Investigating Lemur Microbiomes Across Scales and in Relation to Natural and Anthropogenic Variation

by

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
of Philosophy in the Department of
Evolutionary Anthropology in the Graduate School
of Duke University

2021

ABSTRACT

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Abstract

The composition and function of mammalian gut microbiomes are shaped by complex endogenous and exogenous factors that present on evolutionary and proximate timescales. In the Anthropocene era, host-associated microbiota are inevitably, yet differentially, influenced by natural and anthropogenic factors that vary across individuals and populations. In this dissertation, I used descriptive and experimental approaches, largely within a single species, the ring-tailed lemur (*Lemur catta*), to probe the roles of host physiology, environmental conditions, anthropogenic perturbation, and microbial environment in shaping primate microbiota across scales. First, I conducted a broad investigation of ring-tailed lemur gut microbiota and soil microbiota across 13 lemur populations (n = 209 individuals) spanning this species' natural range in Madagascar, as well as multiple captivity settings in Madagascar and the U.S. By analyzing the lemur and soil microbiota, I showed that lemur gut microbiota vary widely within and between wild and captive populations, and that lemur and soil microbiota covary, suggesting a role for environmental acquisition in shaping interpopulation variation. Second, I analyzed vaginal, labial, and axillary microbiota of female ring-tailed lemurs and Coquerel's sifakas (*Propithecus coquereli*) at the Duke Lemur Center (DLC) to demonstrate the influences of stable traits (e.g., species identity and mating system) and transient traits (e.g., ovarian hormones and forest access). We found that the effects of transient traits build on underlying differences mediated by

stable traits. Third, and further focusing on DLC lemurs, but with a concentration on anthropogenic influence, I worked with a team of researchers to perform an experimental manipulation in ring-tailed lemurs to determine the influence of antibiotic treatment, with or without subsequent fecal transfaunation, on lemur gut microbiomes. I applied ecological frameworks to show that different facets of lemur microbial communities, such as bacterial diversity and composition, followed different recovery trajectories following antibiotic treatment. Fourth, I expanded my focus back to multiple ring-tailed lemur populations in natural and captivity settings to investigate the links between anthropogenic disturbance and antibiotic resistance genes (ARGs). I analyzed ARGs in wild and captive lemurs and soil from their habitats to show that lemur ARGs were correlated with anthropogenic disturbance and covaried with soil ARGs; lemur resistomes reflects multiple routes of ARG enrichment, including via antibiotic treatment or environmental acquisition. Integrating across these four data chapters, my results reveal that (a) the foundations of lemur-associated microbiomes are structured according to broad environmental conditions (e.g., wild vs. captive populations), but that between and within these broad categories, lemur microbiota are sensitive to more nuanced environmental variation, (b) lemur microbiota and resistomes co-vary with environmental microbiota, demonstrating the potential role of environmental acquisition in shaping host-associated communities across varying environments, and (c) integrating host microbial data across scales (e.g., at the individual and population

level) with data on multiple facets of microbial communities (e.g., diversity, composition, membership, and resistomes), was key to providing a holistic perspective on host-associated and environmental microbe interactions across different microbial landscapes.

Dedication

I dedicate this dissertation to my parents, Libby Lyons and Alan Bornbusch, two biologists whose support and generosity know no bounds. You instilled in me scientific curiosity and a deep love of nature which has guided me personally and professionally to where I am today. Thank you.

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As I began graduate school at Duke in 2015, I aspired to study social behavior and cognition in mouse lemurs. Little did I know that a year later I would be studying ring-tailed lemur microbiomes (and I had never aspired to study anything even remotely close to microbiology). Nevertheless, I could not be more grateful with the direction of my dissertation research. I have two people in my professional life who played vital roles in helping me pursue this research: Christine Drea and Lydia Greene.

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To everyone I forgot to name in these Acknowledgements, I thank you. I promise it is not from lack of appreciation, just from the curdled brains of someone finishing a dissertation.

And always remember, the world is dominated by microbes: Who runs the world? Germs.

Chapter 1. Introduction

Communities of microbes, collectively known as microbiota (or microbiomes if the microbial genomes are considered), inhabit virtually every known habitat on Earth (Staley et al., 1997). Most notably, host-associated microbiota are found on the internal and external surfaces of animal bodies (McFall-Ngai et al., 2013; Zoetendal, Vaughan, & De Vos, 2006) and are now recognized as undeniably important to animal ecology, evolution, and health (Apprill, 2017; Christian, Whitaker, & Clay, 2015; Kolodny, Callahan, & Douglas, 2020). Understanding the factors that govern the structure and function of host-associated microbiota has therefore become a burgeoning area of research with relevance to nearly every branch of biological science (White House Office of Science and Technology Policy, 2016; Wood-Charlson et al., 2020). Because animals, including humans, evolved in a "bacterial world," ecological and evolutionary processes have shaped host-microbe symbiosis (McFall-Ngai et al., 2013; Sieber, Traulsen, Schulenburg, & Douglas, 2021). Comparative studies of hosts that span the tree of life have shown foundational patterns in microbiota that often reflect the ecological and evolutionary history of the hosts (e.g., phylosymbiosis; Brooks, Kohl, Brucker, van Opstal, & Bordenstein, 2016; Lim & Bordenstein, 2020). On more proximate scales, endogenous (e.g., physiology) and exogenous (e.g., environment) factors can rival or even overshadow the influences of evolutionary forces in shaping host-associated

microbiota (Greene et al., 2019; Phillips et al., 2012; Youngblut et al., 2019). In addition to naturally occurring processes, human activity can substantially shape animal microbiota; in the Anthropocene era, anthropogenic influences are nearly ubiquitous (Lewis & Maslin, 2015; Y. Zhu & Penuelas, 2020). Rarely, however, has research been focused on understanding the respective influences of these natural and anthropogenic factors on the microbiomes of a single host species that inhabits a wide range of environments. Even rarer are studies that consider these relationships across different scales (e.g., across individuals and populations). In this dissertation, I apply ecological frameworks to broadly examine variation in the microbiota of a single primate species, the ring-tailed lemur (*Lemur catta*), living in a wide array of environmental settings.

Hosts and their associated microbes represent complex ecosystems that experience similar processes to those seen in macro-ecosystems (e.g., forests or oceans) (Costello, Stagaman, Dethlefsen, Bohannan, & Relman, 2012; McDonald, Marchesi, & Koskella, 2020). Ecological succession, for example, whereby the members of an ecosystem undergo gradual change according to shifting environmental conditions, is reflected in microbial colonization of host body sites (Gilbert & Lynch, 2019; Peterfreund et al., 2012). Similarly, concepts of ecosystem diversity, stability, and resilience are increasingly being applied to better understand the health and function of host-associated microbiota (Coyte, Schluter, & Foster, 2015; Fassarella et al., 2021; Hickey, Zhou, Pierson, Ravel, & Forney, 2012). Importantly, these processes have differential impacts across scales; the

influences of changing ecosystems differ depending on the scale of investigation, e.g., at an individual vs. population level vs. lineage level (Greene et al., 2019; Smith et al., 2015). For example, differences in hosts' endogenous ecosystems (e.g., physiology or health) are drivers of within- or between-individual variation in microbiota (Cheng, Song, Xie, & Song, 2019); however, these factors are less likely to play a role in structuring population-level patterns (Spor, Koren, & Ley, 2011; Zmora, Zeevi, Korem, Segal, & Elinav, 2016; but see Amato et al., 2019) and focusing only on this scale risks the potential to not "see the forest for the trees." Similarly, examinations that rely solely on large-scale, population- or species-level perspectives to explain patterns in microbiota may miss the more nuanced details that influence microbiota on more proximate scales. A comprehensive understanding of the ecological factors that shape host-associated microbiota requires examination of both the "forest" and the "trees."

