and laboratory) and therefore characterized the 'core' vole oral microbiome (Fig. 1a and b; using a 90% threshold following the method used by e.g. Bäckhed et al., 2015; Dashper et al., 2019). Among these core families, the most abundant were Pasteurellaceae (field: mean \pm SD = 7.5 \pm 13.3%; laboratory: 15.7 \pm 16.5%), Enterobacteriaceae (field: mean \pm SD = 13.7 \pm 6.4%; laboratory: 10.76 \pm 7.8%), Lachnospiraceae (field: mean \pm SD = 13.7 \pm 6.7%; laboratory: 10.2 \pm 7.5%) and Veillonellaceae (field: mean \pm SD = 12.9 \pm 6.6%; laboratory: 9.9 \pm 7.2%).

Effects of Transition From Laboratory to Field on the Oral Microbiome

Overall, the alpha diversity (Shannon diversity) of the oral microbiome was significantly higher when voles were in the field enclosures compared to when they were in the laboratory (effect of laboratory: b (effect size) = -0.203, $t = -2.317$, $P = 0.021$; Fig. 2). Sex ($b = -0.0213$, $t = -0.293$, $P = 0.771$) and the interaction between sex and whether the sample was obtained in the laboratory or enclosure ($b = 0.167$, $t = 1.362$, $P = 0.174$) were not significantly associated with oral microbiome diversity.

Although vole oral microbiome alpha diversity was overall lower in the laboratory than in the field, there was a nonsignificant change in oral microbiome alpha diversity as the field season progressed (effect of day from the start of the experiment: $b = 0.00252$, $t = 1.707$, $P = 0.089$; Fig. 3). While the voles were in the field enclosures, there were no effects of body mass ($b = -0.00143$, $t = -0.423$, $P = 0.672$) or sex ($b = 0.105$, $t = 1.185$, $P = 0.239$) on vole microbiome diversity or sex-specific changes as the field study progressed, indicated by a lack of significant interaction between sex and days since the start of the study ($b = -0.00308$, $t = -1.566$, $P = 0.118$).

We used a series of negative binomial mixed-effects models to identify the bacterial families that changed significantly in the oral

