Stable and transient structural variation in lemur vaginal, labial, and axillary microbiomes: Patterns by species, body site, ovarian hormones, and forest access

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Abstract (200w limit): Host-associated microbiomes shape and are shaped by myriad processes that ultimately delineate their symbiotic functions. Whereas a host's stable traits, such as its lineage, relate to gross aspects of its microbiome structure, transient factors, such as its varying physiological state, relate to shorter-term, structural variation. Our understanding of these relationships in primates derives principally from anthropoid studies and would benefit from a broader, comparative perspective. We thus examined the vaginal, labial, and axillary microbiota of captive, female ring-tailed lemurs (*Lemur catta*) and Coquerel's sifakas (*Propithecus coquereli*), across an ovarian cycle, to better understand their relation to stable (e.g. species identity/mating system, body site) and transient (e.g. ovarian hormone concentration, forest access) host features. We used 16S amplicon sequencing to determine microbial composition and enzyme-linked immunosorbent assays to measure serum hormone concentrations. We found marked variation in microbiota diversity and community composition between lemur species and their body sites. Across both host species, microbial diversity was significantly correlated with ovarian hormone concentrations; negatively with progesterone and positively with estradiol. The hosts' differential forest access related to the diversity of environmental microbes, particularly in axillary microbiomes. Such transient endogenous and exogenous modulators have potential implications for host reproductive health and behavioral ecology.

Keywords: strepsirrhine, mating system, progesterone, estradiol, environment

Introduction

Various microbial communities occupy the epithelial surfaces of vertebrate hosts and interact with their physiological systems to mediate host health (Belkaid and Segre 2014; Sharon *et al.* 2016; Gilbert *et al.* 2018; Benavidez *et al.* 2019) and reproductive success (Reid *et al.* 2015; Power, Quaglieri and Schulkin 2017). For example, human vaginal microbiomes mediate pathogen acquisition and immune response to foreign cells, including sperm (Taha *et al.* 1998; Yarbrough, Winkle and Herbst-Kralovetz 2014). External epithelial communities, such as the labial and axillary microbiomes, promote host health and reproduction, respectively, through pathogen resistance or wound healing (Grice 2014; Misic, Gardner and Grice 2014) and through the manufacture of bodily odors used to chemically communicate mate quality (Sharon *et al.* 2010; Ezenwa and Williams 2014). The range and specificity of functions served by microbes owe to differences in their community structure across body sites (Huttenhower *et al.* 2012; Schommer and Gallo 2013). Over evolutionary time, host phylogeny largely predicts microbiome structure (Ley *et al.* 2008; Groussin *et al.* 2017; Nishida and Ochman 2018), whereas over shallower time scales, microbiome structure can reflect more transient characteristics, such as the host's physiology or environmental condition (Phillips *et al.* 2012; Nieuwdorp *et al.* 2014; Greene *et al.* 2019). Here, we compare three microbiomes in the captive females of two lemur species to examine the relative contributions of stable (i.e., species identity or mating system and body site) and transient (i.e., ovarian hormone concentrations and forest access) factors in predicting microbial community structure.

Phylogeny is one of the strongest signals of microbiome structure and function at different body sites (Phillips *et al.* 2012; Council *et al.* 2016). In various mammalian lineages, skin microbiomes are structurally distinct between host species and show patterns of 'phylosymbiosis,' whereby the relatedness of skin microbes recapitulates the hosts' phylogenetic relationships (Brooks *et al.* 2016; Ross *et al.* 2018). Thus, well-established patterns in humans (Grice *et al.* 2009; Peterson *et al.* 2009; Huttenhower *et al.* 2012) may not generalize to other primates (Yildirim *et al.* 2014; Davenport *et al.* 2017). Indeed, within closely related primate species, microbial community structures differ significantly between species at specific body sites (vaginal: Spear *et al.* 2010; Stumpf *et al.* 2010; Rivera *et al.* 2011; axillary: Council *et al.* 2016; forearm: Verhulst *et al.* 2018), presumably reflecting species- and site-specific functions. Notably, the diversity of scent-producing epithelial microbiota (e.g. the axilla, external genitalia, and scent glands) have long been thought to underlie host olfactory communication by contributing to the complex array of volatile organic compounds (VOCs) that emanate from different body sites (Gorman, Nedwell and Smith 1974; Albone and Perry 1976). Minimally, therefore, one would expect a positive relationship between odor-producing bacteria and scent-producing microbiomes.

In conjunction with phylogeny and functional morphology, the socioecological characteristics of hosts, such as their type of mating system, also mediate abundances of commensal and pathogenic microbes (Kokko *et al.* 2002; Sharon *et al.* 2010). For instance, species characterized by promiscuity or diverse network of social connectivity (i.e., numbers of unique sexual partners) show increased vaginal microbial diversity (MacManes 2011; Yildirim *et al.* 2014; Kenyon, Delva, & Brotman, 2019), presumably because sexual contact is a vector for sharing microbes between individuals. Furthermore, because sexually transmitted infections (STIs) follows similar networks (Doherty *et al.*, 2005; Liljeros, Edling, & Amaral, 2003), greater microbial diversity in promiscuous species enhances immune function and disease prevention and, ultimately, reproductive success (Thrall, Antonovics and Dobson 2000; Nunn *et al.* 2014).

By comparison with these 'stable' factors, transient factors influence microbial communities over more proximate timescales. Transient factors endogenous to the host, such as changes in steroid hormone concentrations, alter microbial membership across body sites and sexes, with potential reproductive or health consequences. For example, progesterone (P_4) positively correlates with abundances of gingival pathogens in human oral microbiomes in both sexes (Kornman and Loesche 1982; Nakagawa *et al.* 1994). In women, changes in vaginal microbiota accompany variation in estradiol (E_2) concentrations during ovarian cycles (Mishell Jr *et al.* 1971), pregnancy (Loriaux *et al.* 1972), and menopause (Sherman, West and Korenman 1976; Rothman *et al.* 2011). Indeed, the dominance of *Lactobacillus*, which mediates susceptibility to bacterial vaginosis and HIV, is regulated, in part, by E_2 (Redondo-Lopez, Cook and Sobel 1990; Miller *et al.* 2016). Although vaginal microbiota vary across the ovarian cycles of certain anthropoids (Narushima *et al.* 1997; Miller *et al.* 2017), the roles of P_4 and E_2 in structuring the microbiomes of nonhuman hosts remain largely unexplored.

Transient factors exogenous to the host, such as varying contact with environmental microbes, are also known to impact microbiome structure across host taxa (Lax *et al.* 2014; Walke *et al.* 2014; Council *et al.* 2016; Hyde *et al.* 2016). Although the transmission of pathogenic, environmental microbes can negatively impact host health (Jones *et al.* 2008; Finley *et al.* 2013), the transfer of nonpathogenic, environmental microbes can bolster the diversity and function of commensal microbiomes (Hyde *et al.* 2016). Consistent to prior themes, there has been relatively little attention given to environmental microbes.

In the current study, we describe the vaginal, labial, and axillary microbiota of two strepsirrhine primates – the ring-tailed lemur (*Lemur catta*) and the Coquerel's sifaka (*Propithecus coquereli*).

