Recovered microbiome of an oviparous lizard differs across gut and reproductive tissues, cloacal swabs, and feces

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Abstract

Microbial diversity and community function are related, and can be highly specialized in different gut regions. The cloacal microbiome of *Sceloporus virgatus* females provides antifungal protection to eggshells, a specialized function that suggests a specialized microbiome. Here, we describe the cloacal, intestinal, and oviductal microbiome from *S. virgatus* gravid females, adding to growing evidence of microbiome localization in reptiles and other taxa. We further assessed whether common methods for sampling gastrointestinal (GI) microbes – cloacal swabs and feces – provide accurate representations of these microbial communities. We found that different regions of the gut had unique microbial communities. The cloacal microbiome showed extreme specialization averaging 99% Proteobacteria (Phylum) and 83% *Enterobacteriacaea* (Family). *Enterobacteriacaea* decreased up the GI and reproductive tracts. Cloacal swabs recovered communities similar to that of lower intestine and cloacal tissues. In contrast, fecal samples had much higher diversity and a distinct composition (common Phyla: 62% Firmicutes, 18% Bacteroidetes, 10% Proteobacteria; common Families: 39% *Lachnospiraceae*, 11% *Ruminococcaceae*, 11% *Bacteroidaceae*) relative to all gut regions. The common families in fecal samples made up < 1% of cloacal tissue samples, increasing to 43% at the upper intestine. Similarly, the common families in gut tissue (*Enterobacteriaceae* and *Helicobacteraceae*) made up < 1% of the fecal microbiome. Further, we found that cloacal swabs taken shortly after defecation may be contaminated

with fecal matter. Our results serve as a caution against using feces as a proxy for GI microbes, and may help explain high between-sample variation seen in some studies using cloacal swabs.

Keywords: Microbiome specialization, gut tissue, intestine, feces, cloaca, reptiles, *Sceloporus*

Introduction

As high-throughput sequencing becomes more accessible, microbiome studies are expanding beyond humans and mammalian model systems to non-model organisms. It has become clear that the community of microbes within the gut of all animals plays an important role not only in host physiology but also their ecology and evolution (Archie & Theis, 2011; McFall-Ngai et al., 2013; Moran et al., 2019; Reese & Dunn, 2018). To better understand the wide-ranging implications of host-microbe coevolution and the variety of functions the gut microbiome can provide for the host, it is critical to study a broad diversity of host species with a broad diversity of environmental pressures and adaptations. Conducting such research on wild populations is particularly important because captivity has been shown to change the composition of gut microbiomes (Hird, 2017; Keenan et al., 2013; Kohl et al., 2017).

The function and the composition of the gut microbiome can be highly specialized along different regions of the gastrointestinal (GI) tract, which vary in acidity, specific macromolecules secreted by gut epithelia, oxygen content, and other conditions that affect which microbes can thrive (Beasley et al., 2015; Reese & Dunn, 2018). Regional localization of the gut microbiome is well established in both model and non-model systems (Beasley et al., 2015; Colston et al., 2015; Kohl et al., 2017; Shterzer et al., 2020; Videvall et al., 2018; Yasuda et al., 2015; Zhang et al., 2021). Among oviparous vertebrates, the terminus of the GI tract is shared with the reproductive tract, as the cloaca is the site of defecation, copulatory intromission, and oviposition. Thus, the cloacal microbiome of birds and reptiles likely experiences unique selective pressures relative to the rectal microbiome of mammals. For instance, the cloacal microbiome may be affected by breeding season, sex, degree of promiscuity, and other aspects of host reproduction and behavior (Escallón et al., 2019; Lee, 2015; White et al., 2011).

In the striped plateau lizard, *Sceloporus virgatus*, females lay their eggs in soil burrows at the onset of the summer monsoon rains and then leave the nest site, providing no further parental care (Rose, 1981). Synchronizing oviposition with the monsoon season appears to be unique to *S. virgatus* among temperate lizard species, and has been hypothesized to have a strong selective impact on the species (Vinegar, 1975); this timing may reduce egg mortality by desiccation yet potentially expose eggs to new

fungal pathogens. Thus, we propose a strong selective impact on the species' microbiome as well. Indeed, microbes are transferred from the cloacae of *S. virgatus* females to eggshells during egg laying, reduce fungal attachment to eggs, and improve hatch success (Bunker et al., 2021). Given this specialized ecological function of the cloacal microbiome, we hypothesized that the gut microbiome of *S. virgatus* females will express a high degree of localization along the GI and reproductive tracts. While such localization has been found in several other reptile species (Colston et al., 2015; Costello et al., 2010; Keenan et al., 2013; Kohl et al., 2017), we predicted a pattern in contrast to that found in these other reptiles, which generally show an increase in diversity and abundance of bacteria moving down the GI tract to the cloaca. Specifically, we predicted that the cloaca of gravid *S. virgatus* females will show reduced microbial diversity, with a greater relative abundance of microbes with antifungal capabilities, and a unique community structure relative to upper regions. To test this, we compared the microbiome of gravid females across the cloaca, lower and upper intestinal regions, and the oviduct, described in Study 1.

It is common among studies of reptiles and birds for cloacal swabs and/or fecal samples to be used as a proxy for the gut microbiome as a whole (Colston et al., 2015; Escallón et al., 2019; Hong et al., 2011; Jiang et al., 2017; Kohl et al., 2017; Kreisinger et al., 2015; Zhang et al., 2021). These methods are attractive as they are non-destructive, minimally invasive, and allow for repeat sampling of the same individuals and communities over time (Berlow et al., 2020; Videvall et al., 2018). However, a microbial community recovered from swabs or feces may represent only a portion of the gut due to the regional localization. While only a few studies have directly compared the two non-invasive sampling approaches, unique community structures have been recovered by cloacal swab and fecal sampling in zebra finches and ostriches (Berlow et al., 2020; Videvall et al., 2018). Here, we add to this important work by directly comparing microbial communities collected from cloacal swab and fecal samples of *S. virgatus* individuals. We predict that the communities will be distinct in *S. virgatus*, indicating selection for the particular cohort of microbes that occupy the cloaca, due in part to the natural history of *S. virgatus* (as detailed above). To test this idea, in Study 2 we compared microbial communities recovered from cloacal

swabs collected before and after defecation, and we also described the fecal microbiome itself. Additionally, because previous work on the *S. virgatus* microbiome has been based on cloacal swab samples (Bunker et al., 2021; Martin et al., 2010), we assessed whether the microbiome recovered from cloacal swabs was indeed representative of cloacal tissue or other regions of the GI and reproductive tracts.

Methods

Study 1: Is the cloaca a uniquely specialized region of the gastrointestinal and reproductive tracts?