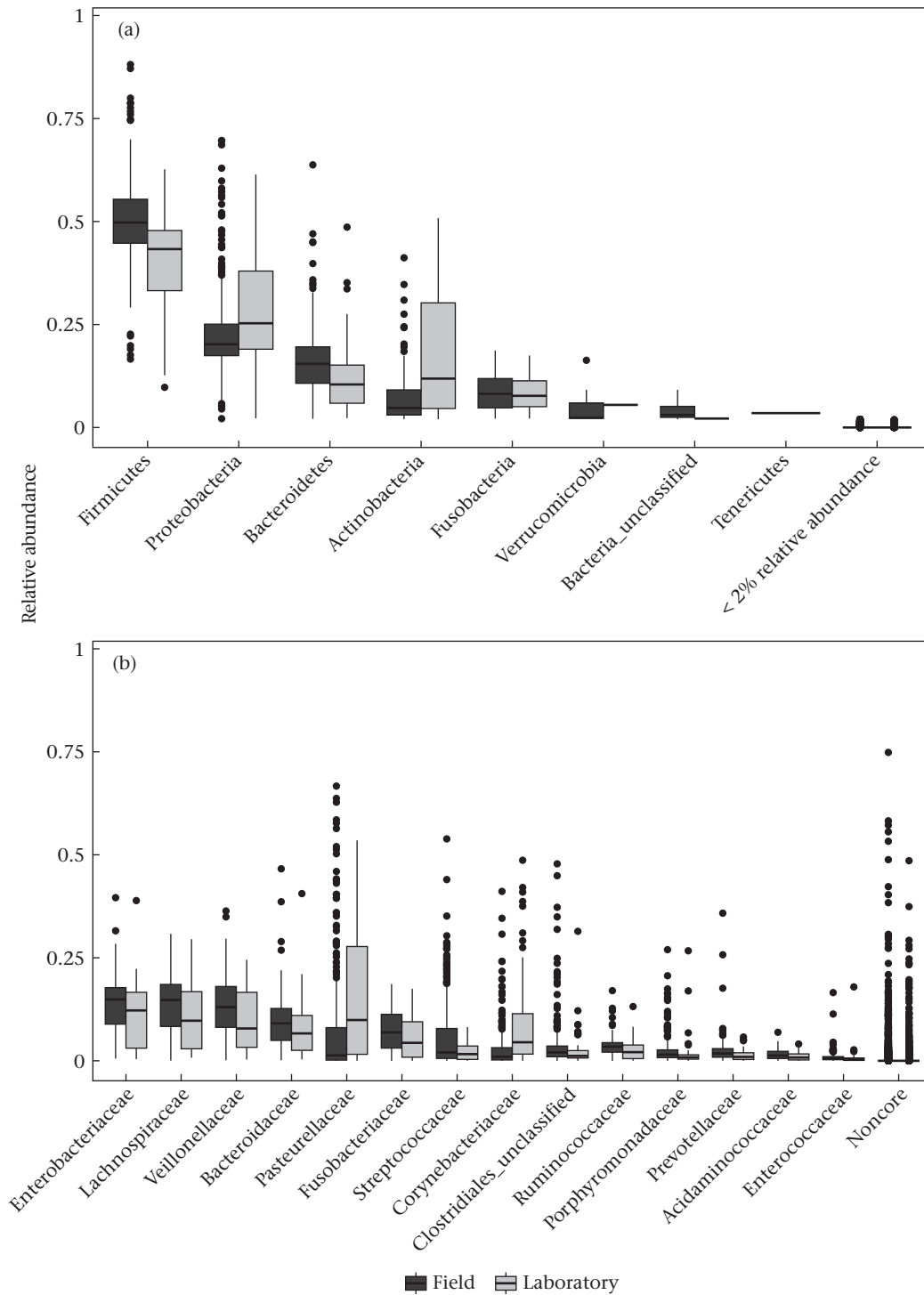


Figure 1. Taxonomic composition of the vole oral microbiome. (a) The seven phyla of bacteria that made up >2% of the relative abundance of the vole oral microbiome (not including unclassified bacteria). (b) Core bacterial families with high prevalence (present in at least 90% of all samples). The box plots show the median and 25th and 75th percentiles; the whiskers indicate the values within 1.5 times the interquartile range and the circles are outliers.

microbiome community after the laboratory to field transition. Models revealed that 34 bacterial families changed significantly in relative abundance as individuals transitioned from the laboratory to the field, controlling for sex, individual identity and enclosure (Fig. 4). Of those taxa that changed significantly, 11 (32%) were core bacterial families. The general trend was an increase in noncore taxa as voles moved from the laboratory to the field, and a decrease in the relative abundance of core taxa. This pattern aligns with our

finding of an increase in oral microbiome alpha diversity in field samples, as voles incorporate more noncore, environmental taxa into their oral microbiome. The relative abundances of 10 of the core families that changed with laboratory versus field enclosure were significantly lower in field than laboratory samples, including Bacteroidaceae ($\beta = -0.23$, $P = 0.03$), Prevotellaceae ($\beta = -0.51$, $P = 0.0003$), Ruminococcaceae ($\beta = -0.40$, $P = 0.0002$) and Streptococcaceae ($\beta = -0.58$, $P = 0.0002$). In contrast, bacteria from the

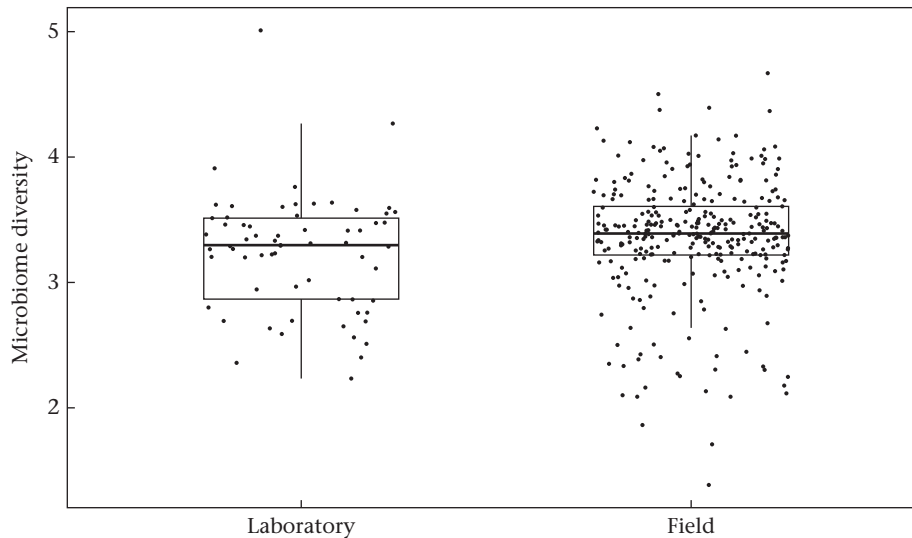


Figure 2. Oral microbiome alpha diversity (Shannon diversity) of prairie voles housed in the laboratory, where they were born, compared to after they were subsequently released into field enclosures. Each data point corresponds to a unique oral microbiome sample obtained from a vole when it was living in the laboratory ($N = 65$ samples from 47 voles) or in the field enclosure ($N = 301$ samples from 50 voles). The box plots show the median and 25th and 75th percentiles; the whiskers indicate the values within 1.5 times the interquartile range.

family *Corynebacteriaceae* increased as voles moved from the laboratory to the field ($\beta = 1.70$, $P < 0.0001$).

Social Interactions and Microbiome Diversity and Dissimilarity

Social network degree, the total number of unweighted social connections an individual vole had, was not significantly related to oral microbiome alpha diversity (effect of degree: $b = 0.00715$, $t = 0.0689$, $P = 0.494$, P values from randomization tests = 0.838, 0.963, 0.948; Fig. 5).

Social network association index, the strength of a pair's social connection, was not significantly related to the pair's oral microbiome dissimilarity (beta diversity) at any time point, whether dissimilarity was quantified in the laboratory sample (enclosure 1 mrqap: $b = -0.224$, $P = 0.999$; Fig. 6a; enclosure 2 mrqap: $b = -0.110$, $P = 0.938$; Fig. 6d), the first field sample (enclosure 1

mrqap: $b = 0.226$, $P = 0.999$; Fig. 6b; enclosure 2 mrqap: $b = 2.167$, $P = 0.449$; Fig. 6e) or the last field sample (enclosure 1 mrqap: $b = 0.0227$, $P = 1$; Fig. 6c; enclosure 2 mrqap: $b = 2.729$, $P = 0.443$; Fig. 6f).