Although understudied relative to anthropoid primates, strepsirrhines are exceptionally diverse ecologically, morphologically, behaviorally, physiologically, and chemically (Martin 1972; Drea 2019), making them ideal subjects for the study of microbial contributions to host health and behavior. Whereas the ring-tailed lemur is primarily terrestrial, lives in multi-male, multi-female groups and mates promiscuously, the arboreal Coquerel's sifaka lives in smaller, family groups, and forms relatively stable pair bonds, although opportunities exist for occasional extra-pair mating. The females of both species differentially rely on labial scent marking relative to urine marking to communicate socio-reproductive information (Hayes, Morelli and Wright 2004; Scordato and Drea 2007; delBarco-Trillo *et al.* 2011; Greene and Drea 2014). Thus, while providing the opportunity to expand our understanding of host-microbe relationships across the primate order, these two host species also motivate distinct expectations about various stable and transient influences over microbial community structures.

More specifically, we expect the microbiomes of these species to reflect stable host traits and to differ structurally by species or mating system and body site. In particular, we expect the promiscuous ring-tailed lemur to harbor more diverse vaginal consortia than the pair-bonded sifaka. We also expect that, compared to vaginal microbiomes, the microbiomes of prominent scent-producing areas, the axilla and labia, will harbor more fermentative, odor-producing bacteria. With regard to transient traits, such as endogenous ovarian hormones, we expect aspects of microbiota richness and composition to vary with P_4 and/or E_2 concentrations. Lastly, with regard to exogenous influences, we expect the hosts' most distally located, labial and axillary microbiomes to harbor greater microbial diversity and abundance of environmental taxa than their more proximally located, vaginal microbiome, particularly when the captive hosts occupy outdoor (versus indoor) enclosures that allow greater contact with environmental microbes.

Methods

Subjects and housing

Our subjects were seven, adult, female lemurs (n = 3 ring-tailed lemurs, n = 4 Coquerel's sifakas), housed socially with conspecifics in different groups at the Duke Lemur Center (DLC; Durham, NC, USA). To avoid the potential confound introduced by pregnancy, the focal females were members of all-female groups or groups in which males were either immature or gonadectomized; they included all available, naturally cycling females at the DLC that, owing to housing constraints, would have no reproductive opportunities during the period of study.

In the Northern Hemisphere, the breeding seasons of captive ring-tailed lemurs and sifakas occur from October-February and July-October, respectively. Both species are polyestrous, with ring-tailed lemurs having up to three cycles within their breeding season (Evans & Goy 1968; Drea 2007) and Coquerel's sifakas having up to two cycles (Greene & Drea 2014; unpublished DLC records). The ovarian cycle of ring-tailed lemurs spans a 35- to 40-day period (Evans and Goy 1968; Bogart, Kumamoto and Lasley 1977; Van Horn and Resko 1977) with a receptivity period of up to 22 hours (Van Horn and Resko 1977), whereas the cycle of Coquerel's sifakas spans a 45- to 60-day period (Robert Schopler, pers. comm. 2018; in sister species *P. verreauxi*, Brockman *et al.* 1995), with a receptivity period of 0.5-96 hours (Brockman 1999).

All of the subjects had access to indoor and outdoor enclosures (146 m^2/animal); a subset of the subjects (n = 1 ring-tailed lemur, n = 2 Coquerel's sifakas) also had access to multi-acre forest enclosures where they semi free-ranged, including with hetero-specific lemurs. Details on their diets, foraging, and social behavior have been reported elsewhere (Starling *et al.* 2010; Greene *et al.* 2019). The subjects were maintained in accordance with the NIH Guide for the Care and Use of Laboratory Animals, and procedures were approved by the Institutional Animal Care and Use Committee of Duke University (protocol A111-16-05).

Study design

Biological sampling, at each of three body sites, occurred repeatedly across one ovarian cycle during each species' peak breeding season. At four, equivalently spaced time points (i.e., every 11-13 days for ring-tailed lemurs and every 14-15 days for sifakas), we concurrently obtained epithelial/secretion and blood samples from the subjects, for microbial and endocrine analyses, respectively. We thus collected 28 microbial samples (7 subjects × 4 collections) per body site (or 84 microbial samples in total) and 28 blood samples. Because these females' cycles are asynchronous and would be sampled relatively infrequently, in addition to absolute hormone concentrations, we also used the natural log of the females' E_2 to P_4 ratios ($\ln(E_2/P_4)$) across the sampling times as a means to align and visualize their cycles. Each female's greatest value of $\ln(E_2/P_4)$, approximating the periovulatory period, was assigned the 'standardized' time point 2 in Figure 5 below. In our results, in addition to assessing microbial patterns associated with individual steroid concentrations, we also examined how any patterns related to values of $\ln(E_2/P_4)$, a hormonal index of conception probability (Baird *et al.* 1991).

Sample collection

We obtained our samples from awake, gently restrained animals that are habituated to collection procedures. Our handling of individually processed individuals occurred in the morning before the animals were fed (between 9:00am and 10:30am). First, within 5 minutes of each animal's capture, we collected 3 mL of whole blood from the femoral vessels (Drea 2007). To collect microbial samples, we used sterile, cotton-tipped swabs (pre-moistened with sterile water for samples taken from the genitals). We collected vaginal swabs from approximately 2-3 cm into the vaginal canal. We collected labial secretions by swabbing the external, labial folds on the right-hand side for 15 sec. For axillary samples, we rubbed the swabs against the skin of each animal's right axilla for 60 sec. We immediately placed the epithelial samples on ice and stored them in a – 80 °C freezer within 45-60 min of collection. After allowing the blood samples to clot at ambient temperatures for approximately 30-45 min, we centrifuged them (2200-2500 RPM for 15 min) and transferred the serum into sterile tubes for storage at – 80 °C.

Microbial DNA extraction, sequencing and bioinformatics

Using the DNeasy Powersoil kit (QIAGAN, Frederick, MD), we extracted microbial gDNA from epithelial/secretion samples. To improve the DNA yield from these low-yield samples, we included additional incubation periods (1) after adding Solution C1 (10 min at 65 °C in a heat-block) and (2) after adding Solution C6 (prior to the final elution step, 10 min at room temperature). We quantified the extracted DNA using a Fluorometer (Qubit 4 with a broad-spectrum kit, Thermo Fisher Scientific, Waltham, MA) and shipped aliquots of extracted gDNA to the Argonne National Laboratory's

Environmental Sequencing facility (Lemont, IL) for library preparation and sequencing. There, the V4 region of the 16S rRNA gene (515F-806R) was amplified via polymerase chain reaction with region-specific primers adapted for the Illumina MiSeq platform (Caporaso *et al.* 2012). Forward primers contained a twelve-base barcode sequence to support pooling of samples in each flow cell lane. Once pooled, amplicon libraries were cleaned using AMPure XP Beads (Beckman Coulter, Pasadena, CA), and quantified using a fluorometer (Qubit 4). Amplicons were sequenced on a 151bp x 12bp x 151bp Illumina MiSeq run (Caporaso *et al.* 2012).