Akin to examinations across scales, investigations of host-associated microbiota must consider the governing influences of both natural and anthropogenic factors. Although it is increasingly difficult to find animal populations that are truly undisturbed by human activity, studies of wildlife have provided insight into the natural factors that shape microbiota (Stothart, Palme, & Newman, 2019; Williams et al., 2018). For example, microbial community structure is correlated with seasonal changes in diet (Baniel et al., 2021; Góngora, Elliott, & Whyte, 2021; Hicks et al., 2018), social interaction between conspecifics (Moeller et al., 2016; Sarkar et al., 2020), and habitat type within a species'

natural range (Bennett et al., 2016; Ellison et al., 2019). Unlike these natural phenomena that shape microbiota structure, presumably in an adaptive manner, anthropogenic factors can detrimentally alter microbiota, with implications for host well-being (Barelli et al., 2015; Hernández-Gómez, 2020). As exemplified in studies of wild vs. captive conspecifics, environments defined by human activity have significant impacts on animal microbiota (McKenzie et al., 2017; Redford, Segre, Salafsky, del Rio, & McAloose, 2012). Nevertheless, studies that rely on a strict dichotomy between wild vs. captive animals report broad patterns; the influences of natural and anthropogenic factors are more likely to occur along more nuanced gradients that vary with the host's environments.

An over-arching theme I investigated throughout my dissertation research was the role of interactions between host-associated and environmental microbiota. Hosts evolved in and, importantly, continue to exist in a "bacterial world" and they are, thus, components of large-scale microbial landscapes with myriad sources of microbes. One widely studied example of the acquisition of external microbes is the vertical transmission of microbes during mammalian birth (Ferretti et al., 2018; Korpela et al., 2018); the contribution of maternal microbes to infant microbiota colonization is a vital component of infant development (Mueller, Bakacs, Combellick, Grigoryan, & Dominguez-Bello, 2015; Wang et al., 2020). Similar to the sharing of microbes between mothers and infants, the horizontal transmission of microbes between conspecific hosts

can result in group ‘signatures’ that are hypothesized to play a role in social behavior and communication (Archie & Tung, 2015; Grieneisen, Livermore, Alberts, Tung, & Archie, 2017; Perofsky, Lewis, Abondano, Di Fiore, & Meyers, 2017). Although host-host microbial transmission has received significant scientific attention, the interactions between host-associated and environmental consortia, outside of epidemiological contexts, is relatively understudied. However, there is increasing recognition that commensal microbiota are amalgamations of microbes that stem from multiple sources experienced by the host over their lifetime (Adair & Douglas, 2017; Selway et al., 2020). For example, there is mounting evidence that relationships between diet and gut microbiota stem not only from the input of dietary nutrients, but the introduction of food-associated microbes (Smith, Snowberg, Caporaso, Knight, & Bolnick, 2015). Moreover, many animals practice forms of geophagy (i.e., earth-eating) and/or coprophagy (i.e., the consumption of feces), which are suggested to have adaptive purposes related to nutrient and microbial supplementation (Borruso et al., 2021; Johns & Duquette, 1991; Krishnamani & Mahaney, 2000); the consumption and incorporation of external microbes may play an important role in governing the structure and function of host-associated microbiota. That multiple sources contribute to host consortia is further evidenced by a burgeoning interest in microbial “source tracking” and the associated development of “source-sink” analysis tools (Knights et al., 2011; Shenhav et al., 2019). Ultimately, a comprehensive understanding of host-microbe relationships,

particularly in the Anthropocene era, must consider how these ever-changing microbial landscapes may be shaping host-associated communities.

The effects of the Anthropocene era on natural environmental are particularly evident in Madagascar, one of the world's greatest biodiversity hotspots. Over Madagascar's ~80 million years of geographical isolation, diverse habitats and climatic variation facilitated wide-spread adaptive radiations (Ganzhorn, Lowry, Schatz, & Sommer, 2001; Herrera, 2017; Reddy, Driskell, Rabosky, Hackett, & Schulenberg, 2012), resulting in rates of endemism ranging from 85-100% across floral and faunal groups (Goodman & Benstead, 2005). Contrasting with its rich biodiversity, Madagascar is also consistently ranked as one of the economically poorest countries in the world (Paternostro, Razafindravonona, & Stifel, 2001). This combination of rich natural resources and widespread poverty has led to vast exploitation of natural ecosystems, with much of Madagascar's natural habitat being degraded or destroyed (Harper, Steininger, Tucker, Juhn, & Hawkins, 2007; Vieilledent et al., 2018). This system is an exemplary, albeit unfortunate, example of the interplay between natural and anthropogenic factors; Malagasy wildlife and their microbes co-evolved without human influence for millions of years but those relationships are now invariably influenced by human activity (Bublitz et al., 2015; Junge, Barrett, & Yoder, 2011; Larsen et al., 2016).

There are five reasons I focused on a single host species, particularly ring-tailed lemurs, for my dissertation research: (1) By studying a single host species, I was able to

avoid the confound of variation in host phylogenetic placement, a factor that underlies significant variation in microbiota (Nishida & Ochman, 2019; Youngblut et al., 2019); (2) Because ring-tailed lemurs are highly omnivorous (Gould, 2006; Sauther, Sussman, & Gould, 1999), they are less reliant on specialized gut microbiota to process dietary nutrients. Their gut microbes are thus less sensitive to natural variation in diet (compared to e.g., dietary specialists; Frankel, Mallott, Hopper, Ross, & Amato, 2019; Greene et al., 2019; Greene et al., 2019; Youngblut et al., 2019), another factor that is widely studied for its relationships with gut microbiota. These first two reasons allowed me to focus on the influences of non-phylogenetic and non-diet factors that have previously been underrepresented in primate microbiota literature; (3) The ecological flexibility of ring-tailed lemurs allowed me to study of populations across environments that ranged from relatively undisturbed forests to degraded natural habitats and multiple captivity settings; (4) Because ring-tailed lemurs have long been the subjects of scientific study (Jolly, 1964, 1966a, 1966b), including multiple investigations of microbiota (Bennett et al., 2016; Fogel, 2015; Modesto et al., 2018; Villers, Jang, Lent, Lewin-Koh, & Norosoarinaivo, 2008), previous studies have provided important foundations for my research; (5) Ring-tailed lemurs are endangered (Gould & Sauther, 2016; LaFleur, Clarke, Reuter, & Schaeffer, 2016) and, as their population numbers continue to decline, research on their microbiota in natural and captivity settings has valuable potential to inform *in situ* conservation efforts and *ex situ* care and husbandry.

Under the broad goal of using ecological frameworks to examine the factors that govern host-associated microbiota, I had three main aims: (1) to describe the variation in microbiota diversity, composition, membership, and resistomes within a single host species, (2) to test the extent to which microbiota variation is shaped by natural and anthropogenic factors, and (3) to investigate the role of environmental microbes in shaping host-associated bacterial communities and resistomes. Ultimately, my research is firmly grounded in the concepts of One Health: animal, including human, and environmental systems are inextricably intertwined and an understanding of one should be considered in the context of the others.