Mating Patterns, Genetic Relatedness and Microbiome Dissimilarity

Oral microbiome dissimilarity (Bray–Curtis dissimilarity) was not related to whether the pair of voles produced offspring together ($b = 0.0118$, $t = 0.276$, $P = 0.783$; Fig. 7). Whether the laboratory or the last field sample was used was also not significant ($b = -0.0294$, $t = -0.564$, $P = 0.573$; Fig. 7). The interaction between whether or not the pair produced offspring together and whether the laboratory or the last field sample was used was also not significant ($b = -0.0205$, $t = -0.372$, $P = 0.710$). Therefore, voles that had offspring together did not have significantly more

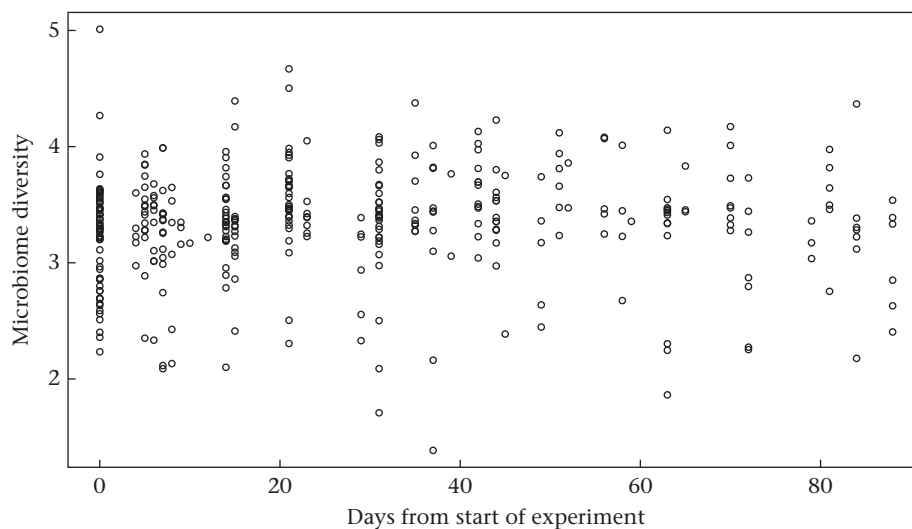


Figure 3. Oral microbiome alpha diversity (Shannon diversity) of prairie voles according to the number of days they had been living in the field enclosures. Data points correspond to the data of oral microbiome samples ($N = 367$ samples from 50 voles) obtained from when the voles were first released into the enclosures (0 days) until the end of the field experiment approximately 3 months later.

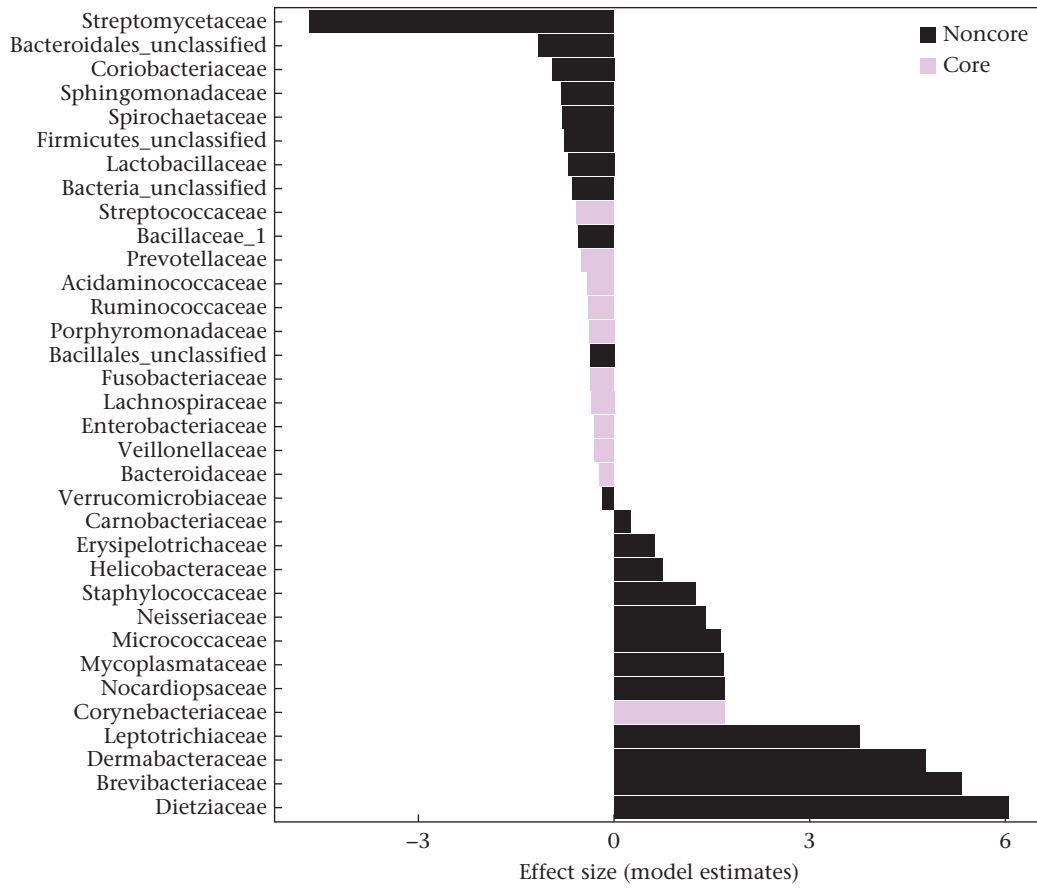


Figure 4. Bar plots reflect results from negative binomial linear mixed-effects models testing the effects of site of sample collection (laboratory or field) on the relative abundance of bacterial families. Families depicted here were significantly associated with the site of sample collection (FDR-adjusted $P > 0.05$); magnitudes of the effects are represented as model estimates on the x axis. Positive effects reflect an increase in relative abundance in laboratory samples compared to field samples; negative effects reflect a decrease in relative abundance. Bacterial families that make up the core vole oral microbiome are coloured pink.

similar microbiomes, and the similarity of their microbiomes did not change whether we compared samples from the laboratory (as a control) or their last samples in the field.