Sceloporus virgatus lizards are small insectivorous animals found in the Madrean Sky Islands of the southwestern United States and south into Mexico (Stebbins, 2003). Our study population is at the northern reach of their range, in Cochise County, Arizona near the American Museum of Natural History's Southwestern Research Station (SWRS). To address the adaptive hypothesis that the cloacal microbiome will be unique from that of the upper GI and reproductive tracts, we collected gravid *Sceloporus virgatus* females (n = 8) between 28 June and 01 July 2019 using a loop of fishing line tied to a retractable fishing pole. Female cloacae were swabbed (BD ESwab™) immediately following capture. Microbes from these field swabs were eluted into an Amies solution by manual shaking, frozen within 24 h of collection, transported on dry ice to Tacoma, WA on 02 July, and then stored at -80°C until DNA extraction. The lizards were kept in large outdoor enclosures at SWRS with access to water bowls and naturally occurring prey until 02 July when they were shipped overnight to Tacoma, WA in individual plastic containers on ice packs. Upon arrival, we euthanized females using buffered MS-222 (Conroy et al., 2009) and collected transverse sections of tissue (~2-4 mm long) from the cloaca (expanded tissue immediately above the vent), lower intestine (~10 mm above cloaca), upper intestine (~5 mm below stomach), and oviduct (at the position of the lowest egg on the left side) using heat sterilized instruments.

We did not attempt to separate or isolate mucosal vs. luminal contents. Tissue samples were frozen at -80°C until DNA extraction.

Study 2: How does sampling methodology (swab vs feces) affect recovered community composition?

To assess the efficacy of cloacal swab and fecal sampling in *S. virgatus*, we collected 8 adult lizards (n = 4 females and n = 4 males) on 30 May 2019 using the same sites and methodology described above. Animals were housed individually in sterile 15 X 23 cm plastic tanks lined with paper towels that had also been sterilized with 70% ethanol on a west-facing screened porch of SWRS's Live Animal Holding Facility. They were offered a single cricket on the day of capture that was removed if not eaten by ~1200 the next day. Sterile water was provided *ad libitum*, and one heat lamp on a 14:10 light cycle was shared between two adjacent tanks.

Lizard cloacae were swabbed first at ~0500 on 31 May, before animals had woken up and defecated; this is considered our pre-defecation cloacal swab sample. Beginning at 0600, the tanks were checked for feces every 30 min, until 1830, when the heat lamps had been off for 30 min and the lizards were no longer active. When a fecal pellet was found, it was collected with sterile forceps and the lizard's cloaca was immediately swabbed. Based on this method, all post-defecation cloacal swabs were taken within 30 min of the defecation event. We also collected a control swab, which we used to sample the researchers' hands, a lizard's external vent and belly, and the air of the porch, to collect any microbes that may have contaminated cloacal swabs during sampling.

DNA Extraction

DNA from swab, tissue, and fecal samples were extracted using the Qiagen DNEasy® Blood and Tissue Kit (Qiagen, Inc). For the cloacal swab samples, we used the manufacturer protocol for

Purification of Total DNA from Animal Blood or Cells with the optional lysis buffer incubation for Gram-positive bacteria. For tissue and fecal pellets, we included the optional lysis buffer incubation for 30 min at 37°C. After incubation, Buffer AL and proteinase K were added to the tubes. The samples were then incubated at 56°C for 180 minutes (for tissue) or 90 min (for feces) while shaking at 500 RPM (USA Scientific Mixer HC- 8012-0000, Ocala, FL, USA). From here, the extraction was completed according to the Purification of Total DNA from Animal Blood or Cells protocol, beginning at Step 3 (addition of pure ethanol). An extraction blank was included in each extraction. DNA in all samples and blanks was quantified via Qubit (Invitrogen, Waltham, MA) to ensure extraction was successful.

Illumina Library Prep

We used a two-step polymerase chain reaction (PCR) procedure modified from the IBest Genomics Core at University of Idaho to amplify the 16s V4 region of the microbial DNA in each sample (Taylor et al., 2019). PCR1 utilized 515F/806R primer pairs to amplify the region of interest, and PCR2 extended the amplicons with sample-specific barcodes. For PCR1, we used 7.5 uL Phusion Flash High Fidelity Master Mix (ThermoFisher, Waltham, MA), 0.15 uL of the forward and reverse 50 µM primers, 0.75 uL molecular grade Bovine Serum Albumen (BSA, 20 mg/mL, New England BioLabs), 5.45 uL purified water, and 1 uL template DNA. The thermal cycling protocol was: initial denaturation at 98°C for 10 sec; 28 cycles of denaturation at 98°C for 1 sec, annealing at 57°C for 5 sec, and extension at 72°C for 20 sec, with a final extension at 72°C for 60 sec (Taylor et al., 2019). We visualized PCR products on a 2% agarose gel using 10x Sybr Green (Molecular Probes, Invitrogen; Carlsbad, CA). All iterations of PCR1 included a positive (*Serratia* genomic DNA) and a negative (purified water) control. Mock communities (BEI Resources, ATCC, Manassas, VA) were also included in some PCR1 runs, as a further measure of quality control.

We performed PCR1 in triplicate for each sample, then pooled those replicates for PCR2. Samples (including positive controls) that showed strong bands in at least two of three PCR1 replicates

were diluted 1:4 in purified water before being used as template DNA in PCR2, and all others were undiluted. In addition to pooling replicates of the negative controls, negative controls from different PCR1 runs were further pooled to reduce the number of control samples sent for sequencing. Because of this, some iterations of PCR2 contained a pooled PCR1 negative control, and some contained a new PCR2 negative control with purified water instead of DNA template.

For PCR2, we used 10 uL Phusion Flash High Fidelity Master Mix, 0.24 uL BSA, 8.01 uL of purified water, 0.75 uL of unique barcoded primer pairs (2 μM; supplied by IBest Genomics Core, at the University of Idaho; see Taylor et al., 2019), and 1 uL of the PCR1 pooled product. The thermal cycling protocol was the same as the protocol for PCR1, but was run for only 8 cycles (for 36 cycles total). We visualized PCR2 products on a 2% agarose gel with 10x Sybr Green and confirmed that each sample had undergone a band shift compared to PCR1 (indicative of attachment of barcode primers). We then pooled all samples, with pool volume based on the intensity of each sample band, and shipped them to IBest Genomic Core for purification, DNA quantification, and sequencing on the Illumina MiSeq platform. All negative controls and mock communities were included in the sequencing process, but positive controls were not.

Illumina Raw Data Processing and Statistical Analysis

Sequences were received demultiplexed, with adapters and primers removed. Quality analysis for each sample was performed using FastQC (Andrews, 2010) and those results were consolidated using MulitQC (Ewels et al., 2016). Mean quality scores and length distribution for the whole dataset were manually inspected and used to determine a cutoff length of 250 bp for forward reads and 150 bp for reverse reads. Samples were then processed in R v3.1.6 (R Core Team, 2020) using the DADA2 (Callahan et al., 2016) pipeline based on this tutorial: https://benjjneb.github.io/dada2/tutorial.html. Samples were trimmed as described above and filtered with a max expected error of 2. An average of 91.6% of reads were kept in all experimental samples after processing. Taxonomic classification of

amplified sequence variants (ASVs) was performed through the assignTaxonomy function, using the Silva database (Quast et al., 2013), release 132. Potential contaminants were removed with the Decontam package (Davis et al., 2018), using the "prevalence" method with a threshold of 0.1. Control samples, including a control swab (n = 1), extraction blanks (n = 9), and PCR negatives (n = 24), were used for comparison. Any ASV that had fewer than 27 reads across all samples was discarded. One cloacal tissue sample only kept 12 reads after processing and was not included in analyses. Finally, read numbers were log transformed to account for differences in read depth. All parameters were determined based on an analysis of the mock communities.