We analyzed the raw sequence data using a bioinformatics pipeline generated in QIIME2 (ver. 2019.2, Bolyen *et al.* 2019). We first joined paired-end sequences, demultiplexed, and discarded sequences that did not assign to a sample. Using the DADA2 plugin (q2-dada2, Callahan *et al.* 2016), we denoised, quality filtered, and removed phiX and chimeric sequences from the demultiplexed reads. Using the resulting sequences, we compiled a QIIME2 feature table, from which we discarded a single sample that had fewer than 10,000 assigned sequences. To generate a midpoint-rooted phylogenetic tree, we used the mafft program (Katoh *et al.* 2002) and fasttree2 (Price, Dehal and Arkin 2010) to perform a multiple-sequence alignment, remove highly variable sequence positions, and generate phylogenetic relationships. To assign taxonomy to our sequence features, we *de novo* trained the Naive Bayes classifier using the SILVA 132 database at 97% sequence similarity (ver. 132, Quast *et al.* 2012; Yarza *et al.* 2014) and tested the classifier using our representative sequences. We removed features classified as mitochondria or chloroplasts from downstream analyses.

We used the resulting taxonomy in combination with the feature table to calculate three metrics of alpha diversity (the logarithm of the number of observed operational taxonomic units, Shannon-Weaver, and Faith's phylogenetic diversity). Because all three metrics were highly, positively correlated ($0.85 < r < 0.97$), we performed a principal components analysis on these metrics (prcomp {stats}, R ver. 3.6.1) and extracted the first principal component as a composite metric of alpha diversity. We created alpha rarefaction plots for each metric of alpha diversity and used the inflection points in the plots to determine the cutoff of 10,000 reads per sample (above which, alpha diversity plateaued). To assess microbial composition, we calculated beta diversity using unweighted UniFrac, a metric that is well-suited to detect variation in communities with numerous rare taxa and those with distinct bacterial membership between groups. After calculating metrices of diversity, we combined features without assigned taxonomy below the Kingdom level into an "Unassigned". We also included the conglomerate "Other" to visually represent the rare taxa that had relative abundances lower than 1%.

Enzyme-linked immunosorbent assays

We measured the concentrations of P_4 and E_2 in serum samples using commercial, competitive enzyme immunoassay (EIA) kits (ALPCO diagnostics, Salem, NH, USA). The P_4 and E_2 assays have sensitivities of 0.1 ng/mL and 0.01 ng/mL, respectively. For P_4, the inter-assay coefficients of variation (CVs) were 5.86% and 6.99% for low and high controls, respectively. The intra-assay CV, calculated as the mean CV of duplicate samples, averaged 6.12%. For E_2, the inter-assay CVs were 7.47% and 13.27% for low and high controls, respectively, and the intra-assay CV averaged 4.83%. Concentrations of P_4 and E_2 are reported in pg/mL.

Statistical analyses

To test for stable differences in alpha diversity across host species and body sites, we used Kruskall-Wallis tests with Dunn's multiple comparison corrections in GraphPad's Prism software (following Greene and McKenney 2018). To test for variation in microbial composition, or beta diversity, we used permutational multivariate analyses of variance (PERMANOVAs; adonis{vegan} in R ver 3.6.1) on unweighted Unifrac distances (Lozupone & Knight, 2005; Luzopone *et al*., 2011; following Amato *et al.* 2019). Our PERMANOVA was stratified by animal and included the following explanatory variables: species, body site, and their interaction, P_4, E_2, forest access, and body site nested within individual. To compare beta diversity between the body sites of each species, we implemented post-hoc tests of the PERMANOVA using the pairwise.Adonis package in R (Martinez Arbizu 2017). We analyzed within body-site variation for each species by calculating pairwise comparisons of beta diversity distances with Bonferroni-corrected student *t*-tests (QIIME, ver13.8; see Greene *et al.* 2019).

To test for transient relationships between microbial community structure, species identity, body sites, hormone concentrations, and forest access, we used linear mixed models (LMMs) in the lmer package of R. We first tested for relationships between hormone concentrations (of P_4 or E_2, individually) and our composite measure of alpha diversity across the different microbial communities. In this first LMM (LMM1; Table 1), we included species, body site, and their interaction, forest access, P_4 and E_2 as fixed effects, with body site nested within individual included as a random effect. Our second LMM (LMM2), substituted $\ln(E_2/P_4)$ (i.e., our proxy of ovarian phase) for the individual hormone concentrations, but was otherwise identical to LMM1. To assess stable differences in alpha diversity across host species and body sites, we report the results of pairwise comparisons from our LMM1, adjusting *p*-values for multiple comparisons using Tukey's method.

We also report LMMs specifically predicting the relative abundances of two groups of bacterial taxa. The first bacterial group, determined *a priori*, consisted of five taxa identified in previous studies (typically focused on humans) as being associated with reproductive hormones at different body sites: Lactobacillales (Ferris *et al.* 2007; Jakobsson and Forsum 2007; Mirmonsef *et al.* 2014; Miller *et al.* 2016); *Streptococcus* (Cowley and Heiss 1991; Noguchi *et al.* 2004; Bezirtzoglou *et al.* 2008); *Bacteroides* (Larsen, Markovetz and Galask 1977; Kornman and Loesche 1982; Eschenbach *et al.* 2000; Bezirtzoglou *et al.* 2008); *Prevotella*, (Nakagawa *et al.* 1994; Kumar 2013; Miller *et al.* 2017); and

Family XI (Miller *et al.* 2017). For this group, we ran a third set of LMMs (LMM3) predicting the relative abundance of each taxon. Additionally, because the results from microbial studies on humans or even anthropoids may not be representative of strepsirrhine microbiomes, we ran a fourth set of exploratory LMMs (LMM4) predicting each of the five most abundant taxa we found within each strepsirrhine body site, as well as the category "Other" as a measure of rare taxa. For LMM4, we corrected for multiple hypothesis testing using the Benjamini-Hochberg method (Benjamini and Hochberg 1995). All of our analysis scripts, as well as the data and metadata files needed to reproduce our results in R are available on Open Science Framework at https://osf.io/usynq/. Sequencing reads are also available on the National Center for Biotechnology Information's Sequence Read Archive (BioProject ID #TBD, BioSample accession #s TBD).

Results

Stable traits: Patterns in relation to species, mating system, and body site

General species patterns

After bioinformatic analyses, our 83 remaining microbial samples yielded 9,238,389 sequences with an average of 38,018 reads per sample. The sequences were classified into 1,556 genera within 34 identified bacterial phyla. Across both host species, the microbiota at all three body sites were dominated by taxa in the Bacteroidetes, Firmicutes, Fusobacteria, and Proteobacteria phyla, with smaller, site-specific contributions from the Acidobacteria, Actinobacteria, Epsilonbacteraeota, Spirochaetes, Tenericutes, and Verrucomicrobia phyla (Figure 1). Although the phyla represented in the microbiota of both species were similar across the three body sites, as anticipated, the identified genera and overall alpha and beta diversity metrics varied significantly by host species, body site, and their interaction terms (Table 1). We present results on the microbial membership for each species by body site, below.

Vaginal microbiota

The vaginal microbiota of ring-tailed lemurs and sifakas were dominated by three phyla; Bacteroides, Firmicutes, and Fusobacteria (Figure 1). Of the genera that accounted for minimally 1% of each species' vaginal microbiota, only six were shared by both species: *Bacteroides* and *Porphyromonas* (phyla Bacteroidetes), *Peptoniphilus* and *W5053* (Firmicutes), *Campylobacter* (Epsilonbacteraeota), and *Fusobacterium* (Fusobacteria). Both lemur species harbored a diverse array of taxa in the Clostridiales order. The vaginal microbiota of sifakas were dominated by three of the shared genera, *Bacteroides*, *Porphyromonas*, and *Fusobacteria*, which had a combined, mean relative abundance of 66%. These three genera are the only abundant taxa found across all three body sites in both host species.