Oral microbiome dissimilarity changed more over the course of the field season for sibling pairs than nonsibling pairs, indicated by the significant interaction between whether the pair were siblings or not and whether the samples were taken in the laboratory or at the end of the field season (laboratory versus last field sample * sibling or not: $b = 0.0724$, $t = 2.263$, $P = 0.024$; Fig. 8). This indicated that the oral microbiome of sibling pairs (which were initially housed together in the laboratory before being released into the field) became more dissimilar from the laboratory to the field while nonsibling pairs did not change significantly during this period. Neither the main effects of sibling or nonsibling ($b = -0.0311$, $t = -1.302$, $P = 0.193$) nor whether we compared laboratory samples or the last field samples ($b = -0.00610$, $t = -0.761$, $P = 0.447$) were significant.

DISCUSSION

Our study investigated the links between social behaviour and the oral microbiome in prairie voles. We first described the composition of the prairie vole oral microbiome and found that it had a higher alpha diversity in the experimental field enclosures than in the laboratory, although there was no consistent change over time. We found no significant relationship between the alpha diversity of the oral microbiome and the number of social connections of that vole. A pair's oral microbiome similarity did not

have any significant relationship to the strength of that pair's social connection nor whether that pair produced offspring together. However, the oral microbiome became more dissimilar after the transfer to the field enclosures in pairs of siblings while the dissimilarity did not change in nonsibling pairs.

Since most studies of the microbiome have examined the gut microbiome, we compared the composition of the oral microbiome in our study to the prairie vole gut microbiome (from faecal samples) as described by Curtis et al. (2018) to see how they compare, although note that Curtis et al. (2018) only used female prairie voles and we used both females and males. Firmicutes was the most common phylum in both the oral and gut microbiome. The abundance of bacteria from the phyla Proteobacteria (a group that includes pathogens and species associated with dysbiosis but that is extremely diverse and can be found in normally functioning mammals, potentially maintaining an anaerobic environment; Moon et al., 2018) and Actinobacteria was lower in the gut microbiome than the oral microbiome. In contrast, bacteria from the phylum Bacteroidetes, a group that often specializes in breaking down different carbohydrates and that is commonly found in the microbiomes of mammals, including other rodents, rabbits and humans (Curtis et al., 2018), was more abundant in the gut microbiome than the oral microbiome. Fusobacteria were present in the oral microbiome but not the gut microbiome, consistent with studies that found that some Fusobacteria species are important in the development and persistence of periodontal bacterial communities (Hendrickson et al., 2014). Saccharibacteria was third most abundant in the gut microbiome (although only at 5% of total

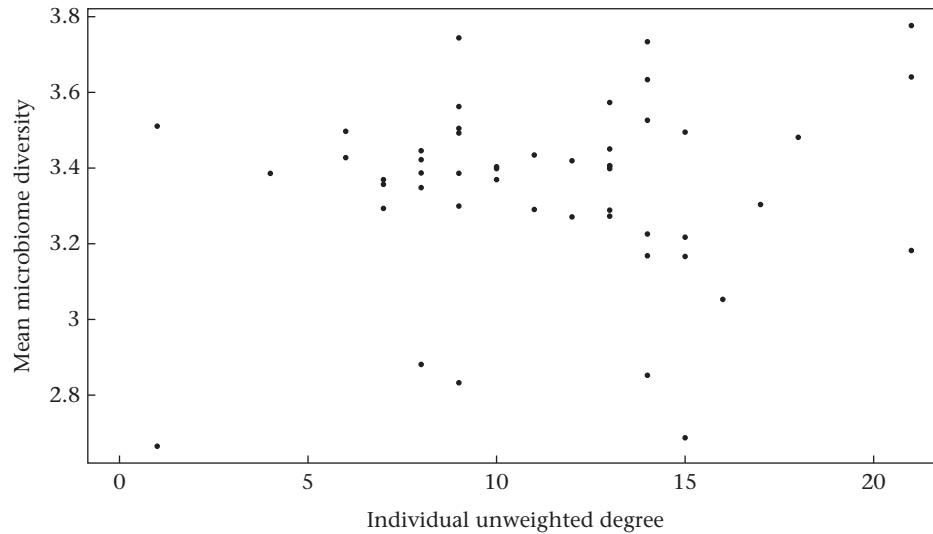


Figure 5. Association between mean oral microbiome alpha diversity (Shannon diversity) and the frequency of social interactions (quantified as the unweighted degree from social network analyses) in free-living prairie voles in the field enclosures. Each data point represents the average oral microbiome diversity for all samples obtained from each vole while it was in the field enclosure ($N = 50$ voles).