Once samples had been processed, the phyloseq package (McMurdie & Holmes, 2013) was used to organize and store data of different types for analyses. Shannon diversity index values and richness were then calculated with the "estimate_richness_ function" from phyloseq, and Faith's phylogenetic diversity index values were calculated using the Picante package (Kembel et al., 2010) based on phylogenetic trees created and optimized with Phangorn (Callahan et al., 2016; Schliep et al., 2016). The alignment was created with the DECIPHER package (Wright, 2016). We examined how these variables differed across tissue types and swab samples using mixed-effects ANOVAs with lizard ID as a random effect. We compared fecal and pre-defecation cloacal swab communities, as well as pre- and post-defecation cloacal swab communities using separate paired t-tests. Richness was log-transformed to account for non-normal distribution when comparing pre- and post-defecation swabs. Phylogenetic diversity was log transformed when comparing tissue types.

Pairwise distances between samples were calculated by the vegan package (Oksanen et al., 2019) using Bray-Curtis distances, and these distances were then used to generate non-metric multidimensional scaling (NMDS) plots. Using the trees described above, we used phyloseq to generate weighted UniFrac distances to compare phylogenetic community composition, and we used unweighted UniFrac distance to compare community membership of groups; both metrics were used to generate Principle Coordinate Analysis (PCoA) plots. For all distance metrics, dispersion between groups was first tested with the betadisper function from the vegan package, and then a PERMANOVA test (Adonis function from the

vegan package) was performed. Differential abundance was tested using the Corncob package (Martin et al., 2020). All plots were made with the GGplot2 package (Wickham, 2017).

Results

Study 1: Is the cloaca a uniquely specialized region of the gastrointestinal and reproductive tracts?

The Shannon diversity value was lowest in the cloacal tissue, on average, but was not statistically different across any tissue types or cloacal swabs ($F = 1.32$, $df = 4,26$, $p = 0.425$, Fig 1a). Similar non-significant patterns were found when comparing richness ($F = 2.07$, $df = 4,26$, $p = 0.114$; Fig 1b) and phylogenetic diversity across tissue types ($F = 1.30$, $df = 4,26$, $p = 0.297$; Fig 1c). When comparing beta diversity, measured by Bray-Curtis distance, there was a significant difference in composition between all groups ($F = 1.52$, $df = 4,33$, $p = 0.014$), although the groups were dispersed similarly ($F = 1.04$, $df = 4,33$, $p = 0.400$, Fig 2a). When clustered using weighted UniFrac distances, community composition tended to differ across all tissue types ($F = 1.87$, $df = 4,33$, $p = 0.060$, Fig 2b), although the groups were dispersed differently ($F = 4.12$, $df = 4,33$, $p = 0.008$), which could account for the difference in distances. When clustered using unweighted UniFrac distances, community membership differed significantly between tissue types ($F = 1.66$, $df = 4,33$, $p = 0.008$, Fig 2c), and the tissue types showed similar dispersion ($F = 1.16$, $df = 4,33$, $p = 0.346$).

The most abundant family in all tissue types was *Enterobacteriaceae* (Figure 3; supplemental information). In the cloacal tissue, on average, *Enterobacteriaceae* made up 82.7 ± 10.8% (SE) of the whole community, while the next most abundant family (*Helicobacteraceae*) only made up 16.6 ± 10.9% and was > 0.1% in only 2 individuals. No other families made up more than 1% of the composition of the cloacal tissue. The lower intestine showed similar patterns: *Enterobacteriaceae* made up 64.7 ± 11.5% and the next most abundant family was *Helicobacteraceae.* However, the lower intestine had several families that made up between 1-5% of the community, including *Bacteroidaceae*, *Ruminococcaceae*,

Tannerellaceae, *Lachnospiraceae*, and *Desulfovibrionaceae*. The upper intestine had the lowest percent composition of *Enterobacteriaceae* with only 34.9 ± 9.1%, closely followed by *Ruminococcaceae* which made up 32.9 ± 12.8% on average, and both *Bacteroidaceae* and *Burkholderiaceae* made up ~9% (± 7.5, ± 9.0). The oviduct community was 54.9 ± 9.3% *Enterobacteriaceae* on average and had many families that were between 1-5%, but no others higher than 10%.

A differential abundance analysis showed that the percentage of *Enterobacteriaceae* found in the cloacal tissue microbial community was similar to that in lower intestine ($t = 0.156$, $p = 0.372$) communities, and was significantly greater than that in the upper intestine ($t = -3.09$, $p = 0.005$) and oviduct ($t = -2.06$, $p = 0.049$) communities (supplemental information). It also showed that the cloacal tissue community had distinctly low percentages of *Ruminococcaceae* (lower intestine: $t = 4.38$, $p < 0.001$; upper intestine: $t = 7.94$, $p << 0.001$; oviduct: $t = 2.49$, $p = 0.019$) and *Bacteroidaceae* (lower intestine: $t = 4.88$, $p < 0.001$; upper intestine: $t = 5.07$, $p << 0.001$; oviduct: $t = 3.59$, $p = 0.001$).

Study 2: How does sampling methodology (swab vs feces) affect recovered community composition?

Shannon diversity index values were significantly higher in fecal pellets than in pre-defecation cloacal swabs ($t = 16.29$, $df = 7$, $p < 0.001$; Fig 4a), as was richness ($t = 8.39$, $df =,7$, $p < 0.001$; Fig 4b) and phylogenetic diversity ($F = 10.94$, $df = 7$, $p < 0.001$; Fig 4c). Beta diversity analysis using Bray-Curtis distance showed that the microbial composition of fecal pellets was significantly more similar to other fecal pellets than it was to that of the pre-defecation cloacal swabs ($F = 9.65$, $df = 1,14$, $p = 0.001$; Fig 5a). The groups were also dispersed differently ($F = 5.54$, $df = 1,14$, $p = 0.034$). Fecal pellets also differed from pre-defecation cloacal swabs in both UniFrac metrics (weighted: $F = 18.91$, $df = 1,14$, $p = 0.001$, Fig 5b; unweighted: $F = 15.13$, $df = 1,14$, $p = 0.001$, Fig 5c), and dispersion of the communities also varied for each measurement (weighted: $F = 13.83$, $df = 1,14$, $p = 0.002$; unweighted: $F = 4.78$, $df = 1,14$, $p = 0.046$).