For my first data chapter (Chapter 2), I performed a large-scale, comparative investigation of ring-tailed lemur gut microbiota in thirteen populations (n = 209 individuals) across eight natural habitats in Madagascar and in two captivity settings in Madagascar and three captivity settings in the U.S. Simultaneously, I collected soil microbiota from eight of the lemurs' habitats. For this project, I aimed to (a) show that lemur microbiota variation reflects environmental variability that is underrepresented by a simple wild vs. captive dichotomy and (b) test whether exposure to and incorporation of environmental microbes contributed to interpopulation variation in lemur gut microbiota. As a large-scale description of gut microbiota in wild and captive ring-tailed lemurs, this chapter provides important foundations for me to build the next three data chapters.

In my second data chapter (Chapter 3), I investigated host stable traits (species identity, mating system, and body site traits) and transient traits (reproductive hormones and forest access) as mediators of lemur microbiota that could reflect evolutionary vs. more proximate timescales, respectively. Unlike in my other chapters, I focused on females of two lemur species, ring-tailed lemurs and Coquerel's sifaka (*Propithecus coquereli*) and characterized microbiota at three body sites: armpit (axilla), labia, and vagina. To test whether ovarian hormones (estradiol and progesterone) related to aspects of lemur microbiota, I sampled the lemurs longitudinally across a single ovarian cycle. Finally, I examined relationships between the host's forest access and the various microbiota. Beyond expanding my research to other lemur species and non-gut microbiota, this chapter highlighted the complexity of factors that can influence a single population under human care.

In my third data chapter (Chapter 4), I applied classical ecological principles (i.e., the 'diversity begets stability' and 'keystone species' hypotheses; Banerjee, Schlaeppi, & van der Heijden, 2018; Berry & Widder, 2014; Konopka, 2009; Wohl, Arora, & Gladstone, 2004) to better understand short- and long-term microbial dynamics during community perturbation recovery. Specifically, as part of a large-scale project examining relationships between microbiota, condition-dependent signals (e.g., olfactory communication), and host health, we used an experimental manipulation in male, ring-tailed lemurs at the DLC, to test the influences of antibiotic treatment and subsequent

fecal transfaunation on the diversity, composition, and resistomes of lemur gut microbiota. Through this targeted, experimental approach, I focused on the causal effects of antibiotic treatment, a specific, but impactful, factor that influences host-associated microbiota, particularly in captive settings. In this chapter, we also broaden the examination of microbiota to include antibiotics resistance genes (ARGs), a component of microbiota with significant implications for lemur health and care.

In my final data chapter (Chapter 5), I continue my investigation of ARGs in lemur microbiota by re-analyzing (using shotgun metagenomics) a subset of lemur fecal and soil samples from Chapter 2. Although antibiotic treatment is the most direct route by which ARGs can develop (as examined in Chapter 3), contact with ARG reservoirs in e.g., non-wildlife animals, humans, or the environment, can facilitate the transmission of ARGs to wildlife hosts. In this chapter, I (a) characterized ARGs in the gut microbiota of 100 ring-tailed lemurs from multiple wild and captive populations, (b) determined whether lemur ARGs correlate with components of anthropogenic disturbance faced by the lemur populations, and (c) tested for covariation between ARGs in wild lemurs and their environments (e.g., soil). With these data, I aimed to highlight that antibiotic resistance is not solely a threat to human health, but also a potential ecological and conservation concern that extends well outside traditionally studied clinical settings.

Thesis format

This thesis comprises an introduction (Chapter 1), four data chapters (Chapters 2-5), and a conclusion (Chapter 6). Chapters 2 and 4 are under review and revision, respectively, in *Animal Microbiome*; Chapter 3 has been published in the FEMS Microbiology Ecology; and Chapter 5 is in preparation as an invited submission under a Special Research Topic in Frontiers: Ecology and Evolution. Although each chapter represents my own work as a graduate student, all data chapters represent collaborative efforts. Chapter 4, in particular, stemmed from a large-scale, integrative study conceived by my advisor, Christine Drea, and carried out by myself and other lab members. All studies were accomplished through guidance from my advisor.

Chapter 2. Gut microbiota of ring-tailed lemurs (*Lemur catta*) vary across natural and captive populations and correlate with environmental microbiota

The content of this chapter has been submitted for publication in *Animal Microbiome* under the special collection "Microbiomes in Wild Animals: In captivity and the field." The full reference is as follows:

Bornbusch, S.L., Greene, L.K., Rahobilalaina, S., Calkins, S., Rothman, R.S., Clarke, T.A., LaFleur, M., & Drea, C.M. *Under review*. Gut microbiota of ring-tailed lemurs (*Lemur catta*) vary across natural and captive populations and correlate with environmental microbiota

Introduction

The structure of gut microbial communities within vertebrates is influenced in part by endogenous host factors, such as genotype and physiology (Amato et al., 2019; Hansen, Gulati, & Sartor, 2010; Milani et al., 2020), and in part by exogenous factors, such as sociality, seasonality, habitat quality, and diet (Rothschild et al., 2018; Tasnim, Abulizi, Pither, Hart, & Gibson, 2017; Tung et al., 2015). These exogenous factors can influence which microbial taxa in a gut community thrive or become depauperate, as amply demonstrated in dietary studies (David et al., 2014; Greene, McKenney, O'Connell, & Drea, 2018; Kartzinel, Hsing, Musili, Brown, & Pringle, 2019; Youngblut et al., 2019), or they can provide opportunities for more direct routes of microbial acquisition (Cardona et al., 2018; Hyde et al., 2016; Kuthyar, Manus, & Amato, 2019; Peccia & Kwan, 2016). For example, the transmission of microbes between hosts, including horizontal pathogen transfer (Durrer & Schmid-Hempel, 1994; Kulkarni & Heeb, 2007; A. B. Pedersen & Davies, 2009) or vertical transmission during the birthing process and nursing (Arora, Sadovsky, Dermody, & Coyne, 2017; Funkhouser & Bordenstein, 2013), are significant drivers of host health and development. There is, likewise, the potential for horizontal acquisition of microbes via exposure to environmental consortia on natural (e.g., soil) and man-made surfaces, plus on food and in water (Hyde et al., 2016; Seedorf et al., 2014; Selway et al., 2020; Smith et al., 2015; Walke et al., 2014); however, this latter route to shaping host-associated communities,

hereafter referred to as 'environmental acquisition,' remains understudied. Here, we match-sampled ring-tailed lemur (*Lemur catta*) feces and soil from 13 settings, representing both a large portion of the lemurs' natural habitat range in Madagascar and a range of captive housing conditions in Madagascar and the U.S. (Table 1), to (a) characterize variation in host gut microbiota, (b) characterize variation in soil microbiota, and (c) test for any covariation between host and soil communities. Examining environmental microbes alongside host-associated
communities is a first step to understanding the role of environmental acquisition in population-level differences between host microbiomes.

Previous studies of intraspecific variation in gut microbiota, often framed using a 'wild vs. captive' comparison, have provided valuable descriptions of differences in presumed extremes (Greene et al., 2019; McKenzie et al., 2017; E. Schmidt, Mykytczuk, & Schulte-Hostedde, 2019). For example, researchers often report a 'signal of captivity,' whereby the gut microbiota of captive hosts differ significantly from those of wild conspecifics, converging on a perturbed or 'humanized' composition (Clayton et al., 2016; McKenzie et al., 2017; Yao et al., 2019). Perturbations of this nature are generally attributed to commercial diets that include manufactured chow and cultivated produce (Bornbusch et al., 2019; Clayton et al., 2016; Frankel et al., 2019); nevertheless, studies of captive populations have been focused on accredited zoos or rescue facilities that may not represent the range of captive conditions or may be confounded by within-species

comparisons across continents (Frankel et al., 2019; Gibson et al., 2019; Greene et al., 2019). Even comparative field studies have been limited in the number of populations per species studied, typically to a few populations that differ on a given metric of interest (e.g., season, habitat type or quality; Baniel et al., 2021; Hicks et al., 2018; Ren et al., 2017)). Because hosts experience a wider range of environmental settings than is typically encompassed within wild vs. captive comparisons, a broader comparative approach is necessary to provide a more comprehensive and nuanced understanding of gut microbiota variation.