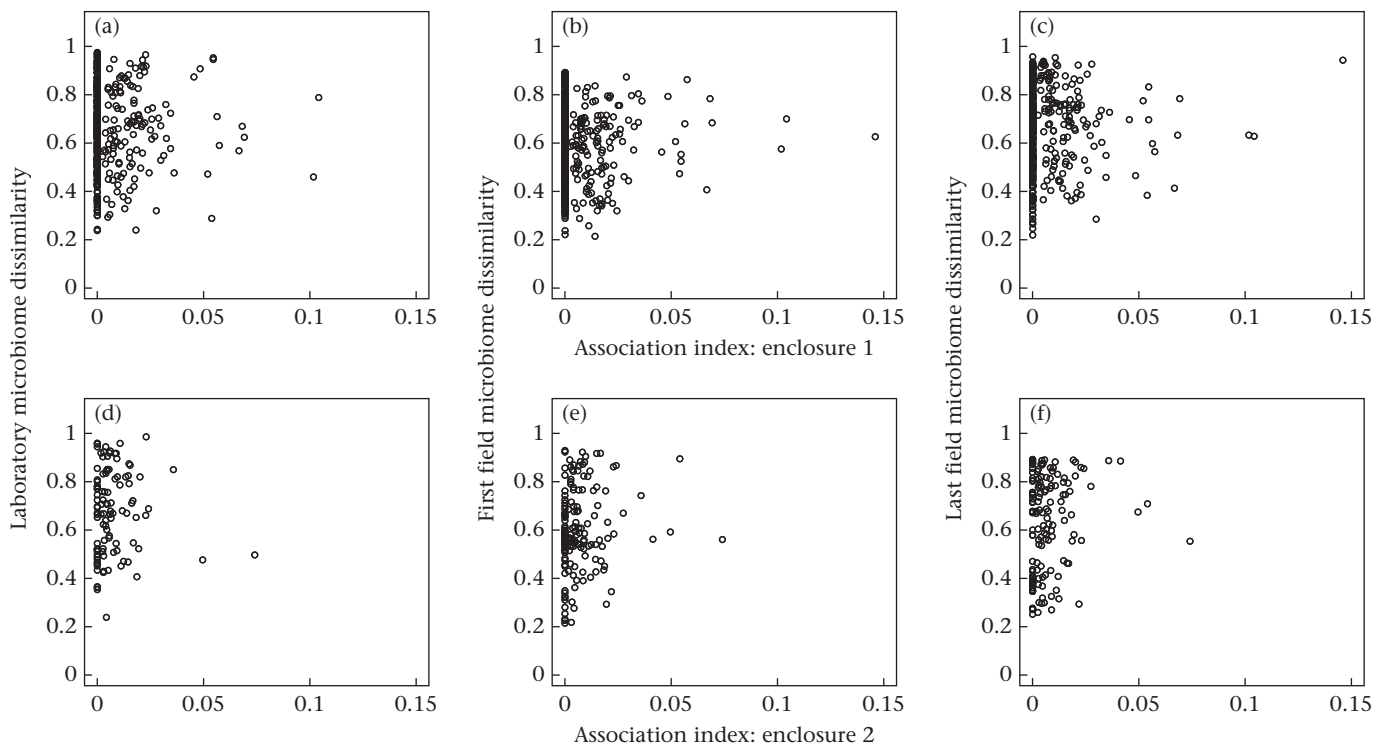


Figure 6. Association between the strength of each vole pair's social connection (quantified using the social network association index) and oral microbiome dissimilarity (Bray-Curtis) in prairie voles (a, d) when they were initially in the laboratory, (b, e) the first sample collected once when they were released into the field enclosures and (c, f) at the end of the field experiment. Data points represent the oral microbiome diversity of voles at different time points in two different field enclosures (enclosures 1 and 2).

composition) but was absent from the oral microbiome; the function of *Saccharibacteria* in voles is currently unknown (Curtis et al., 2018), so the impact of this difference is unclear. Note that because we used corn as bait for the live traps, the oral microbiome composition we measured may not completely reflect that of a wild vole or of a vole kept in the laboratory. Corn is a low-quality food and the voles would have needed to eat wild vegetation as well, so while no vole would have exclusively eaten corn, it was likely to be present in their diets and this would affect their oral microbiome.

The oral microbiome of voles was more diverse when the voles were in the field enclosures than when they were in the laboratory, but there was no significant change in diversity as the time spent in the enclosures increased. Thirty-four bacterial families changed significantly in abundance from the laboratory to the field enclosures, with 11 of these being core bacterial families. This increase in oral microbiome diversity from the laboratory to the field is likely due to a combination of factors. Voles went from living only with their siblings in the laboratory to being able to freely choose with