The most abundant taxa recovered from fecal pellets was *Lachnospiraceae,* which made up 38.7 ± 5.2% on average (Fig 6). *Enterobacteriaceae, Bacteroidaceae,* and *Ruminococcacea* each accounted for ~10% of the recovered communities, and several others made up between 1-5% of the recovered community including *Tannerellaceae, Erysipelotrichaceae, Eggerthellaceae, Marinifilaceae,* and *Akkermansiaceae.* Pre-defecation cloacal swab communities were dominated by *Enterobacteriaceae* (84.9 ± 5.3%), and the next most abundant taxa was *Helicobacteraceae,* which only accounted for 5.1 ± 2.2% of the community. A corncob analysis confirmed that *Lachnospiraceae* was significantly more abundant ($t = -7.20$, $p < 0.001$) and *Enterobacteriaceae* was significantly less abundant ($t = 3.83$, $p < 0.001$) in fecal samples compared to pre-defecation cloacal swabs.

There was no significant difference between pre- and post-defecation cloacal swabs in any alpha diversity metric (Shannon: $t = 1.40$, $df = 7$, $p = 0.204$; Richness: $t = 1.45$, $df = 7$, $p = 0.190$; Phylogenetic diversity: $t = 1.72$, $df = 7$, $p = 0.129$; Fig 4), although the post-defecation swabs tended to have higher diversity. Swab types did not cluster separately based on Bray-Curtis distances ($F = 1.24$, $df = 1,14$, $p = 0.231$, Fig 5a), and the groups were dispersed similarly ($F = 2.57$, $df = 1,14$, $p = 0.131$). Pre-defecation swab communities differed from post-defecation swab communities in dispersion based on weighted ($F = 7.08$, $df = 1,14$, $p = 0.019$, Fig 5b) and unweighted ($F = 36.14$, $df = 1,14$, $p < 0.001$, Fig 5c) UniFrac distances, as well as composition of unweighted UniFrac distance ($F = 2.20$, $df = 1,14$, $p = 0.035$), although this latter result could be due to differences in dispersion rather than true clustering of groups. The different swab types did not cluster separately based on weighted UniFrac distance ($F = 1.14$, $df = 1,14$, $p = 0.281$). While the same two taxa dominated the pre- and post-defecation swabs, post-defecation *Enterobacteriaceae* was reduced to only 44.8 ± 13.2%, and *Helicobacteraceae* increased to 19.0 ± 9.5%. There was also an increase in the abundance of feces-associated taxa following defecation, particularly *Lachnospiraceae* which accounted for 15 ± 7.4% on average. However, there was a distinct bifurcation in the post-defecation cloacal swab samples. In five samples, *Enterobacteriaceae* and *Helicobacteraceae* account for >75% of the community, similar to the pre-defecation swabs, while the remaining three

samples had a more “feces-like” community, with a higher abundance of *Lachnospiraceae* and *Bacteroidaceae*.

How well does each sampling method represent the gut microbiome?

Cloacal swabs recovered similar communities to that of the cloacal tissue and lower intestine: *Enterobacteriaceae* made up 69.8% ± 14.2, with *Helicobacteraceae, Ruminococcaceae, Bacteroidaceae, Lachnospiraceae* and *Tannerellaceae* all making up between 2-10% on average. A differential abundance analysis showed similar abundances of *Enterobacteriaceae* between cloacal swabs and cloacal tissue ($t = -1.05$ $p = 0.304$). In contrast, as described above, tissue from the oviduct and upper intestine had different major taxa and different relative abundances of *Enterobacteriaceae* relative to the cloacal swabs.

Even though fecal pellets and tissues were collected from different animals, we compare their microbial communities descriptively. The three most abundant families in fecal pellets (*Lachnospiraceae*, *Ruminococcaceae*, and *Bacteroidaceae*) combined made up an average of 60% of the fecal microbial community, 43% of the upper intestine community, and less than 1% of the cloacal community (supplemental information). The similarity between fecal and the upper intestine communities is predominantly due to the high abundance of *Ruminococcaceae* and *Bacteroidaceae* in the upper intestine. However, despite the high abundance of *Lachnospiraceae* in fecal pellets, only about 1% of the upper intestine community was made up of *Lachnospiraceae*.

At the phylum level, fecal pellet communities averaged 62% Firmicutes, 18% Bacteroidetes, and 11% Proteobacteria (Fig 7). As we sampled from the oviduct and upper intestine to the lower intestine and cloaca, the percentage of Firmicutes and Bacteroidetes steadily decreased and the percentage of Proteobacteria increased, to the point of nearly totally dominating the cloacal community at an average of 99% Proteobacteria found in cloacal tissues (Fig 7).

Discussion

We found clear localization of the microbiome in the gut and reproductive tracts of gravid *S. virgatus* females. While localization has been seen in other reptiles (Colston et al., 2015; Costello et al., 2010; Keenan et al., 2013; Kohl et al., 2017; Zhang et al., 2021), the cloaca in previously sampled reptiles was found to have relatively high diversity, in part because it contained bacterial species from all upper regions of the gut. This pattern may be expected as the cloaca is the terminus of the GI and reproductive systems, and is often assumed to be both inoculated by feces that pass through and influenced by sexual transmission during copulation (Videvall et al., 2018; Wen et al., 2021; White et al., 2011). Similarly, the reproductive tract of chickens shows increasing levels of diversity from oviducts to cloaca, which has the highest diversity (Wen et al., 2021). In contrast to these previously described patterns, the cloacal microbiome of *S. virgatus* lizards has low diversity, especially at the Family and Phylum level, at which it was dominated by *Enterobacteriaceae* and Protobacteria. It is distinct from upper intestine, oviductal, and fecal microbiomes. We propose strong selection for the specific cohort of bacteria found in the *S. virgatus* cloaca, as females deposit beneficial microbes on their eggs during oviposition that facilitate egg survival (Bunker et al., 2021). This function could be the driving force behind the winnowing of the microbiome in the cloaca, especially considering that many members of the *Enterobacteriaceae* family are known to have antifungal properties (Dhar Purkayastha et al., 2018; Gutiérrez-Román et al., 2015; Kalbe et al., 1996).

As we only have tissue from gravid females sampled immediately after shipment, it is possible that the localization of the gut microbiome is specific to the gravid condition or an artifact of stress. Although the degree of GI localization in response to these factors has not been well-examined, previous studies have found that fecal microbial complexity is reduced in females during pregnancy and gravidity (Koren et al., 2012; Trevalline et al., 2019b). However, we found similar microbial community structure recovered from swabs in Study 1 (gravid females) and Study 2 (pre-ovulation females and males) suggesting the *S. virgatus* cloacal microbiome is not highly dependent on reproductive condition (see

supplemental information). A more direct assessment of the seasonality of the cloacal microbiome is in progress.