As noted, differential exposure to environmental microbes provides potential for horizontal transmission and environmental acquisition (Chen, Chen, Weng, Shaw, & Wang, 2017; Guarner et al., 2006; Mushegian, Arbore, Walser, & Ebert, 2019; Selway et al., 2020; Smith et al., 2015; Walke et al., 2014), with the ingestion of specific microbes being linked to novel digestive functions of the gut microbiota (Borruso et al., 2021; Li et al., 2016; Sylvain & Derome, 2017). Under certain conditions, environmental acquisition has been shown to outweigh vertical transmission as the main mode of microbial colonization (Inoue & Ushida, 2003; Kikuchi, Hosokawa, & Fukatsu, 2007). Although environmental acquisition may promote heterogeneity within and between hosts (Leftwich, Edgington, & Chapman, 2020), its role rarely has been considered a differentiating factor between wild and captive hosts. Husbandry practices and veterinary care, for example, introduce cleaning products and antibiotics to the

microbial environment of captive animals (Hartmann et al., 2016; Maamar, Hu, & Hartmann, 2020), further differentiating it from natural habitats (Thompson et al., 2017), with potentially critical consequences to microbiome structure and function.

Ring-tailed lemurs are semi-terrestrial, omnivorous strepsirrhine primates (Gould, 2006; Jolly, Sussman, Koyama, & Rasamimanana, 2006b) that occupy various habitats across southern Madagascar (Gabriel, 2013) and also survive well in captivity (Mason, 2010). Their ecological flexibility, coupled with existing knowledge about their gut microbiome (Bennett et al., 2016; Bornbusch el al., *in prep*; Fogel, 2015; Greene et al., 2019), motivates broader comparative study of intraspecific variation that takes environmental acquisition of microbes into consideration. Here, we combine 16S rRNA sequencing and statistical tools based on source-sink ecological theory (Dias, 1996) to analyze covariation between lemur gut microbiota (e.g., sink communities) and soil microbiota (e.g., source communities). Given the variability of environmental factors across our multiple settings, we expect the diversity, membership, and composition of lemur gut microbiota and soil microbiota to differ within and between three, broad 'environmental' conditions: wilderness, captivity in Madagascar, and captivity in the U.S (Table 1).

If diet or habitat quality were the main driver of gut microbiota composition, we would expect (a) wild lemurs to show variation between their natural settings, (b) captive lemurs, regardless of continent, to show similar gut microbiota between their

Table 1. Research settings (names, descriptions, and locations) and samples collected under wilderness conditions and under captivity conditions in Madagascar and the U.S. A subset of the samples collected were omitted from analyses owing to low-yield extractions or low-quality sequencing. Soil samples could not always be obtained. Maps show locations of each setting; the gray shaded area shows the natural range of wild ring-tailed lemurs in Madagascar.

Condition	Setting (abbreviation)	Setting description	Samples: collected (analyzed)	
			fecal	soil
wilderness	Amoron'I Onilahy (AMO)	Riverine gallery forest, dry scrub forest	20	4 (3)
	Berenty Reserve (BER)	Semi-arid dry forest, spiny forest	22 (19)	4
	Beza Mahafaly Special Reserve (BEZ)	Riverine gallery and semi-arid spiny forest	26	4 (3)
	Fiheranana (FIH)	Dry forest and spiny forest	2	-
	Isalo National Park (ISO)	Dry deciduous forest	18 (16)	3
	Ivohiboro (IVO)	Humid forest, grassland	15	-
	Ranomay (RAN)	Dry forest	13	2 (1)
	Tsimanampetsotsa National Park (TSI)	Dry forest and spiny forest	29 (28)	8
captivity: Madagascar	Lemur Rescue Center (Toliara, Madagascar; LRC)	Outdoor enclosures in dry and spiny forest	33	2 (1)
	Various towns (pets)	Pets housed in human dwellings	8	-
captivity: U.S.	Duke Lemur Center (Durham, NC; DLC)	Indoor and outdoor enclosures, including free ranging in semi-deciduous forest	22	3 (2)
	National Zoological Park (Washington, DC; NZP)	Indoor and outdoor enclosures, moated island with vegetation	4	-
	North Carolina Zoo (Asheboro, NC; NCZ)	Indoor and outdoor enclosures, moated island with vegetation	3	-
		Total samples:	215 (209)	30 (25)

Madagascar
U.S.
Legend:
wilderness: BEZ, FIH, AMO, BER, RAN, TSI, IVO, ISA
captivity: Madagasacar: pet, LRC
captivity: U.S.: NCZ, DLC, NZP

settings (reflecting commercial diets and perturbed habitats), and (c) wild and captive lemurs to differ most drastically from one another, in line with prior studies (Clayton et al., 2016). If, however, environmental acquisition were to play a major role in shaping lemur gut microbiota, we would again expect (a) wild lemurs to show variation between their natural settings (reflecting the soil microbiota of the lemurs' habitat), but we would expect (b) Malagasy lemurs (wild and captive) to share certain soil-derived microbiota, differing most drastically from lemurs in captivity in the U.S., and (c) differential access to soil within captive conditions to correlate with differential soil-associated microbes present in hosts. With regard to the latter, for example, we might expect greater proportions of soil-associated microbes in captive lemurs that gain access to natural enclosures compared to their counterparts that are housed indoors.

Results

Lemur gut microbiota: Variation in diversity, membership, and composition

Alpha diversity. Across the gut microbiota of all ring-tailed lemurs sampled in this study, metrics of alpha diversity differed significantly by environmental condition (Generalized Linear Models or GLM; Shannon: $F = 23.773$, $p < 0.001$; Faith's phylogenetic: $F = 4.415$, $p = 0.013$; Figures 1a, b) and by setting (GLM; Shannon: $F = 13.157$, $p < 0.001$; Faith's phylogenetic: $F = 5.628$, $p < 0.001$; Figures 1c, d; Appendix A.1.1; Tables 5-8). The gut microbiota of wild lemurs and captive lemurs in the U.S. (hereafter,