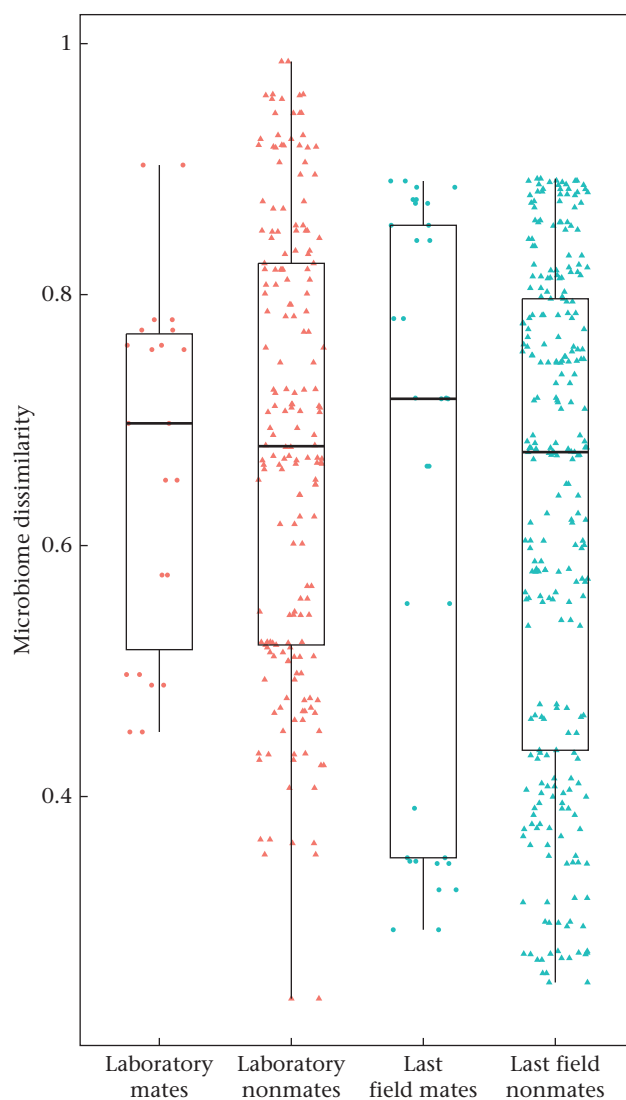


Figure 7. Oral microbiome dissimilarity (Bray–Curtis) of prairie voles in relation to whether or not the pair produced offspring together and whether the sample was obtained in the laboratory (before release into the enclosures) or was the last sample obtained in the field ('Last Field'). Voles were classified as 'mates' (circles) if they successfully produced offspring with one another whereas those opposite-sex dyads not producing offspring together were considered 'nonmates' (triangles). This was only tested for individuals in enclosure 2 due to the low number of pairs that produced offspring in enclosure 1. The box plots show the median and 25th and 75th percentiles; the whiskers indicate the values within 1.5 times the interquartile range.

which individuals they interacted (23 or 47 other voles depending on the enclosure). This increase in the number of social contacts could have led to an increase in oral microbiome diversity (similar to [Raulo et al., 2021](#)); however, owing to the lack of association between diversity and the number of social connections, this change in the microbiome could be due to increased exposure to diverse microbes in the natural environment in the enclosures. Voles went from being fed rodent chow to foraging on their own for a variety of wild plants that changed over time. The oral microbiome should rapidly respond to this shift in diet as much starch and carbohydrate processing takes place in the oral cavity ([Sedghi et al., 2021](#)). Additionally, once released into the enclosures, voles likely acquired microbes from the soil through foraging or burrowing. Finally, voles engage in coprophagy ([Kenagy & Hoyt, 1980](#); [National Research Council, 1995](#)), which could be another method

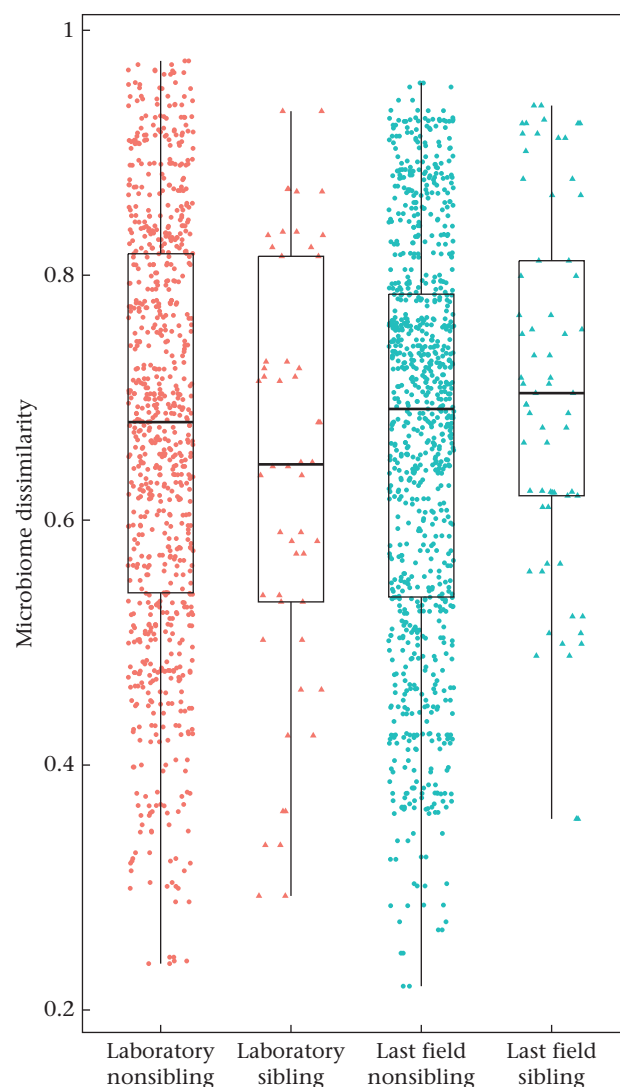


Figure 8. Oral microbiome dissimilarity (Bray–Curtis) in nonsiblings (circles) and siblings (triangles) from samples obtained in the laboratory and from the last sample obtained in the field ('Last field'). This was only tested for enclosure 1 due to the low number of sibling pairs in enclosure 2. The box plots show the median and 25th and 75th percentiles; the whiskers indicate the values within 1.5 times the interquartile range.

for voles to alter oral microbiota without directly interacting with or grooming other individuals. For example, [Bo et al. \(2020\)](#) showed that voles had lower alpha diversity and a different composition in the gut microbiome when coprophagy was prevented. We cannot distinguish among these possibilities, but our results highlight that the environment strongly influences oral microbiome composition, composition can change rapidly, and animals in more natural environments have more diverse microbiomes than when they are in the laboratory (see also [Schmidt et al., 2019](#) for similar results in the gut microbiome for wild mice).