Cloacal swabs taken from a given individual lizard resulted in communities similar to the lower intestine and the cloacal tissue, and distinct from higher regions of the gut and reproductive tract. The cloacal swab community was predominantly *Enterobacteriaceae* but, like the lower intestine, had several families that made small but noticeable contributions to the community and was dispersed slightly differently than the cloacal tissue microbiome. The upper intestine and oviduct have different microbial communities than the lower sections of the intestinal tract but also have differences in major families from one another. Recent evidence has contradicted the long-accepted idea that embryonic development is sterile (Funkhouser & Bordenstein, 2013). Microbial communities similar to gut microbes have been found in chicken oviducts (Shterzer et al., 2020), and Trevaline et al. (2019a) found that microbes may colonize bird and lizard eggs from within the oviduct before the shell develops. The unique community in the oviduct here could indicate an internal egg microbiome which is seeded during egg development; the functional significance of this microbiome should be investigated further.

In contrast to the adequate sampling of the cloacal and lower intestinal microbiome by cloacal swabs, fecal pellets contain strikingly distinct communities from all gut regions. Distinct fecal and GI tract microbiomes have also been found in mammals (Lkhagva et al., 2021; Sugden et al., 2021; Zoetendal et al., 2002). Additionally, fecal samples and pre-defecation cloacal swabs taken from the same *S. virgatus* individuals were found to have different microbial communities from one another by every metric we examined, aligning with evidence from birds that the two non-invasive techniques recover different communities (Berlow et al., 2020; Videvall et al., 2018). As described above, the microbiome of swabs was more similar to the cloaca and lower intestine, and largely dominated by *Enterobacteriaceae*. The microbiome of fecal samples was most similar to that found in the upper intestine, with a relatively high abundance of *Ruminococcaceae* and *Bacteroidaceae,* but was dominated by *Lachnospiraceae* which was largely absent from all regions of the gut, and lacked the high abundance of *Enterobacteriaceae* that

was found across the gut. Overall, these patterns caution against the common practice of using fecal samples as a proxy for the gut microbiome without first validating this approach.

While we detected some changes between pre- and post-defecation swab samples, those changes were largely non-significant, and were likely due to increased variation within the post-defecation swabs, as indicated by the samples differing more in dispersion than in composition. The variation is due to a distinct bifurcation in the communities recovered from post-defecation swabs, with most of these swabs mirroring cloacal tissue, with its high abundance of Proteobacteria, and a smaller portion displaying a more "feces-like" community. The variation seen in post-defecation cloacal swabs was also seen in the field swabs of gravid females; 5 of the 8 cloacal swab communities from Study 1 were made up of >95% *Enterobacteriaceae* and *Helicobacteraceae*, while other samples were dominated by *Lachnospiraceae* and *Bacteroidaceae*, two feces-associated taxa. Because recovered communities from feces and pre-defecation cloacal swabs were distinct, it seems unlikely that the cloaca is being inoculated with fecal microbes. Rather, a more likely explanation is that small amounts of fecal material may attach to swabs if they are taken shortly after defecation, which then masks the much less diverse cloacal community when it is sequenced. The idea of temporary contamination is further supported by the fact that the same lizards, when re-swabbed for a different study, often had comparatively lower diversity and a greater relative abundance of *Enterobacteriaceae*-associated ASVs (supplemental information). Some animals continued to vary between the feces-like and cloacal tissue phenotype when they were resampled by swabbing, likely due to repeated defecation events. Cloacal swabs often have been found to be inconsistent and unreliable (Videvall et al., 2018; Williams & Athrey, 2020), and potential contamination by fecal material could account for some of this variation. Indeed, cloacal swabs have been used to collect feces in the past (Stanley et al., 2015). The fact that these fecal microbes do not colonize and grow in the *S. virgatus* cloaca supports the hypothesis that the low microbial diversity of the cloaca is due to selection, and that there is a mechanism to maintain stability of the microbiome in that region. This type of selection has been seen in other species (Nyholm & McFall-Ngai, 2004; Zhang et al., 2016), and supports a model in which function is the driving force of microbiome diversity (Reese & Dunn, 2018).

At the phylum level, Bacteroidetes and Firmicutes are often considered to represent the core gut microbiome across vertebrates (Colston & Jackson, 2016; Ley et al., 2008). However, this dogma is based largely on studies that rely on fecal sampling in mammals and so may not accurately represent the GI microbiome of diverse vertebrate taxa. For instance, Bacteroidetes have been found to be relatively rare in some gut regions of wild reptiles and birds (Colston et al., 2015; Hird, 2017; Keenan et al., 2013; Kreisinger et al., 2015), and were relatively uncommon across all *S. virgatus* gut regions and even in fecal pellets, which were dominated by Firmicutes. Firmicutes and Proteobacteria were more equally represented in the *S. virgatus* upper intestine, though Proteobacteria were more common, and Proteobacteria increased in dominance down the GI tract to the cloaca, which was nearly entirely composed of this phylum. Proteobacteria have been found to be highly abundant in the gut of other non-mammalian vertebrates (Colston & Jackson, 2016), particularly in studies that sampled directly from the GI tract rather than relying on fecal sampling alone, but its dominance in the *S. virgatus* cloaca appears to be extreme even in comparison to those systems. In gravid females, the lowest relative abundance of this cloacal community member was 96.9% with greater than 99% on average, and the majority of those reads belonged to a single family. The cloacal swabs were less consistent, as discussed above, although still 82% Proteobacteria on average (across all samples), similar to that of the lower intestinal tissue.

Variation in the gut microbiome across taxa has been considered in relation to diet and other aspects of animal life history and ecology (Colston & Jackson, 2016; Ley et al., 2008). As descriptions of vertebrate microbiomes continue to accumulate, it will be interesting to examine the potential influence of reproductive mode (i.e., viviparity vs. oviparity), as this separates mammals from most non-mammalian vertebrates. We propose that oviparous species, and especially those without egg-tending, may have unique selective pressures on the cloacal microbiome to transfer antifungal or otherwise egg-protective bacteria to eggshells during oviposition. Future research will compare the cloacal microbiome of oviparous and viviparous *Sceloporus* lizards.

Conclusions

The community structure and composition of the microbiome varies depending on tissue type in the gastrointestinal and reproductive tracts of *S. virgatus*, and is highly specialized at the cloaca. While environmental factors have been known to cause regional variation in the gut microbiome, in this case the difference may be due, in part, to selection for cloacal microbes that increase host fitness via the transfer of antifungal microbes from mother to eggshells during oviposition (Bunker et al., 2021). Additionally, although cloacal swabs and fecal samples are generally accepted methods of sampling the microbiome, neither was able to fully represent the entire community of the gut, and the two methods provided unique results. The cloacal swabs adequately sampled communities of cloacal and lower intestine tissues, whereas the fecal microbiome was a distinct community not representative of any sampled gut tissue. We also found that fecal microbes do not seed the cloaca after defecation. While there were few differences between swabs taken before and after defecation overall, there was evidence of possible fecal contamination of individual swabs. Care should be taken to account for this in future research by excluding samples with visible feces from analyses, and additionally by sequencing fecal pellets to identify a "fecal signature" that may be present on swabs even with no visible contamination. Studies that intend to use cloacal swabs or feces as a proxy for the gut microbiome must think carefully on whether these methods will accurately answer their question; in this study, biologically relevant variation in the microbiome could have been masked due to sampling method.