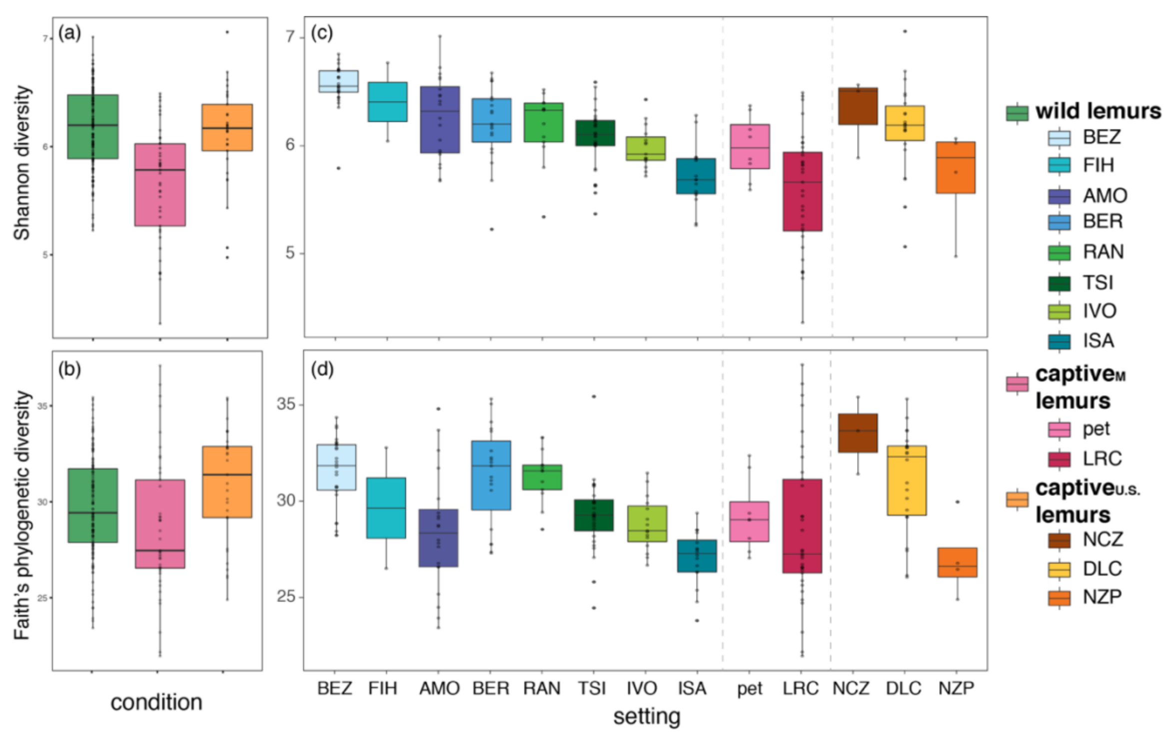

Figure 1. Alpha diversity of gut microbiota in wild and captive ring-tailed lemurs. Alpha diversity metrics of lemur gut microbiota (a, b) collapsed by environmental condition, including wilderness (wild lemurs; green), captivity in Madagascar (captive$_M$ lemurs; pink), and captivity in the U.S. (captive$_{U.S.}$ lemurs; orange), and (c, d) averaged across individuals for each of the 13 different settings inhabited (reprising the color codes of each condition, delineated by dashed vertical lines). Shown are both (a, c) Shannon diversity and (b, d) Faith's phylogenetic diversity. Across the (c, d) settings within a condition (see Table 1 for names of abbreviated study sites), the data are plotted in descending order of mean Shannon diversity.

'captive$_{U.S.}$ lemurs') were similarly diverse overall (pairwise Wilcoxon test; Shannon: $p = 0.635$; Faith's phylogenetic: $p = 0.056$; Figures 1a, b), whereas those of captive lemurs in Madagascar (hereafter, 'captive$_M$ lemurs') were significantly less diverse (pairwise Wilcoxon test; Shannon, wild vs. captive$_M$ lemurs: $p < 0.001$; wild vs. captive$_{U.S.}$ lemurs: $p < 0.001$; Faith's phylogenetic, wild vs. captive$_M$ lemurs: $p = 0.022$; wild vs. captive$_{U.S.}$

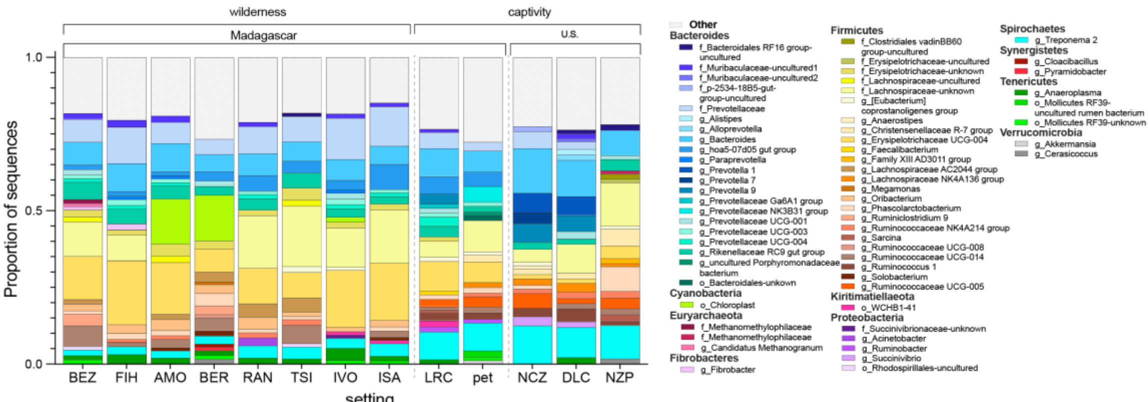

Figure 2. Gut microbiota membership in wild and captive ring-tailed lemurs. Mean proportion of sequences assigned to microbial taxa across lemurs at each of the 13 different settings, with the three conditions (wilderness, captivity in Madagascar, and captivity in the U.S.) delineated by dashed vertical lines (see Table 1 for names of abbreviated study sites). Taxa are identified by phylum and deepest possible taxonomic level (i.e., genus level or above); those representing < 1% of the microbiomes were combined into the category "Other."

lemurs: $p = 0.021$; Figures 1a, b). Within environmental condition, however, both metrics of alpha diversity varied widely between the different settings (Figures 1c, d; Appendix A.1.1; Tables 5-8). For example, among wild lemurs, setting was a significant predictor of both metrics of alpha diversity (GLM; Shannon diversity: $F = 20.768$, $p < 0.001$; Faith's phylogenetic: $F = 11.104$, $p < 0.001$). Sex was not a significant predictor in any models of either alpha diversity metric (Appendix A.1.1; Tables 5-8).

Community membership. The membership of lemur gut microbiota included 64 abundant taxa (i.e., those that accounted for >1% of sequences). Of these 64 taxa, only four (6.2%) were shared across lemurs from all settings: the genera *Bacteroides* (phylum