Social network degree and association index were not associated with oral microbiome alpha diversity in free-living prairie voles, indicating a lack of association between sociality and microbiome diversity in a nongroup-living species. This result differs from a recent study on another species of nongroup-living rodent, the wood mouse. [Raulo et al. \(2021\)](#) showed that gut microbiome diversity in free-living wood mice was related to the individual's unweighted degree (number of social connections) where more social

individuals (i.e. with a higher degree) had a more diverse gut microbiome. However, other studies on a variety of species, but all on the gut microbiome, have found mixed results. Some studies showed that more social individuals had higher (Johnson 2020; Moeller et al., 2016; Perofsky et al., 2017; Singh et al., 2019) or lower (Raulo et al., 2018) gut microbiome diversity whereas in others the relationship was sex specific (Levin et al., 2016). In one of the few studies to focus on different microbiomes from the same individual (oral, nasal, ear, anal scent gland, prepuce and rectum), Rojas et al. (2020) did not find that social behaviour (here social rank) was related to alpha or beta diversity in the microbiome of spotted hyenas, *Crocuta crocuta*, from any part of the body. We note that the causal role of sociality in determining microbiome community diversity is also not clear (Davidson et al., 2020). Given that our study is one of the first to focus on this relationship in the oral microbiome, it is unclear whether this trend between social connections and oral microbiome diversity does not exist or whether it is species and context dependent as in the gut microbiome. We also acknowledge that our study has a somewhat smaller sample size than several of these other studies, which could explain why we did not find a relationship.

Contrary to our results on the oral microbiome, many studies of the gut microbiome have found a link between social association and microbiome similarity where individuals that exhibit stronger social associations have more similar gut microbiomes. In group-living primate species, group membership is related to microbiome similarity (Moeller et al., 2016; Perofsky et al., 2017; Tung et al., 2015; Wikberg et al., 2020), although it can be difficult to distinguish between the effects of close social contact and shared group environment on the microbiome. Within social groups of red-bellied lemurs, *Eulemur rubriventer*, individuals that spent more time in social contact and proximity had more similar microbiomes (Amato et al., 2017). Ambrosini et al. (2019) found that cloacal microbiomes in barn swallow, *Hirundo rustica*, mating pairs were more similar than expected by chance. Additionally, Wikberg et al. (2020) found that the gut microbiome of colobus monkeys, *Colobus vellerosus*, was more similar between individuals of different groups that had higher social connectedness. Similarly, Raulo et al. (2021) found that social association strength was the best predictor of gut microbiome similarity among free-living wood mice.

Given that our study is one of the first to investigate this relationship in the oral microbiome, it is unclear whether this relationship exists in the oral microbiome or is simply different in prairie voles compared to other species. Our association index does not distinguish between positive and negative interactions occurring among voles, only spatiotemporal relationships. Therefore, some voles may have a higher social association, but this relationship could primarily be territorial/aggressive in nature rather than affiliative. This could potentially influence the similarity of oral microbiomes if, for instance, frequent but negative interactions result in less similar microbiomes while frequent positive interactions, such as allogrooming, result in more similar microbiomes. However, we did find that our association indices were related to other measures of preference such as partner–pair preference tests (Sabol et al., 2018). Future studies in prairie voles using similar methods but measuring the gut microbiome will be crucial to understanding how the impacts of sociality differ in different parts of the microbiome.

Previous studies showed that relatedness can have some impact on gut microbiome composition (Goodrich et al., 2016; Grieneisen et al., 2021; McLean et al., 2019). We found that the vole oral microbiome became more dissimilar over time in sibling pairs than nonsibling pairs. Note that siblings would have been in the same cage in the laboratory and provided with the same diet. When voles were released into the seminatural field enclosures, the

microbiomes of siblings became more dissimilar, while the microbiomes of nonsiblings did not change significantly, suggesting there is a stronger impact of environment or behaviour than relatedness. Any similarities in relatedness would have been equally as strong in the field and laboratory, while the shared environment, diet and behavioural interactions from the laboratory were reduced or eliminated in the field. Given that relatedness did not change over the course of the experiment, but siblings went from living together in a smaller, identical environment in the laboratory to a larger, more heterogeneous environment in the enclosures, the increase in sibling oral microbiome dissimilarity when voles were released into the enclosures suggests that sharing the same environment or continuous close contact may be more important than relatedness.

Our results support those from previous studies that the oral microbiome is sensitive to the environment even among highly related individuals. Curtis et al. (2018) found that sibling pairs of female prairie voles had more similar faecal microbiomes than nonsibling pairs; however, same-sex sibling pairs were housed together, so it is difficult to separate environment from relatedness. Ambrosini et al. (2019) found that sibling barn swallow nestlings had more similar cloacal microbiomes than nonsiblings, although they did not distinguish between the effects of relatedness and shared environment. Teyssier et al. (2018) did distinguish between these impacts through a cross-fostering experiment on great tits, *Parus major*, and found that the effect of shared environment was more important than relatedness on the composition of nestling cloacal microbiomes, as full siblings raised in separate nests had more different microbiome compositions while cross-fostered siblings raised in the same nest had more similar microbiome compositions. The oral microbiome in humans has a genetic component based on relatedness, but the inherited oral microbiota often decrease in abundance with age and due to exposure to different environments over time (Gomez et al., 2017). In addition, the environment also has an impact on the oral microbiome composition (Gomez et al., 2017). Mukherjee et al. (2021) and Shaw et al. (2021) found that shared environment was more important than genetic relatedness for acquiring oral microbes in humans and that the environment was more important than relatedness in determining the human salivary microbiome. Finally, Raulo et al. (2021) found no strong influence of relatedness on the gut microbiome of free-living wood mice when they determined the full pedigree for all individuals in their study. Overall, our results support the idea that even if there is a genetic component to the oral microbiome, it seems to be lost over time or the environment is more important than relatedness.