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References

Archie, E. A., & Theis, K. R. (2011). Animal behaviour meets microbial ecology. *Animal Behaviour*, *82*(3), 425–436. https://doi.org/10.1016/j.anbehav.2011.05.029

Beasley, D. E., Koltz, A. M., Lambert, J. E., Fierer, N., & Dunn, R. R. (2015). The Evolution of stomach acidity and its relevance to the human microbiome. *PLOS ONE*, *10*(7), e0134116. https://doi.org/10.1371/journal.pone.0134116

Berlow, M., Kohl, K. D., & Derryberry, E. P. (2020). Evaluation of non-lethal gut microbiome sampling methods in a passerine bird. *Ibis*, *162*(3), 911–923. https://doi.org/10.1111/ibi.12807

Bunker, M. E., Elliott, G., Heyer-Gray, H., Martin, M. O., Arnold, A. E., & Weiss, S. L. (2021). Vertically transmitted microbiome protects eggs from fungal infection and egg failure. *Animal Microbiome, 3*, 43. https://doi.org/10.1186/s42523-021-00104-5

Callahan, B., McMurdie, P., Rosen, M., Han, A., Johnson, A., & Holmes, S. (2016). DADA2: High resolution sample inference from Illumina amplicon data. *Nature Methods*, *13*(7).

Callahan, B., Sankaran, K., Fukuyama, J. A., McMurdie, P., & Holmes, S. (2016). Bioconductor Workflow for Microbiome Data Analysis: From raw reads to community analyses. *F1000Research*, *5*, 1492. https://doi.org/10.12688/f1000research.8986.2

Colston, T. J., & Jackson, C. R. (2016). Microbiome evolution along divergent branches of the vertebrate tree of life: What is known and unknown. *Molecular Ecology*, *25*(16), 3776–3800. https://doi.org/10.1111/mec.13730

Colston, T. J., Noonan, B. P., & Jackson, C. R. (2015). Phylogenetic analysis of bacterial communities in different regions of the gastrointestinal tract of *Agkistrodon piscivorus*, the cottonmouth snake. *PLOS ONE*, *10*(6), e0128793. https://doi.org/10.1371/journal.pone.0128793

Conroy, C., Papenfuss, T., Parker, J., & Hahn, N. (2009). Use of tricaine methanesulfonate (MS222) for euthanasia of reptiles. *Journal of the American Association for Laboratory Animal Science : JAALAS*, *48*(1), 28–32.

Costello, E. K., Gordon, J. I., Secor, S. M., & Knight, R. (2010). Postprandial remodeling of the gut microbiota in Burmese pythons. *The ISME Journal*, *4*(11), 1375–1385. https://doi.org/10.1038/ismej.2010.71

Davis, N. M., Proctor, D. M., Holmes, S. P., Relman, D. A., & Callahan, B. J. (2018). Simple statistical identification and removal of contaminant sequences in marker-gene and metagenomics data. *Microbiome*, *6*(1), 226. https://doi.org/10.1186/s40168-018-0605-2

Dhar Purkayastha, G., Mangar, P., Saha, A., & Saha, D. (2018). Evaluation of the biocontrol efficacy of a *Serratia marcescens* strain indigenous to tea rhizosphere for the management of root rot disease in tea. *PLoS ONE*, *13*(2). https://doi.org/10.1371/journal.pone.0191761

Escallón, C., Belden, L. K., & Moore, I. T. (2019). The Cloacal microbiome changes with the breeding season in a wild bird. *Integrative Organismal Biology*, *1*(1). https://doi.org/10.1093/iob/oby009

Ewels, P., Magnusson, M., Lundin, S., & Käller, M. (2016). MultiQC: Summarize analysis results for multiple tools and samples in a single report. *Bioinformatics*, *32*(19), 3047–3048. https://doi.org/10.1093/bioinformatics/btw354

Funkhouser, L., & Bordenstein, S. (2013). Mom Knows Best: The Universality of Maternal Microbial Transmission. *PLoS Biology*, *11*(8), e1001631. https://doi.org/10.1371/journal.pbio.1001631

Gutiérrez-Román, M. I., Holguín-Meléndez, F., Dunn, M. F., Guillén-Navarro, K., & Huerta-Palacios, G. (2015). Antifungal activity of *Serratia marcescens* CFFSUR-B2 purified chitinolytic enzymes and prodigiosin against *Mycosphaerella fijiensis*, causal agent of black Sigatoka in banana (*Musa* spp.). *BioControl*, *60*(4), 565–572. https://doi.org/10.1007/s10526-015-9655-6

Hird, S. M. (2017). Evolutionary biology needs wild microbiomes. *Frontiers in Microbiology*, *8*. https://doi.org/10.3389/fmicb.2017.00725

Hong, P.-Y., Wheeler, E., Cann, I. K. O., & Mackie, R. I. (2011). Phylogenetic analysis of the fecal

492 microbial community in herbivorous land and marine iguanas of the Galápagos Islands using 16S
493 rRNA-based pyrosequencing. *The ISME Journal*, *5*(9), 1461–1470.
494 https://doi.org/10.1038/ismej.2011.33
495 Jiang, H.-Y., Ma, J.-E., Li, J., Zhang, X.-J., Li, L.-M., He, N., Liu, H.-Y., Luo, S.-Y., Wu, Z.-J., Han, R.-
496 C., & Chen, J.-P. (2017). Diets alter the gut microbiome of crocodile lizards. *Frontiers in*
497 *Microbiology*, *8*, 2073. https://doi.org/10.3389/fmicb.2017.02073
498 Kalbe, C., Marten, P., & Berg, G. (1996). Strains of the genus *Serratia* as beneficial rhizobacteria of
499 oilseed rape with antifungal properties. *Microbiological Research*, *151*(4), 433–439.
500 https://doi.org/10.1016/S0944-5013(96)80014-0
501 Keenan, S. W., Engel, A. S., & Elsey, R. M. (2013). The alligator gut microbiome and implications for
502 archosaur symbioses. *Scientific Reports*, *3*(1), 2877. https://doi.org/10.1038/srep02877
503 Kembel, S. W., Cowan, P. D., Helmus, M. R., Cornwell, W. K., Morlon, H., Ackerly, D. D., Blomberg, S.
504 P., & Webb, C. O. (2010). Picante: R tools for integrating phylogenies and ecology.
505 *Bioinformatics*, *26*(11), 1463–1464. https://doi.org/10.1093/bioinformatics/btq166
506 Kohl, K. D., Brun, A., Magallanes, M., Brinkerhoff, J., Laspiur, A., Acosta, J. C., Caviedes-Vidal, E., &
507 Bordenstein, S. R. (2017). Gut microbial ecology of lizards: Insights into diversity in the wild,
508 effects of captivity, variation across gut regions and transmission. *Molecular Ecology*, *26*(4),
509 1175–1189. https://doi.org/10.1111/mec.13921
510 Koren, O., Goodrich, J. K., Cullender, T. C., Spor, A., Laitinen, K., Kling Bäckhed, H., Gonzalez, A.,
511 Werner, J. J., Angenent, L. T., Knight, R., Bäckhed, F., Isolauri, E., Salminen, S., & Ley, R. E.
512 (2012). Host remodeling of the gut microbiome and metabolic changes during pregnancy. *Cell*,
513 150(3), 470–480. https://doi.org/10.1016/j.cell.2012.07.008
514 Kreisinger, J., Čížková, D., Kropáčková, L., & Albrecht, T. (2015). Cloacal microbiome structure in a
515 long-distance migratory bird assessed using deep 16sRNA pyrosequencing. *PloS One*, *10*(9),
516 e0137401. https://doi.org/10.1371/journal.pone.0137401
517 Lee, W. Y. (2015). Avian gut microbiota and behavioral studies. *Korean Journal of Ornithology. 22*, 1–