The alpha diversities of the vaginal microbiota differed between host species. More specifically, as predicted by differences in the hosts' mating systems, the promiscuous ring-tailed lemurs harbored significantly more diverse communities than did the pair-bonded sifakas (Figure 2; $p = 0.009$). Beta diversity also differed significantly between the two species (Figure 3; visualized in PCoA plot, pairwise adonis, $R^2 = 0.38$, $p < 0.05$). In both species, inter-individual variation in vaginal microbiota composition was significantly less than in the other two body sites (Figure 4; *t*-tests with Bonferroni corrections, ring-tailed lemurs: vaginal vs. labial $t = 4.11$, $p = 0.017$, vaginal vs. axillary $t = 7.19$, $p < 0.001$; sifakas: vaginal vs. labial $t = 5.97$, $p < 0.001$, vaginal vs. axillary $t = 8.93$, $p < 0.001$), indicating that vaginal consortia may be more constrained or stable across individuals than are labial or axillary consortia.

Labial microbiota

Reflecting their physical proximity on the host, the labial microbiomes of both host species shared the same six, abundant taxa as those shared by their vaginal microbiomes, plus one additional genus, *Murdochiella* (Firmicutes; Figure 1). The labial communities of ring-tailed lemurs included a large proportion of *Treponema*, a genus of anaerobic spirochetes often associated with infection or disease (Simonson *et al.* 1988; Radolf and Lukehart 2006), plus members of Corynebacteriaceae and Gammaproteobacteria. By contrast, the labial communities of sifakas were dominated by members of the Lactobacillales and Clostridiales orders.

The alpha diversity of labial microbiomes was similar between the two host species (p = .783) and, within species, only differed significantly from those of vaginal communities in sifakas (Figure 2; ring-tailed lemur: vaginal vs. labial, $p = 0.970$; sifaka: vaginal vs. labial, $p = 0.004$). Only in sifakas did beta diversity in the labial microbiome differ significantly from that of the vaginal microbiome (pairwise adonis, $R^2 = 0.09$, $p < 0.05$). Within each species, interindividual variation in labial microbiome composition was significantly greater than that in the vaginal microbiome (Figure 4, see above), but was similar to that in the axillary microbiome (Figure 4; t-tests with Bonferroni corrections; ring-tailed lemurs: labial vs. axillary $t = -1.56$, $p > 0.99$; sifakas: labial vs. axillary $t = -1.82$, $p > 0.99$).

Axillary microbiota

The axillary microbiomes of ring-tailed lemurs harbored balanced microbial communities, with representation from Bacteroidetes, Firmicutes, Fusobacteria, and Proteobacteria phyla, whereas those of sifakas were dominated by the Proteobacteria phyla and harbored numerous rare or ‘other’ taxa (Figure 1).

Alpha diversity of the axillary microbiomes was not significantly different between species (p = 0.136; Figure 2). Across species, axillary microbiomes had the greatest alpha diversity of the three body sites, but differences between sites were much stronger in sifakas (Figure 2; sifaka: axillary vs. vaginal, $p < 0.001$, axillary vs. labial, $p < 0.001$; ring-tailed lemurs: axillary vs. vaginal, $p = 0.125$, axillary vs. labial, $p = 0.040$). This pattern of alpha diversity in sifakas likely relates to the greater relative abundances of rare taxa in sifaka axillary consortia (Figure 1). The beta diversity of axillary microbiomes differed between species (pairwise adonis, $R^2 = 0.37$, $p < 0.05$) and was significantly different from each species' vaginal and labial consortia (pairwise adonis; ring-tailed lemur: axillary vs. vaginal $R^2 = 0.41$, $p < 0.05$, axillary vs. labial $R^2 = 0.39$, $p < 0.05$; sifaka: axillary vs. vaginal $R^2 = 0.40$, $p < 0.05$, axillary vs. labial $R^2 = 0.26$, $p < 0.05$).

Transient factors: Patterns in relation to reproductive hormones and forest access

Endogenous factors: Cyclical, progestogenic, and estrogenic patterns

Based on the 28 serum samples, sifakas had greater mean concentrations of P_4 and E_2 than did ring-tailed lemurs, although these differences did not reach statistical significance (Figure 5a; P_4: $t = 1.84$, $p = 0.077$; E_2: $t = 1.25$, $p = 0.266$). The ovarian cycles of each lemur, as represented by $\ln(E_2/P_4)$ across the four collection times, are illustrated in Figure 5b.

Across species and body sites, we found that alpha diversity was significantly and negatively related to P_4 concentrations (Figure 6; LMM1, $t = -2.18$, $p = 0.033$; Table 1), but significantly and positively related both to E_2 concentrations (Figure 6; LMM1, t = 2.83, $p = 0.018$) and to $\ln(E_2/P_4)$ (Figure 6; LMM2, t = 2.62, $p = 0.011$). Neither P_4 nor E_2 were significantly related to beta diversity (PERMANOVA, ps = 0.151 and 0.398, respectively).

Based on planned comparisons, we found mixed results when using absolute ovarian hormone concentrations to predict relative abundances of specific taxa of interest. We did not find any significant correlations between P_4 concentrations and the relative abundances of five taxa that are prominent in the literature; however, E_2 concentrations were significantly and positively correlated with the relative abundances of *Streptococcus* in the vaginal microbiomes (LMM3; $t = 2.54$, $p = 0.018$) and of Lactobacillales (i.e., the order of lactic-acid producing bacteria that includes *Lactobacillus*) in the vaginal and labial microbiota (Figure 7b; LMM3; vaginal: $t = 3.94$, $p < 0.001$; labial, $t = 2.50$, $p = 0.046$) of both host species. The relationship between microbial composition and E_2 concentrations varied between body sites, but the predicted patterns of correlation were similar between host species (Figure 7a).

Regarding the most abundant taxa at each host species' body site, once analyses were corrected for multiple comparisons, we found just a single statistically significant result: P_4 concentrations were significantly and positively correlated with the relative abundances of *Treponema* in ring-tailed lemur vaginal microbiomes (LMM4; $t = 6.013$, $p < 0.001$).

Exogenous factors: Environmental taxa and forest access

The lemurs' use of their available habitat, as reflected by differential access to forest enclosures, impacted microbial membership, particularly in the axillary microbiomes. Specifically, the axillary microbiomes of ring-tailed lemurs harbored several abundant taxa in the family Ruminococcaceae, which are common soil microbes (Wegner and Liesack 2016; Vo *et al.* 2017). In addition, prior to bioinformatic filtering, the axillary consortia of both species included large proportions of the order Chloroplasts or family Mitochondria, two groups that are commonly omitted from analyses of host-associated microbes, but include several known environmental taxa. These groups were particularly prevalent in sifaka axillary microbiomes; had members of Mitochondria and Chloroplasts been included in downstream analyses, they would have been the first and sixth most abundant groups, respectively. Within the identified

Mitochondria, there were three abundant fungal taxa, *Zasmidium cellare*, *Annulohypoxylon stygium*, and *Pyronema omphalodes*, which are found on environmental substrates, such as soil and trees (Seaver 1909; Moore and Korf 1963; Tribe, Thines and Weber 2006; Abdullah *et al.* 2010; Fournier and Lechat 2016; Wingfield *et al.* 2018). Across both species, individuals that gained forest access harbored greater abundances of environmental taxa. Although forest access was not significantly correlated with alpha diversity while adjusting for the other factors in our overall model (LMM1; $t = 0.43$, $p = 0.686$), forest access did significantly predict beta diversity across all body sites (PERMANOVA; $R^2 = 0.021$, $p \leq 0.001$; Table 1), even if accounting for only a small percent of the variance.