Most previous studies on social behaviour and the microbiome were conducted on the gut microbiome, so while our results on the oral microbiome provide a nice comparison, comparing them to studies using the gut microbiome may have some limitations given the potential differences between the gut and oral microbiomes. For instance, Kropáčková et al. (2017) found that the oral microbiome of free-living great tits was more diverse than the faecal microbiome, perhaps because only some of the oral microbiome made it to the gut and were then passed through to the faeces, but the oral microbiome varied less between individuals than the faecal microbiome potentially due to direct transfer of oral microbes through preening or allofeeding. Some studies showed that the oral microbiome was more diverse than the rectal microbiome (Bik et al., 2016) whereas others showed that it was less diverse than the lower gastrointestinal tract (Costello et al., 2009; Suzuki & Nachman, 2016). Kropáčková et al. (2017) also found a low correlation between the composition of the oral and faecal microbiomes within great tit individuals, although there is evidence for more overlap in the composition of these microbiomes in humans and

California sea lions, *Zalophus californianus* (Bik et al., 2006, 2016). Although we did not compare microbiomes between different body parts, it is important to consider the above complexities when comparing our results for the associations between the oral microbiome and social behaviour with those of previous studies on the association between the gut microbiome and social behaviour discussed above.

Overall, we did not find a relationship between social behaviour and the oral microbiome at the community level of alpha and beta diversity in prairie voles living under seminatural field conditions. We did find that voles in the seminatural field enclosures had higher oral microbiome alpha diversity than when housed in the laboratory, suggesting the importance of environmental factors like diet on the diversity of the oral microbiome. Given that the majority of other studies have focused on the links between social behaviour and the gut microbiome or examined these relationships in group-living primates, it is important to expand the investigation of the relationship between the microbiome and social behaviour to study the potential benefits or consequences of social behaviour on other parts of the microbiome or in other species, as the gut microbiome is not the only component of the microbiome that is important to an organism's health and survival.

Author Contributions

Anne C. Sabol: Conceptualization, Data collection, Social network statistical analysis, Writing—original draft, Writing—review & editing. **William L. Close:** Laboratory processing of microbiome samples, Microbiome data processing, Writing—original draft, Writing—review & editing. **Lauren Petruccio:** Statistical analysis of exploratory microbiome results, Writing—original draft, Writing—review & editing. **Connor T. Lambert:** Parentage analyses and Writing—review & editing. **Brian Keane:** Supervision, Writing—review & editing. **Nancy G. Solomon:** Supervision, Writing—review & editing. **Patrick D. Schloss:** Supervision, Funding acquisition, Writing—review & editing. **Ben Dantzer:** Conceptualization, Funding acquisition, Supervision, Writing—original draft, Writing—review & editing.

Data Availability

Data are available on figshare: https://figshare.com/projects/Sociality_does_not_predict_oral_microbiome_composition_or_diversity_in_free-living_prairie_voles/152637.

Declaration of Interest

None.

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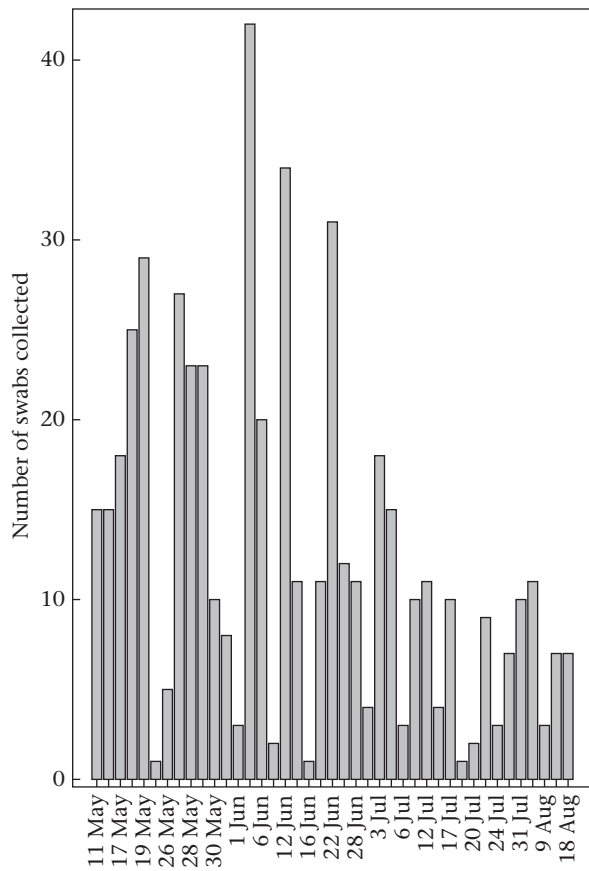
Appendix

Figure A1. The number of oral swab samples collected on each day throughout the study.