11.

Ley, R. E., Lozupone, C. A., Hamady, M., Knight, R., & Gordon, J. I. (2008). Worlds within worlds: Evolution of the vertebrate gut microbiota. *Nature Reviews Microbiology*, *6*(10), 776–788. https://doi.org/10.1038/nrmicro1978

Lkhagva, E., Chung, HJ., Hong, J., Wilson Tang, W. H., Lee, S. I., Hong, S. T., Lee, S. (2021). The regional diversity of gut microbiome along the GI tract of male C57BL/6 mice. *BMC Microbiology, 21,* 44. https://doi.org/10.1186/s12866-021-02099-0

Martin, B., Witten, D., & Willis, A. (2020). *corncob: Count Regression for Correlated Observations with the Beta-binomial* (0.1.0) [R]. https://github.com/bryandmartin/corncob

Martin, M., Gilman, F. R., & Weiss, S. L. (2010). Sex-specific asymmetry within the cloacal microbiota of the striped plateau lizard, Sceloporus virgatus. *Symbiosis*, *51*(1), 97–105. https://doi.org/10.1007/s13199-010-0078-y

McFall-Ngai, M., Hadfield, M. G., Bosch, T. C. G., Carey, H. V., Domazet-Lošo, T., Douglas, A. E., Dubilier, N., Eberl, G., Fukami, T., Gilbert, S. F., Hentschel, U., King, N., Kjelleberg, S., Knoll, A. H., Kremer, N., Mazmanian, S. K., Metcalf, J. L., Nealson, K., Pierce, N. E., … Wernegreen, J. J. (2013). Animals in a bacterial world, a new imperative for the life sciences. *Proceedings of the National Academy of Sciences*, *110*(9), 3229–3236. https://doi.org/10.1073/pnas.1218525110

McMurdie, P., & Holmes, S. (2013). phyloseq: An R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS ONE*, *8*(4), e61217. https://doi.org/10.1371/journal.pone.0061217

Moran, N, McCutcheon, J, & Nakanachi, A. (2008). Genomes and evolution of heritable bacterial symbionts. *Annual Review Genetics*, *42*, 165–190.

Nyholm, S. V., & McFall-Ngai, M. (2004). The winnowing: Establishing the squid–vibrio symbiosis. *Nature Reviews Microbiology*, *2*(8), 632–642. https://doi.org/10.1038/nrmicro957

Oksanen, J., Blanchet, F. G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, P. R., O'Hara, R., Simpson, G. L., Solymos, P., Stevens, M. H. H., Szoecs, E., & Wagner, H. (2019). *vegan:*

Community Ecology Package (2.5-6) [Computer software]. https://CRAN.R-project.org/package=vegan

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., & Glöckner, F. O. (2013). The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. *Nucleic Acids Research*, *41*(D1), D590–D596. https://doi.org/10.1093/nar/gks1219

R Core Team. (2020). *R: A language and environment for statistical computing.* R Foundation for Statistical Computing. https://www.R-project.org/

Reese, A. T., & Dunn, R. R. (2018). Drivers of microbiome biodiversity: A review of general rules, feces, and ignorance. *MBio*, *9*(4). https://doi.org/10.1128/mBio.01294-18

Rose, B. (1981). Factors Affecting Activity in Sceloporus Virgatus. *Ecology*, *62*(3), 706–716. https://doi.org/10.2307/1937739

Sarmiento, C., Zalamea, P.-C., Dalling, J. W., Davis, A. S., Stump, S. M., U'Ren, J. M., & Arnold, A. E. (2017). Soilborne fungi have host affinity and host-specific effects on seed germination and survival in a lowland tropical forest. *Proceedings of the National Academy of Sciences*, *114*(43), 11458–11463. https://doi.org/10.1073/pnas.1706324114

Schliep, K., Potts, A. J., Morrison, D. A., & Grimm, G. W. (2017). Intertwining phylogenetic trees and networks. *Methods in Ecology and Evolution, 8,* 1212-1220. https://doi.org/10.1111/2041-210X.12760

Shterzer, N., Rothschild, N., Sbehat, Y., Stern, E., Nazarov, A., & Mills, E. (2020). Large overlap between the intestinal and reproductive tract microbiomes of chickens. *Frontiers in Microbiology*, *11*, 1508. https://doi.org/10.3389/fmicb.2020.01508

Stanley, D., Geier, M. S., Chen, H., Hughes, R. J., & Moore, R. J. (2015). Comparison of fecal and cecal microbiotas reveals qualitative similarities but quantitative differences. *BMC Microbiology*, *15*, 1-11.

Stebbins, R. C. (2003). *A Peterson Field Guide to Western Reptiles and Amphibians* R. T. Peterson, Ed.; 3rd edition. Houghton Mifflin Harcourt.

Sugden, S., St. Clair, C.C., & Stein, L.Y. (2021). Individual and site-specific variation in a biogeographical profile of the coyote gastrointestinal microbiota. *Microbial Ecology, 81*, 240–252. https://doi.org/10.1007/s00248-020-01547-0

Taylor, M. J., Mannan, R. W., U'Ren, J. M., Garber, N. P., Gallery, R. E., & Arnold, A. E. (2019). Age-related variation in the oral microbiome of urban Cooper's hawks (*Accipiter cooperii*). *BMC Microbiology*, *19*(1), 47. https://doi.org/10.1186/s12866-019-1413-y

Trevelline, B. K., Fontaine, S. S., Hartup, B. K., & Kohl, K. D. (2019a). Conservation biology needs a microbial renaissance: A call for the consideration of host-associated microbiota in wildlife management practices. *Proceedings of the Royal Society B: Biological Sciences*, *286*(1895), 20182448. https://doi.org/10.1098/rspb.2018.2448

Trevelline, B. K., MacLeod, K. J., Langkilde, T., & Kohl, K. D. (2019b). Gestation alters the gut microbiota of an oviparous lizard. *FEMS Microbiology Ecology*, 95(7). https://doi.org/10.1093/femsec/fiz086

Videvall, E., Strandh, M., Engelbrecht, A., Cloete, S., & Cornwallis, C. K. (2018). Measuring the gut microbiome in birds: Comparison of faecal and cloacal sampling. *Molecular Ecology Resources*, *18*(3), 424–434. https://doi.org/10.1111/1755-0998.12744

Vinegar, M. (1975). Demography of the striped plateau lizard, *Sceloporus virgatus*. *Ecology*, *56*(1), 172-182.