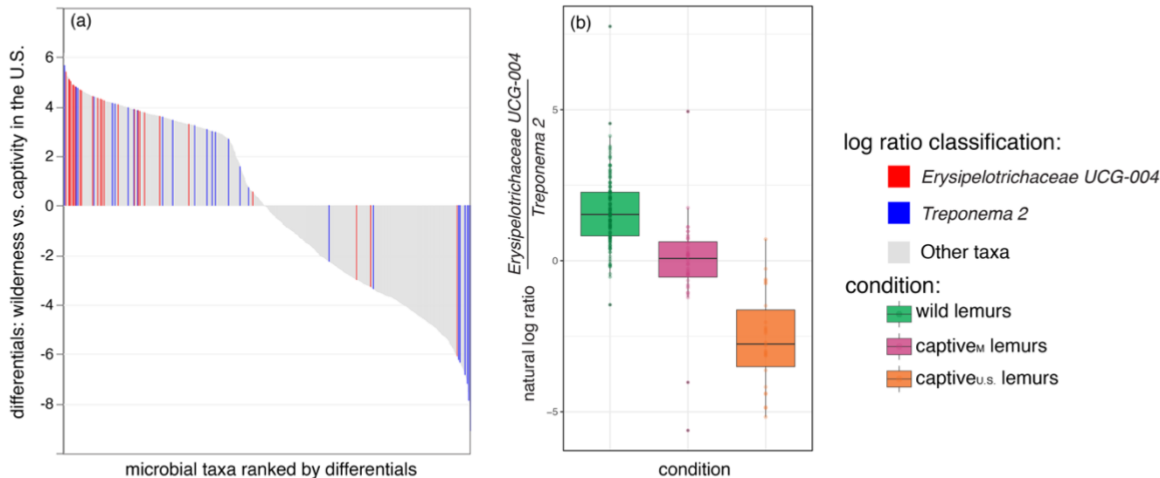

Figure 3. Differential abundance of *Erysipelotrichaceae UCG-004* and *Treponema* 2 in the gut microbiota of wild and captive ring-tailed lemurs.(a) Differential rank plot showing lemur gut microbial taxa (x axis) ranked by their differentials (y axis; the estimated log-fold changes for taxa abundances across sample groups) for lemurs in the wilderness vs. captivity settings in the U.S. Those taxa that are more abundant in the wild lemurs compared to captive lemurs in the U.S. appear on the right side of the plot whereas those that are less abundant in wild lemurs appear on the left side. *Erysipelotrichaceae UCG-004* and *Treponema* 2 differentials are highlighted in red and blue, respectively, with other taxa represented in gray. (b) Natural log ratios of *Erysipelotrichaceae UCG-004* vs. *Treponema* 2 in lemurs across all three environmental conditions.

Bacteroidetes), *Rikenellaceae RC9 gut group* (Bacteroidetes), *Erysipelotrichaceae* UCG-004 (Firmicutes), and *Treponema 2* (Spirochaetes). Within condition, five (7.8%) taxa were shared by all wild lemurs, whereas 10 (15.6%) and six (9.4%) taxa were shared by captive$_{M}$ and captive$_{U.S.}$ lemurs, respectively (Figure 2). Using Analysis of Compositions of Microbiomes (ANCOM), we identified 801 amplicon sequence variants (ASVs) that were differentially abundant across the three conditions. For example, members of the Erysipelotrichaceae family characterized the microbiota of wild lemurs, whereas taxa

from the Spirochaetaceae and Prevotellaceae families were more abundant in the gut microbiota of captive lemurs from both continents. *Erysipelotrichaceae UCG-004* and *Treponema 2*, for example, were abundant in all lemurs (Figure 2), but the log ratios of the two genera distinguished lemur gut microbiota by the three environmental conditions and, in particular, differentiated wild lemurs from $captive_{U.S.}$ lemurs (Figure 3).

Beta diversity. The composition of lemur gut microbial communities was significantly distinct across the three environmental conditions, as revealed by beta diversity (Permutational Multivariate Analysis of Variance or PERMANOVA; wild vs. $captive_{M}$ lemurs: pseudo-F = 30.169, $p < 0.001$; wild vs. $captive_{U.S.}$ lemurs: pseudo-F = 97.912, $p < 0.001$; $captive_{M}$ vs. $captive_{U.S.}$ lemurs: pseudo-F = 20.808, $p < 0.001$). Across all subjects, gut microbiota composition clustered distinctly by condition (principal coordinate analysis of unweighted UniFrac distances; Figures 4a, b). One notable exception, however, owed to a single pet lemur: Unlike its in-country peers (i.e., other $captive_{M}$ lemurs), its microbial community structure matched those of wild lemurs (see arrows in Figures 4a, b).

Across the three environmental conditions, Random Forest Analysis accurately assigned 208 of the 209 gut microbial profiles to the correct condition, with a low (0.48%) out-of-bag (OOB) error rate. Based on its gut microbiota, only the previously mentioned pet lemur (see arrows in Figure 2a, b) was misclassified as a wild lemur. Across the 13

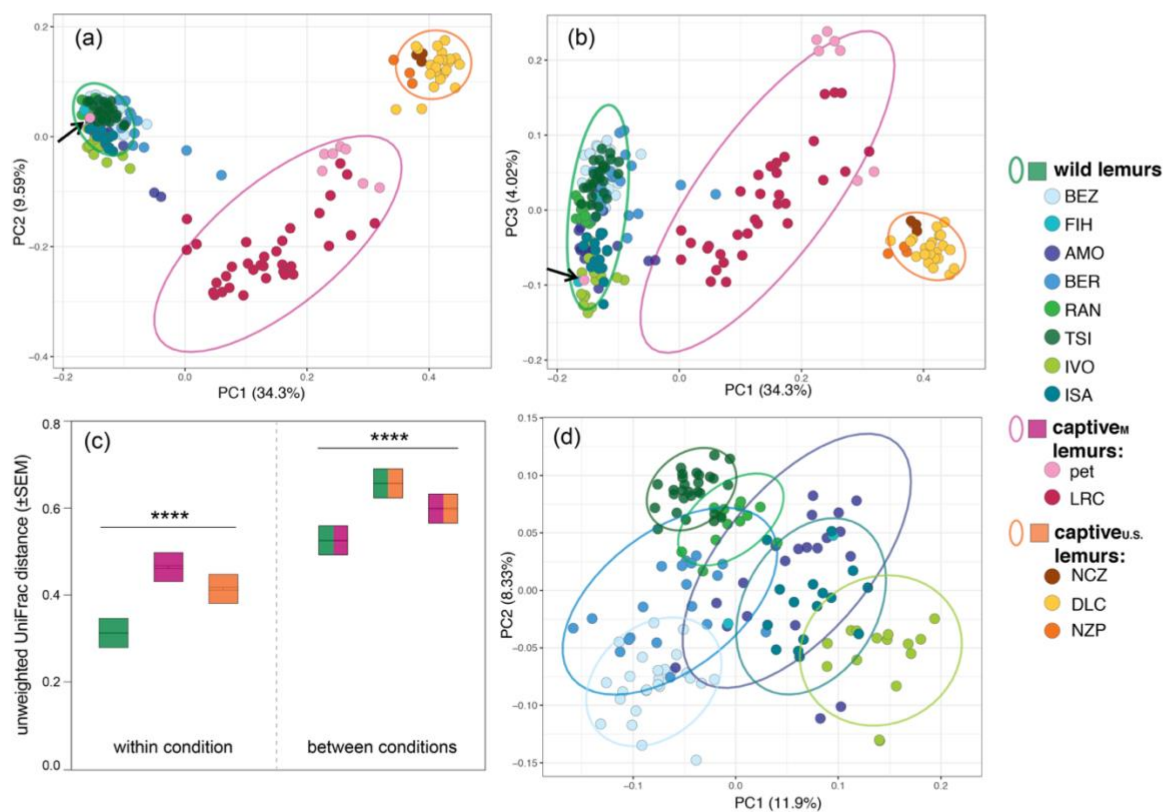

Figure 4. Beta diversity (unweighted UniFrac distances) of lemur gut microbiota across three environmental conditions – wilderness (wild lemurs; green), captivity in Madagascar (captiveM lemurs; pink), and captivity in the U.S. (captiveU.S. lemurs; orange) – that encompass 13 setting (see Table 1 for names of abbreviated study sites). (a, b) Principal coordinate plots, showing axes 1 and 2, or 1 and 3, respectively, of individual gut microbial communities colored by setting and encircled by normal data ellipses reflecting environmental condition. (c) Mean beta diversity distance scores within (single color) and between (two colors) environmental conditions. (d) Principal coordinate plots, showing axes 1 and 2, for the eight settings within the wilderness condition. Kruskal-Wallis test with Benjamini-Hochberg correction; **** $p < 0.0001$.

settings, Random Forest Analysis accurately classified 189 of the 209 microbial profiles (OOB error = 9.57%). The gut microbial communities of wild and captive lemurs in

Madagascar were misclassified at rates of 7.9% and 7.3%, respectively, whereas those of captive lemurs in the U.S. were misclassified at a rate of 20.6%.