Discussion

Our longitudinal and comparative study of multiple strepsirrhine microbiomes both reinforces existing frameworks for understanding gross variation in primates and adds novel perspectives to this framework by shedding light on more discrete patterns of host-microbe interactions. Consistent with findings across primates (Stumpf *et al.* 2010; Council *et al.* 2016), we found that microbial composition, and predictors of that composition, in strepsirrhines differed between host species and their body sites in ways that may reflect distinct functions. Although confounded with species identity, host mating system was predictably reflected in structural aspects of the lemurs' vaginal microbiota, which (if replicated in a larger number species) may indicate differing demands for maintaining host reproductive health. In addition, the labial and axillary microbiomes – potentially crucial to the manufacture of host olfactory signals – were dominated by microbes linked to odor production in the scent glands of other mammals (cite). Transient factors likewise predicted differences in microbiome structure. P_4 and E_2 concentrations yielded opposing associations with alpha diversity, and E_2 concentrations specifically predicted abundance of Lactobacillales and *Streptococcus,* two microbial taxa that are linked to vaginal and reproductive health in humans. Lastly, the relationship between body site and environmental microbes differed between species. Our results are based on a small number of animals and they should be considered preliminary, but collectively, they are consistent with an interpretation to be explored in future studies: in shaping bacterial membership in lemur microbiomes, transient factors build upon foundational differences governed by stable traits.

Consistent with evidence for a 'core microbiome' across closely related host taxa (Henderson *et al.* 2015; Nishida and Ochman 2018), we found that certain abundant genera, such as *Bacteroides*, *Porphyromonas*, and *Fusobacterium*, were shared across the three microbiomes of both species. Perhaps these genera represent a core epithelial microbiome that persists across lemur species despite species-specific patterns in bacterial community structure. Indeed, these three microbial genera were previously found in the genital microbiota of wild lemurs of different species (Yildirim *et al.* 2014; Greene *et al.* 2019). These diverse genera are often considered pathogenic in humans (Slots and Listgarten 1988; Genco, Van Dyke and Amar 1998; Signat *et al.* 2011; Darveau, Hajishengallis and Curtis 2012; Kostic *et al.* 2012); however, their widespread presence across lemurs, in the absence of disease symptomatology, could signal functional importance, rather than pathology.

With regard to their potential role in host olfactory communication, the labial microbiota of lemurs included abundant genera, such as *Porphyromonas*, *Fusobacterium*, *Campylobacter*, and *Anaerococcus*, that have been linked to the chemical signals of various vertebrate taxa (Li *et al.* 2016; Theis *et al.* 2016; Leclaire *et al.* 2017; Greene *et al.* 2019; Yamaguchi *et al.* 2019). *Treponema* and *Porphyromonas*, the two most abundant bacterial genera in the labial microbiomes of ring-tailed lemurs, are also common in human oral microbiomes, where they produce VOCs associated with halitosis (Donaldson *et al.* 2005; Porter and Scully 2006; Aylıkcı and Çolak 2013). These VOCs include dodecanoic, tetradeconoic, and other long-chain, fatty acids that also occur in the labial secretions of ring-tailed lemurs (Scordato, Dubay and Drea 2007). In this species, despite the presence of specialized glandular tissue in the labia (Drea and Weil 2008), the vagina and labia harbored similarly structured microbial consortia. In contrast, female sifakas, had compositionally distinct vaginal and labial consortia. The different relationships between vaginal and labial microbiomes across the two host species may reflect differences in the morphology of their external genitalia (Hill 1953); ring-tailed lemurs have pronounced, deep labial folds that, relative to the superficially distinct labia of sifakas, may create different bioavailable niches between the two species.

Characterizing the gross differences in microbial community structure that accompany stable, host traits provides important context for understanding how transient factors may differentially influence microbiota and their potential functions across hosts. For instance, between ring-tailed lemurs and sifakas, the underlying differences in vaginal microbiota membership and diversity may reflect distinct strategies by which hosts harness vaginal microbiota to prevent disease transmission and promote reproductive health across the breeding season.

In women and other female anthropoids, increased P_4 concentrations in the luteal phase coincide with a 'window of vulnerability,' during which the host is more susceptible to pathogens and STIs (Sodora *et al.* 1998; Wira and Fahey 2008; Vishwanathan *et al.* 2011; Wira, Rodriguez-Garcia and Patel 2015). Although not previously considered a component of this vulnerability, a negative relationship between P_4 and microbial diversity, as suggested by our analyses, may contribute to this increased susceptibility to infection. Nevertheless, the positive relationship between E_2 and microbial diversity could be a countervailing adaptation. Peak E_2 concentrations typically coincide with peak fertility, proceptivity, and receptivity (i.e., sexual activity) (Dixson 1998). Here, in females of both study species, the greatest microbial diversity in the vaginal canal coincided with peak E_2 concentrations. Although these females did not have access to sexually active males, the finding may suggest that natural, hormonally mediated cyclicity in microbial diversity could function to provide protection against the changing risk of disease exposure via sexual relations.

An additional mechanism by which vaginal microbes can protect against pathogens is the production and maintenance of acidic conditions, as exemplified by the dominance of the lactic acid-producing bacteria (LABs), Lactobacillus, in human vaginal microbiota (Kaewsrichan, Peeyananjarassri and Kongprasertkit 2006; Ravel *et al.* 2011). In the human vaginal epithelium, E_2 increases the production of glycogen, which Lactobacillus can metabolize into lactic acid, thereby reducing vaginal pH (Boskey *et al.* 2001; Mirmonsef *et al.* 2014). Similar to patterns seen in humans, the relative abundances of LABs in the vaginal microbiomes of ring-tailed lemurs and sifakas were positively correlated with E_2 concentrations. This This correlation between E_2 concentrations and LAB abundances suggests that E_2 may regulate vaginal microbial structure similarly across different primate hosts.

These influences of stable and endogenous host traits are further combined with influences of exogenous factors, such as the hosts' habitat use and its interactions with environmental taxa. Although the diets of captive animals can homogenize gut microbial communities across host species (Clayton *et al.* 2016; McKenzie *et al.* 2017), the same may not be true of glandular microbial communities (Greene *et al.* 2019). We found that captive lemurs living under similar conditions (and receiving the same diet within species) harbored distinct epithelial microbiota depending on whether or not they had access to forest enclosures: increased forest access was related to increased abundances of environmental microbes across body sites. In particular, identified fungal taxa in the family Mitochondria were abundant in the axillary microbiomes of sifakas that had forest access. That this relationship was most strongly expressed in lemur axillary microbiomes expands on an earlier finding that, relative to humans, nonhuman anthropoids harbor more environmental microbes in their axillary microbiomes than elsewhere across the body (Council *et al.* 2016).