Wen, C., Li, Q., Lan, F., Li, X., Li, G., Yan, Y., Wu, G., Yang, N., & Sun, C. (2021). Microbiota continuum along the chicken oviduct and its association with host genetics and egg formation. *Poultry Science*, 101104. https://doi.org/10.1016/j.psj.2021.101104

White, J., Richard, M., Massot, M., & Meylan, S. (2011). Cloacal bacterial diversity increases with multiple mates: Evidence of sexual transmission in female common lizards. *PLOS ONE*, *6*(7), e22339. https://doi.org/10.1371/journal.pone.0022339

Wickham, H. (2017). ggplot2—Elegant Graphics for Data Analysis (2nd Edition). *Journal of Statistical Software*, *77*.

Williams, T., & Athrey, G. (2020). Cloacal Swabs Are Unreliable Sources for Estimating Lower Gastro-Intestinal Tract Microbiota Membership and Structure in Broiler Chickens. *Microorganisms*, *8*(5), 718. https://doi.org/10.3390/microorganisms8050718

Wright, E., S. (2016). Using DECIPHER v2.0 to Analyze Big Biological Sequence Data in R. *The R Journal*, *8*(1), 352. https://doi.org/10.32614/RJ-2016-025

Yasuda, K., Oh, K., Ren, B., Tickle, T. L., Franzosa, E. A., Wachtman, L. M., Miller, A. D., Westmoreland, S. V., Mansfield, K. G., Vallender, E. J., Miller, G. M., Rowlett, J. K., Gevers, D., Huttenhower, C., & Morgan, X. C. (2015). Biogeography of the intestinal mucosal and lumenal microbiome in the rhesus macaque. *Cell Host & Microbe*, *17*(3), 385–391. https://doi.org/10.1016/j.chom.2015.01.015

Zhang, C., Derrien, M., Levenez, F., Brazeilles, R., Ballal, S., Kim, J., Degivry, M.-C., Quéré, G., Garault, P., van Hylckama Vlieg, J. E. T., Garrett, W. S., Doré, J., & Veiga, P. (2016). Ecological robustness of the gut microbiota in response to ingestion of transient food-borne microbes. *The ISME Journal*, *10*(9), 2235–2245. https://doi.org/10.1038/ismej.2016.13

Zhang, L., Yang, F., Li, N., & Dayananda, B. (2021). Environment-Dependent Variation in Gut Microbiota of an Oviparous Lizard (*Calotes versicolor*). *Animals*, *11*(8), 2461. https://doi.org/10.3390/ani11082461

Zoetendal, E. G., von Wright, A., Vilpponen-Salmela, T., Ben-Amor, K., Akkermans, A. D. L., & de Vos, W. M. (2002). Mucosa-associated bacteria in the human gastrointestinal tract are uniformly distributed along the colon and differ from the community recovered from feces. *Applied and Environmental Microbiology, 68*, 3401–3407.

Data Availability

The sequences and associated metadata used to support the conclusions of this study are available in the sequence read archive of NCBI (BioProject PRJNA687551). R scripts and csv files are available on Dryad: https://doi.org/10.5061/dryad.2547d7wps.

Author Contributions

All authors contributed to the study conception. Marie E. Bunker collected samples, performed all lab work, and drafted the manuscript. Stacey L. Weiss assisted with field work and sample collection. All authors edited drafts and approved of the final manuscript.

Figure Legends

Fig. 1 Mean **(A)** Shannon diversity **(B)** richness and **(C)** Faith's phylogenetic diversity of gravid female *S. virgatus* cloacal swabs and tissue types. Although cloacal tissue diversity was lower than the other tissue types and the swabs, this difference was not statistically significant in any measure. Error bars represent standard error

Fig. 2 Beta diversity of gravid female *S. virgatus* cloacal swabs and tissue types. **(A)** Non-metric multi-dimensional scaling (NMDS) plot created by using Bray Curtis distance to calculate pairwise distances based on community composition; 3 dimensions were used to calculate distances but only the most influential two are pictured here. Samples clustered using (B) weighted and (C) unweighted UniFrac distances were used to create Principal Coordinates Analysis (PCoA) plots. Hulls and ellipses are colored by sample type

Fig. 3 Percent composition of bacterial families in cloacal swabs and tissue types from gravid female *S. virgatus*. Each vertical bar represents one sample. Colored portions of the bars represent the relative

abundance of the top ten most abundant taxa; the remaining taxa were combined into the "other" category. The y-axis indicates percent composition of total reads for that sample. Raw data are provided in the supplemental information

Fig. 4 Effect of defecation on cloacal swab microbiome samples relative to the fecal pellet microbiome. **(A)** Shannon diversity index values, **(B)** richness, and **(C)** Faith's phylogenetic diversity of *S. virgatus* cloacal swabs before and after defecation, and fecal pellets. The fecal pellets have significantly higher diversity than the pre-defecation swabs in all metrics, but there were no differences between pre- and post-defecation swabs. Error bars represent standard error

Fig. 5 Beta diversity of *S. virgatus* cloacal swabs and feces. **(A)** Non-metric multi-dimensional scaling (NMDS) plots created by using Bray Curtis distance to calculate pairwise distances based on community composition of cloacal swabs of *S. virgatus* lizards before and after defecation, as well as fecal samples from the same individuals. 2 dimensions were used to calculate distances. Samples clustered using (B) weighted and (C) unweighted UniFrac distances to create Principal Coordinates Analysis (PCoA) plots. Hulls and ellipses are colored by sample type

Fig. 6. Percent composition of bacterial families in pre- and post-defecation swabs, and fecal samples from *S. virgatus* females (F) and males (M). Each vertical bar represents one sample. Colored portions of the bars represent the relative abundance of the top ten most abundant taxa; the remaining taxa were combined into the "other" category. The y-axis indicates the percent composition of total reads for that sample. Raw data are provided in the supplemental information

Fig. 7. Percent composition of the bacterial phyla known to dominate the vertebrate gut microbiome found in all *S. virgatus* samples from Study 1 and Study 2. Each vertical bar represents the mean percent composition for a given sample type. Raw data are provided in the supplemental information