With respect to uniformity within environmental condition, the composition of gut microbial communities were least dissimilar between wild lemurs and most dissimilar between $captive_{M}$ lemurs (Kruskal-Wallis test; main effect of condition on beta diversity: $\chi^2 = 27487$, $p < 0.0001$; pairwise Wilcoxon test; within wild vs. within $captive_{M}$ lemurs: $p < 0.001$; within wild vs. within $captive_{U.S.}$ lemurs: $p < 0.0001$; Figure 4c). Between conditions, the microbiota of wild and $captive_{M}$ lemurs were the least dissimilar, whereas the microbiota of wild vs. $captive_{U.S.}$ lemurs were the most dissimilar (pairwise Wilcoxon test: 'wild vs. $captive_{M}$' vs. 'wild vs. $captive_{U.S.}$', $p < 0.0001$; 'wild vs. $captive_{M}$' vs. '$captive_{M}$ vs. $captive_{U.S.}$', $p < 0.0001$; Figure 4c). Considering wild lemurs only, microbiota composition clustered by setting (Figure 4d). Although there was some overlap between settings, the patterns are suggestive of microbial 'signatures' across different settings.

Soil microbiota: Variation in diversity, membership, and composition

Alpha diversity. Across the eight settings for which we sampled soil, the alpha diversity of soil microbiota did not vary significantly between conditions (Kruskal-Wallis test; Shannon diversity: $\chi^2 = 3.3457$, $p = 0.187$; Faith's phylogenetic: $\chi^2 = 3.433$, $p = 0.179$; Figure 5) nor between settings (Kruskal-Wallis test; Shannon diversity: $\chi^2 = 7.496$,

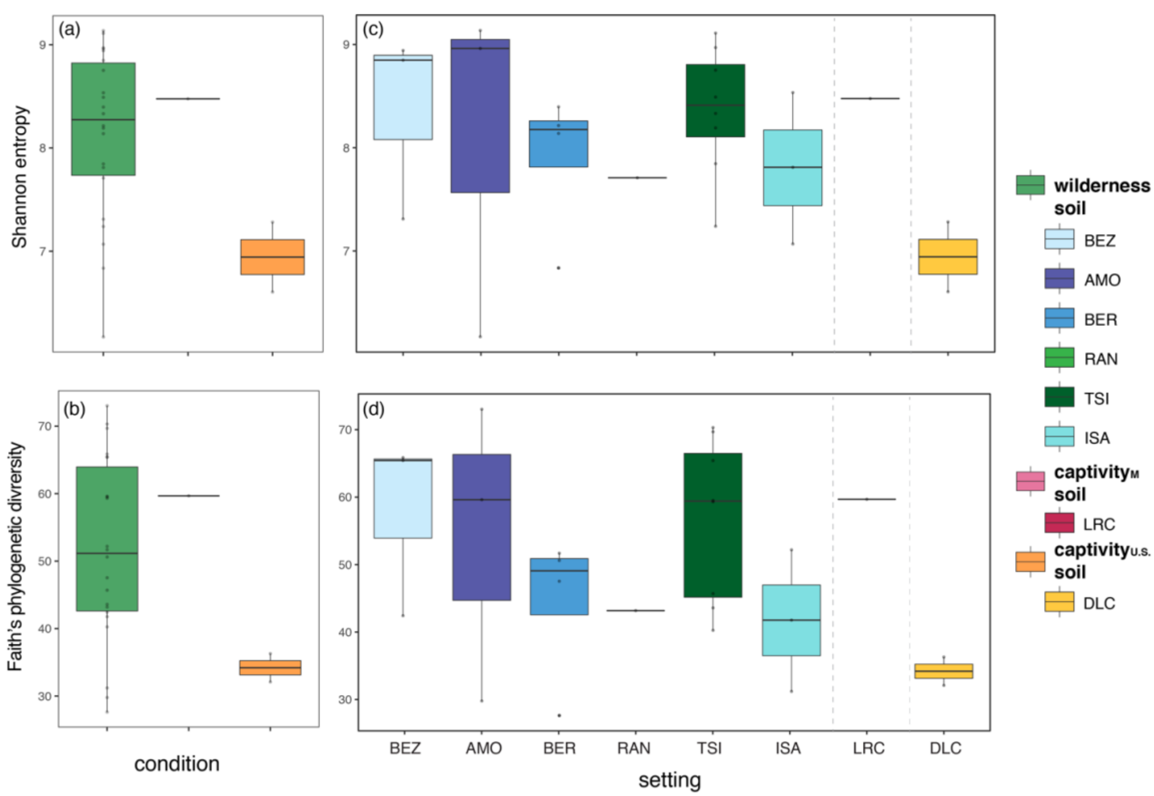

Figure 5. Alpha diversity metrics of soil microbiota (a, b) collapsed by environmental condition, including wilderness (wilderness soil; green), captivity in Madagascar (captivity$_{M}$ soil; pink), and captivity in the U.S. (captivity$_{U.S.}$ soil; orange) and (c, d) averaged across individuals for each of the eight different settings (reprising the color codes of each condition, delineated by dashed vertical lines). Shown are both (a, c) Shannon diversity and (b, d) Faith's phylogenetic diversity. Across the (c, d) settings within a condition (see Table 1 for names of abbreviated study sites), the data are plotted in descending order of mean Shannon diversity.

p = 0.379; Faith's phylogenetic: χ^2 = 8.936, p = 0.257; Figure 5). These null findings may owe to small sample sizes.

Community membership. The membership of soil communities included 77 abundant taxa, of which none were shared across all settings (Figure 6). Of the identified soil microbiota, 78.12% were unique to the soil samples and were not found in any

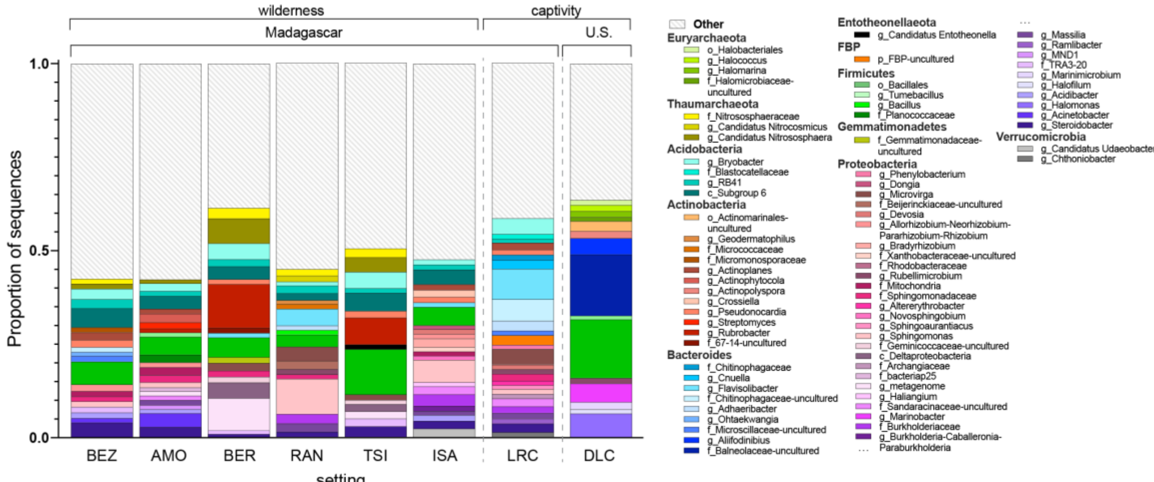

Figure 6. Membership of soil microbiota. Mean proportion of sequences assigned to microbial taxa of soil at each of the eight settings sampled, within the three conditions: wilderness (wilderness soil; green), captivity in Madagascar (captivity$_{M}$ soil; pink), and captivity in the U.S. (captivity$_{U.S.}$ soil; orange), which are delineated by dashed vertical lines (see Table 1 for names of abbreviated study sites). Taxa are identified by phylum and deepest possible taxonomic level (i.e., genus level or above); those representing < 1% of the microbiomes were combined into the category "Other".