Because it is common practice in studies of mammalian microbiota to omit from downstream analyses entire groups of non-bacterial taxa (e.g. Mitochondria), including those that comprise known environmental taxa, relatively little is known about the contribution of these groups to commensal communities. Furthermore, when not removed from analyses, environmental bacteria are often considered contaminants or foreign microbes and are rarely regarded as integrated members of commensal communities. Acknowledging this disparity could be an important step toward understanding how transient, environmental communities influence host-associated microbes. A consideration of both evolutionary and more proximate factors is vital to understanding the dynamic composition of microbiota over time and, ultimately, the functional relevance of microbiomes to their hosts.

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Yildirim S, Yeoman CJ, Janga SC *et al.* Primate vaginal microbiomes exhibit species specificity without universal Lactobacillus dominance. *ISME J* 2014;**8**:2431.

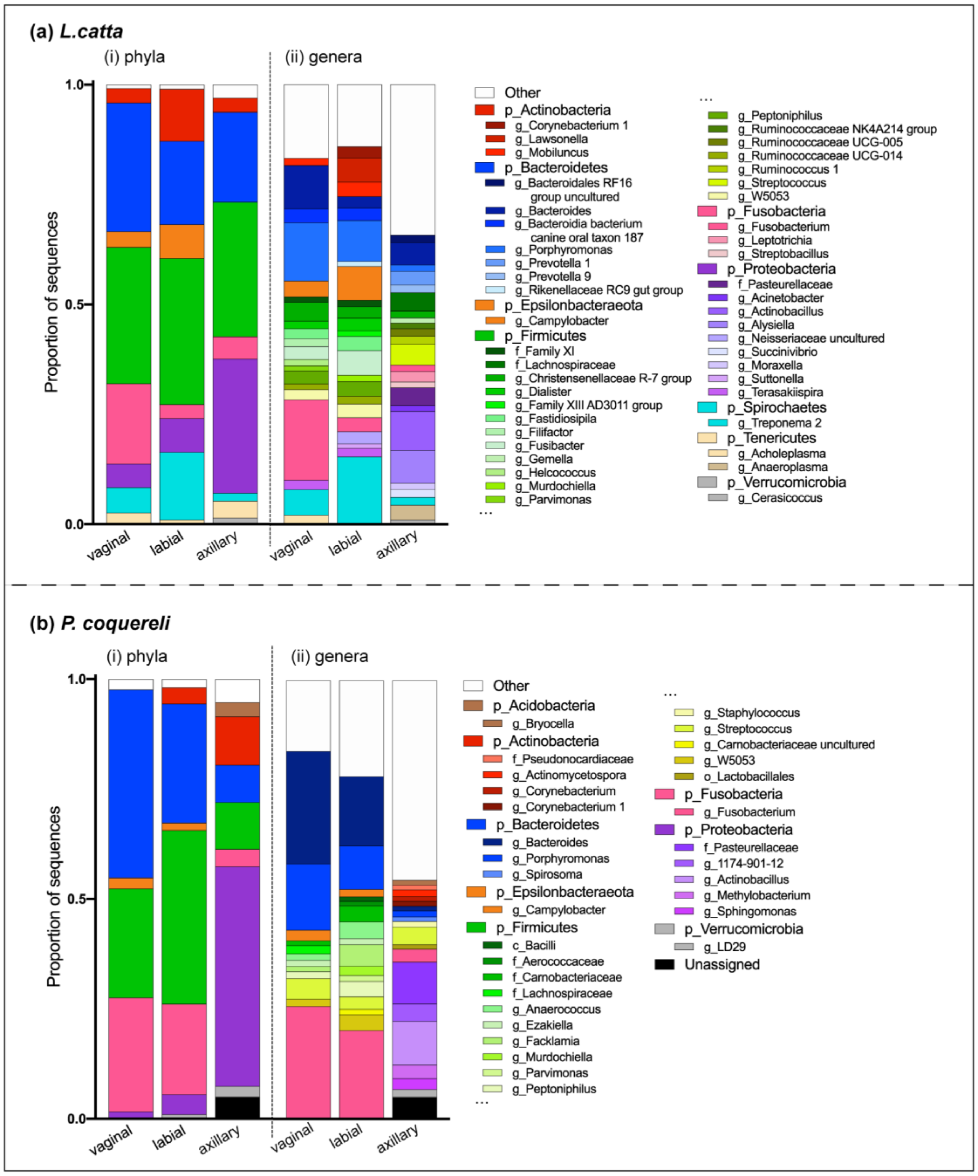

Figure 1. The relative abundances of bacterial taxa in the vaginal, labial, and axillary microbiomes of (a) ring-tailed lemurs (*L. catta*) and (b) sifakas (*P. coquereli*). For each microbiomes, bacterial (i) phyla and (ii) genera representing less than 1% of the microbiomes we combined into "Other".

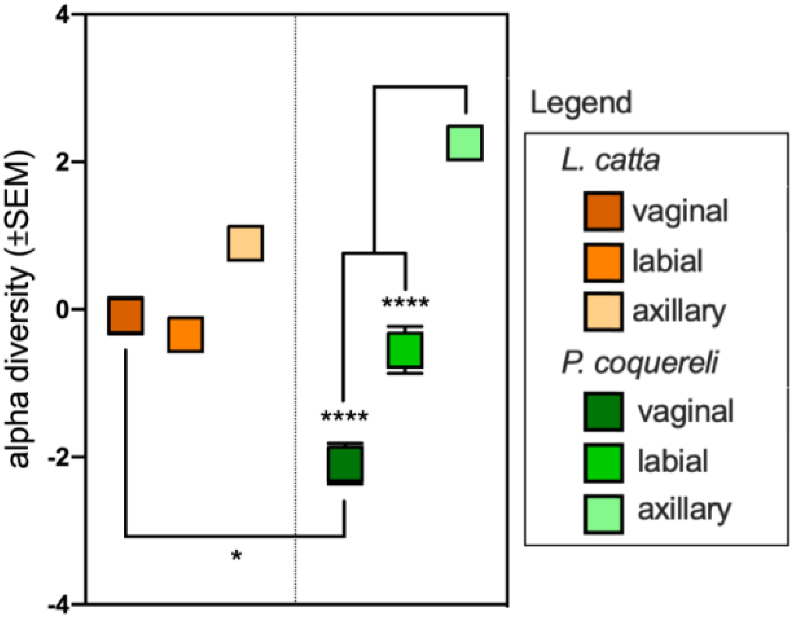

Figure 2. Alpha diversity with standard error means (SEM) across the three body sites in both species. Kruskal-Wallis test with Dunn's multiple comparison corrections; $p < 0.05$*, $p < 0.0001$****.

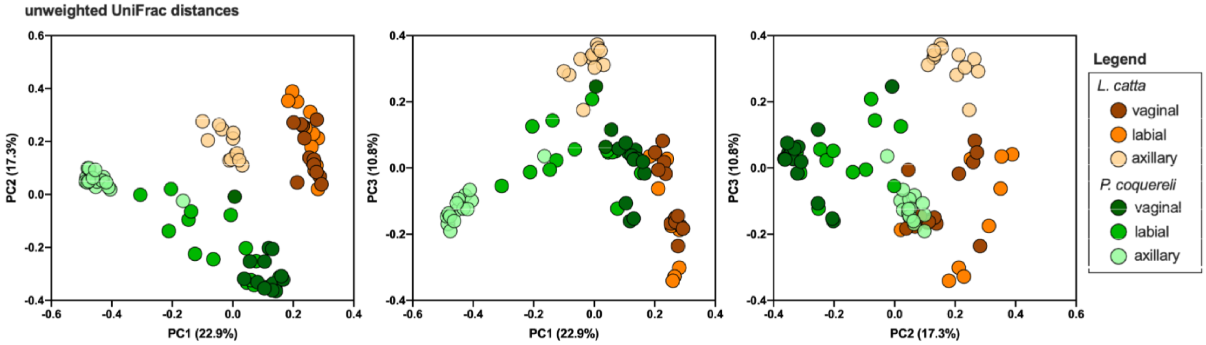

Figure 3. PCoA of unweighted UniFrac distances showing coordinate spaces of principal components 1, 2, and 3.

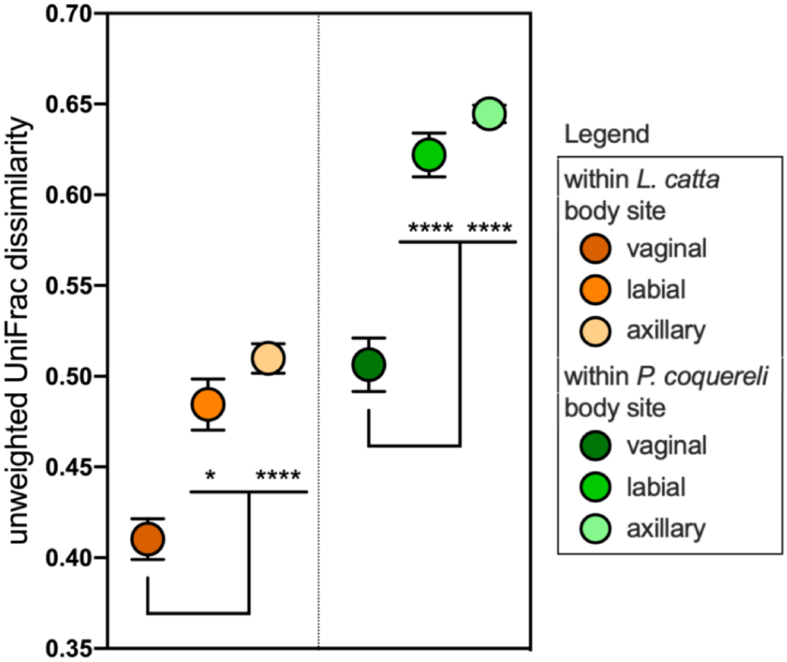

Figure 4. Variation in unweighted UniFrac distances between all samples within a given microbiome. Greater values indicate greater dissimilarity and greater interindividual variation in microbiome composition. Significance determined using *t*-tests with Bonferroni corrections.

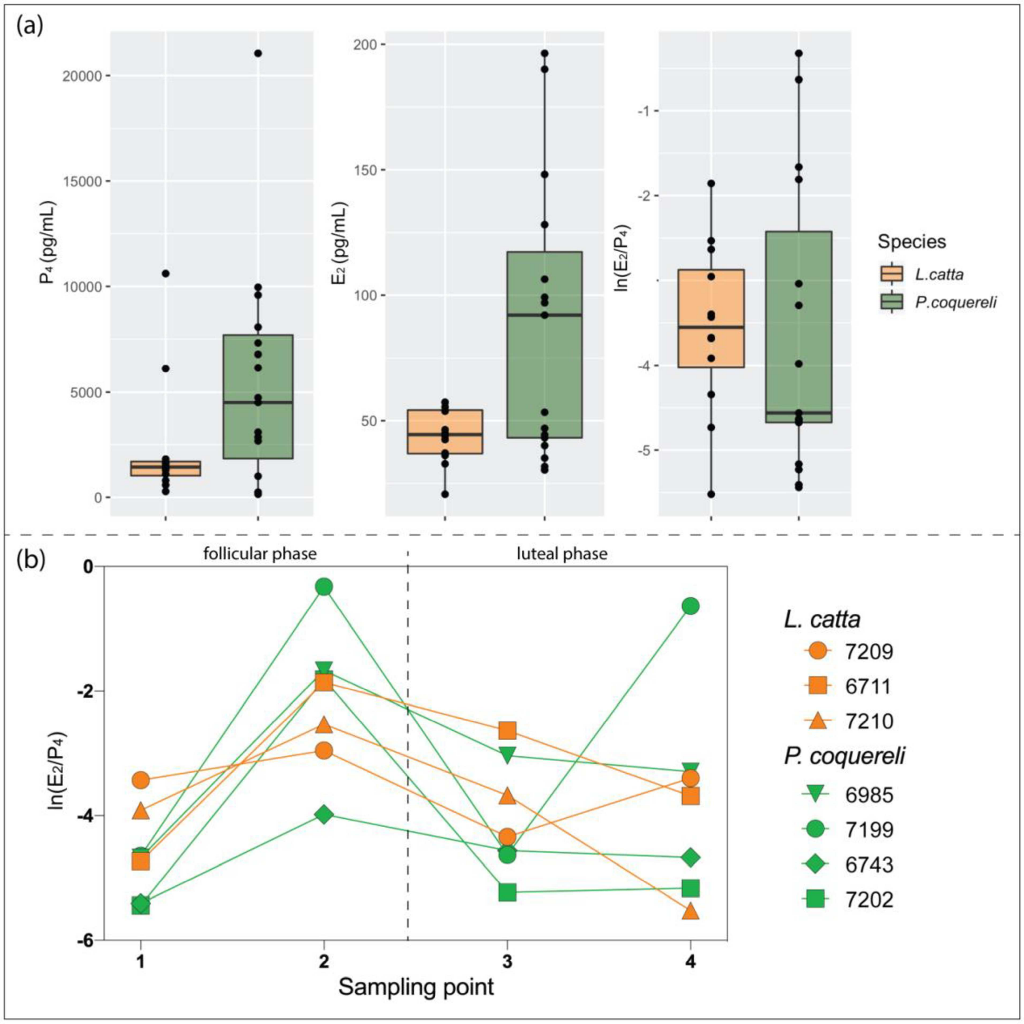

Figure 5. Variation in (a) E_2 and P_4 concentrations and $\ln E_2/P_4$ in both species and (b) variation in each lemur's $\ln E_2/P_4$ across the four sampling points with inferred cycle phase.

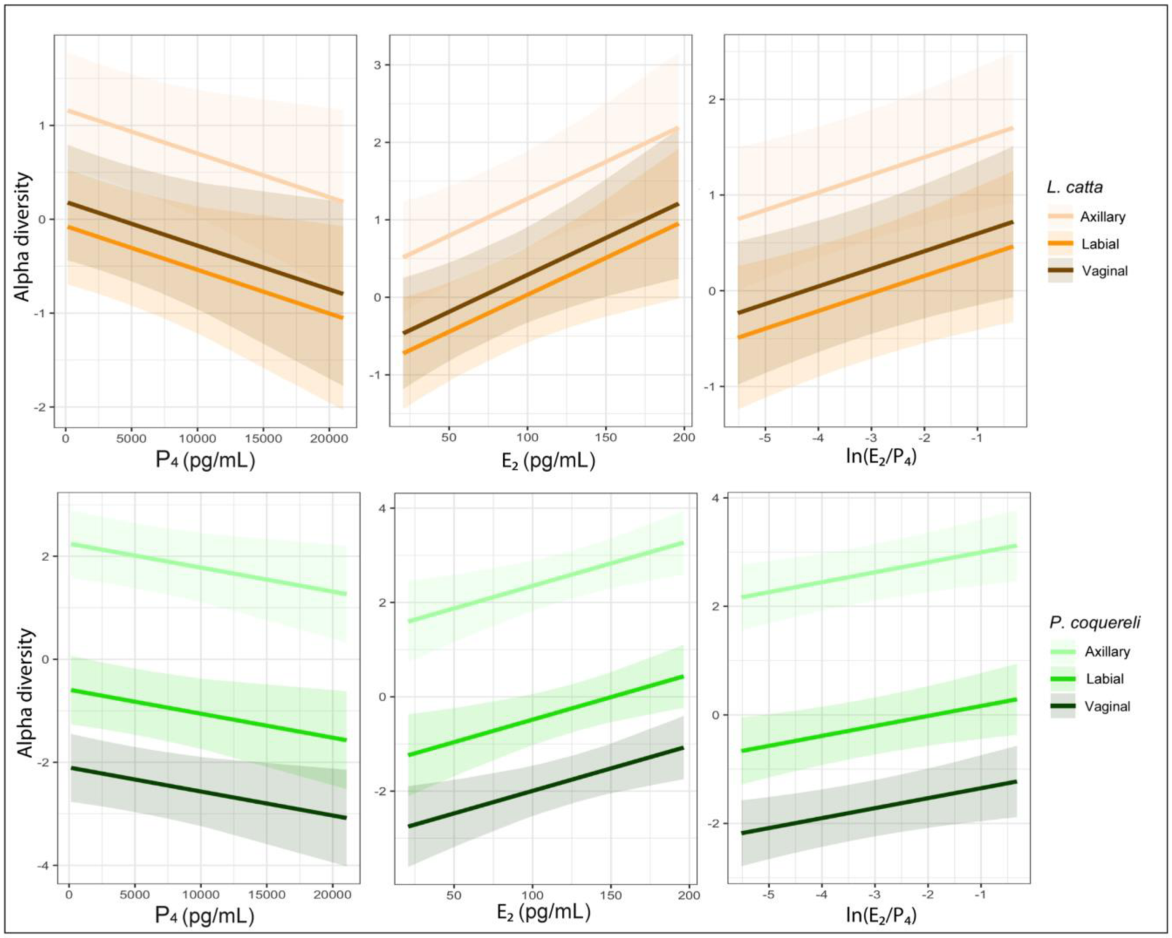

Figure 6. Model-based predictions of alpha diversity from P_4 and E_2 independently (GAM 1), and from $\ln(E_2/P_4)$ (GAM 2).

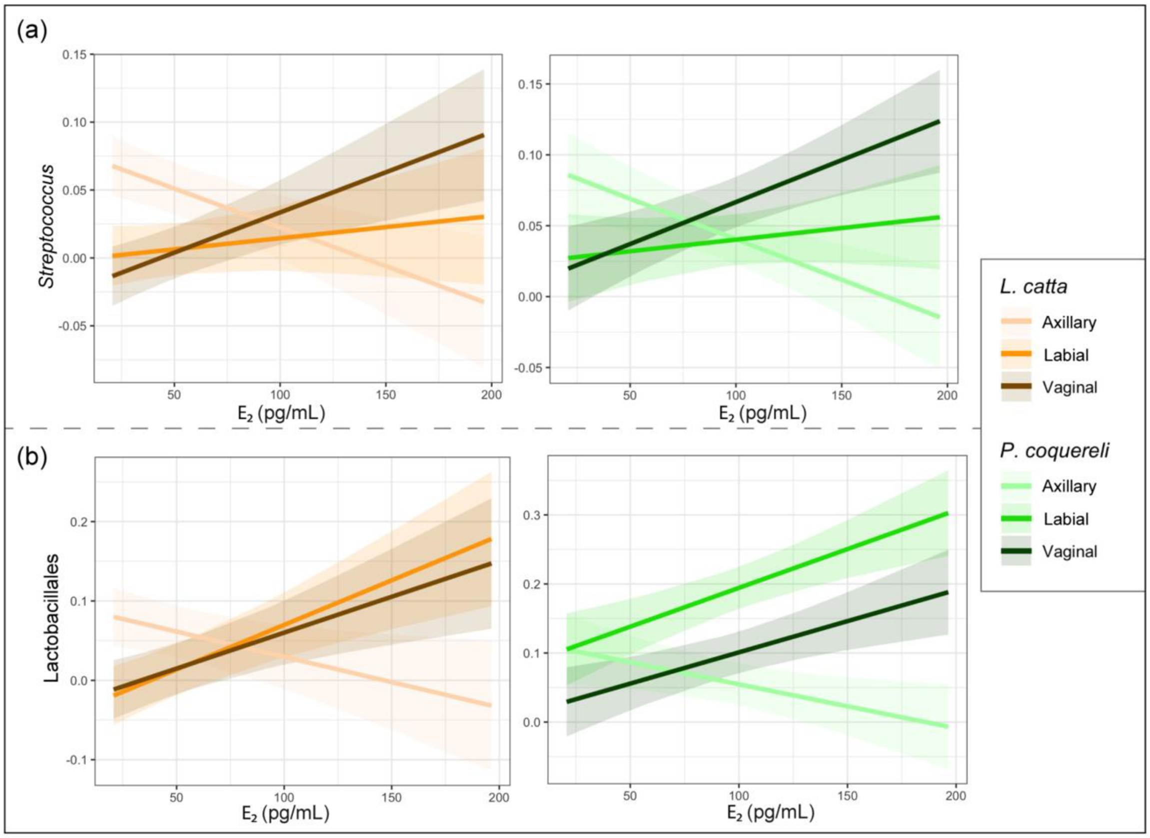

Figure 7. Model-based predictions of (a) *Streptococcus* and (b) Lactobacillales abundances from E_2 concentrations.

Table 1. Results of multivariate analyses of microbiome alpha diversity (GAM 1 & 2), and beta diversity (PERMANOVA). Bolded results are statistically significant ($p < 0.05$).

LMM 1: alpha diversity ~ species*body site + free-ranging + P_4 + E_2 + (1|Animal/Gland)	*F*	*p*
Species	3.745	0.111
Body Site	**75.684**	**<0.001**
Free-ranging status	0.184	0.686
P_4	**4.729**	**0.033**
E_2	**8.028**	**0.081**
Species × Body Site	**27.677**	**<0.001**
LMM 2: alpha diversity ~ species*body site + free-ranging + 1n(E2/P4) + (1|Animal/Gland)	*F*	*p*
Species	0.765	0.436
Body Site	**75.716**	**<0.001**
Free-ranging status	0.482	0.527
$\ln(E_2/P_4)$	**6.873**	**0.011**
Species × Body Site	**27.705**	**<0.001**
PERMANOVA: beta diversity ~ species*body site + animal/body site + free-ranging + P_4 + E_2 (strata = animal)	R^2	*p*
Species	**0.178**	**<0.001**
Body site	**0.179**	**<0.001**
Free-ranging status	**0.021**	**<0.001**
P_4	0.009	0.151
E_2	0.006	0.398
Animal	**0.061**	**<0.001**

Species × Body site	**0.118**	**<0.001**
Animal × Body Site	**0.084**	**0.038**