lemur fecal samples. For the five wild populations for which we sampled soil, only five abundant taxa were shared: the genera *Bacillus* (phylum Firmicutes), *Steroidobacter* (Proteobacteria), *Bryobacter* (Acidobacteria), and *RB41* (Acidobacteria), and an unidentified member of the class Subgroup 6 (Acidobacteria). ANCOM identified nine ASVs that were differentially abundant across all soil samples, five of which (55.6%) belonged to the Balneolaceae family. In addition, compared to soil from Madagascar, the soil communities from the U.S. (hereafter, captivity$_{U.S.}$ soil) were differentially enriched

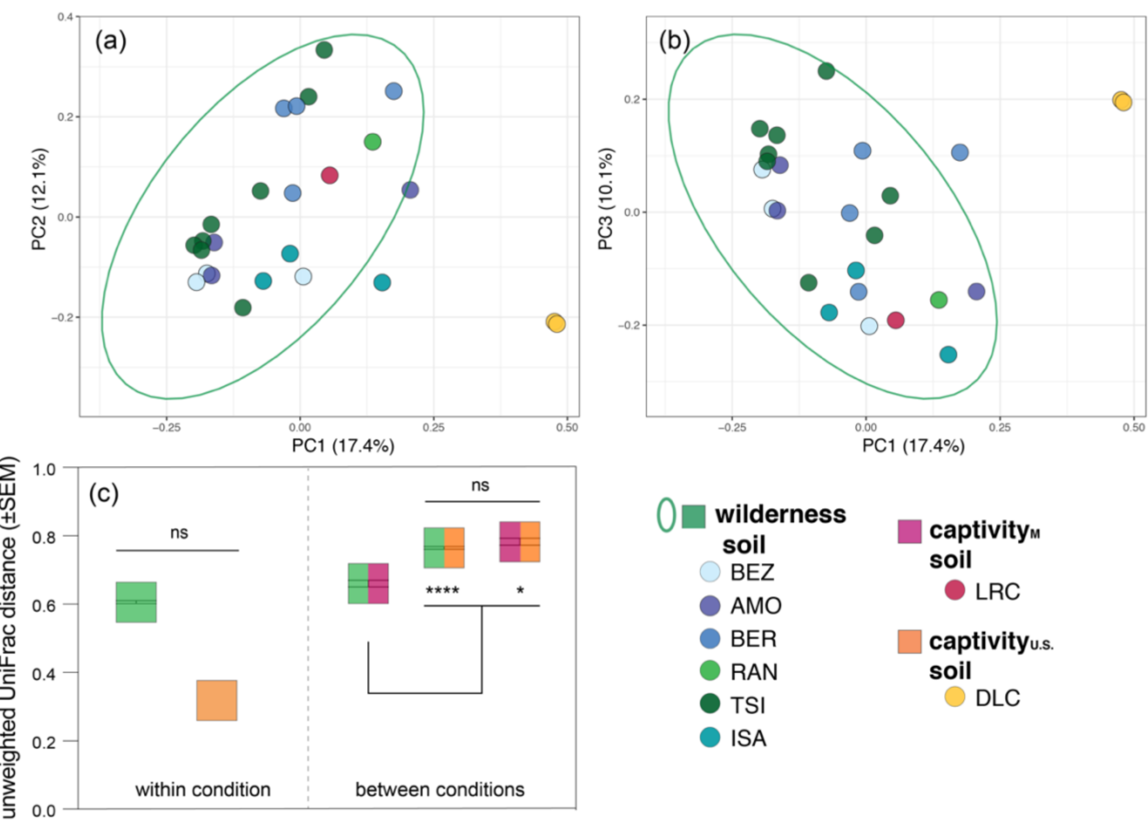

Figure 7. Beta diversity (unweighted UniFrac distances) of soil microbiota across three environmental conditions - wilderness (wilderness soil; green), captivity in Madagascar ($\text{captivity}_{\text{M}}$ soil; pink), and captivity in the U.S. ($\text{captivity}_{\text{U.S.}}$ soil; orange) – that encompass eight setting (see Table 1 for names of abbreviated study sites). (a, b) Principal coordinate plots, showing axes 1 and 2, or 1 and 3, respectively, of soil microbial communities colored by setting and encircled by normal data ellipses reflecting environmental condition. (c) Mean beta diversity distance scores within (single color) and between (two colors) environmental conditions. Kruskal-Wallis test with Benjamini-Hochberg correction; * $p < 0.05$, **** $p < 0.0001$.

for the genus *Bacillus*. By contrast, members of the family Nitrososphaeraceae (Thaumarchaeota) and the genus *Acinetobacter* (Proteobacteria) characterized soil from the wilderness and captivity settings in Madagascar (hereafter, wilderness and $\text{captivity}_{\text{M}}$ soils, respectively; Appendix A.1.3; Figure 31).

Beta diversity. The beta diversity of the soil microbiota varied between conditions (Figure 7), but only significantly so between wilderness and captivity$_{M}$ soils (PERMANOVA; wilderness vs. captivity$_{M}$ soils: pseudo-F = 1.337, p = 0.202; wilderness vs. captivity$_{U.S}$ soils: pseudo-F = 3.897, p = 0.012; captivity$_{M}$ vs. captivity$_{U.S}$ soils: pseudo-F = 7.752, p = 0.329). Variation in soil communities within a condition was not significantly different between wilderness soils and captivity$_{U.S.}$ soils (pairwise Wilcoxon test, p = 0.130; Figure 7c). Between conditions, wilderness and captivity$_{M}$ soils had the lowest dissimilarities (pairwise Wilcoxon test; 'wilderness vs. captivity$_{M}$' vs. 'wilderness vs. captivity$_{U.S}$' soils: p < 0.001; 'wilderness vs. captivity$_{M}$' vs. 'captivity$_{M}$ vs. captivity$_{U.S}$': p = 0.016; 'wild vs. captivity$_{U.S}$' vs. 'captivity$_{M}$ vs. captivity$_{U.S}$': p = 0.338 Figure 7c).

Covariation of lemur gut and soil microbiota

There were 191 ASVs shared between lemur gut and soil microbiota. These were dominated by members of the Firmicutes (75 ASVs or 39.3%), Proteobacteria (49 ASVs or 25.6%), and Bacteroidetes (38 ASVs or 19.9%) phyla. Although many of the shared taxa were abundant (>1%) in either lemur gut or soil microbiota, only one genus, *Acinetobacter* (Proteobacteria), was abundant in both lemur gut and soil microbiota.

As would be predicted if environmental acquisition impacts host microbial communities, there was a significant correlation between the abundances of microbes in lemur feces and soil samples (Mantel test; r = 0.494, p < 0.001). The proportion of 'soil-

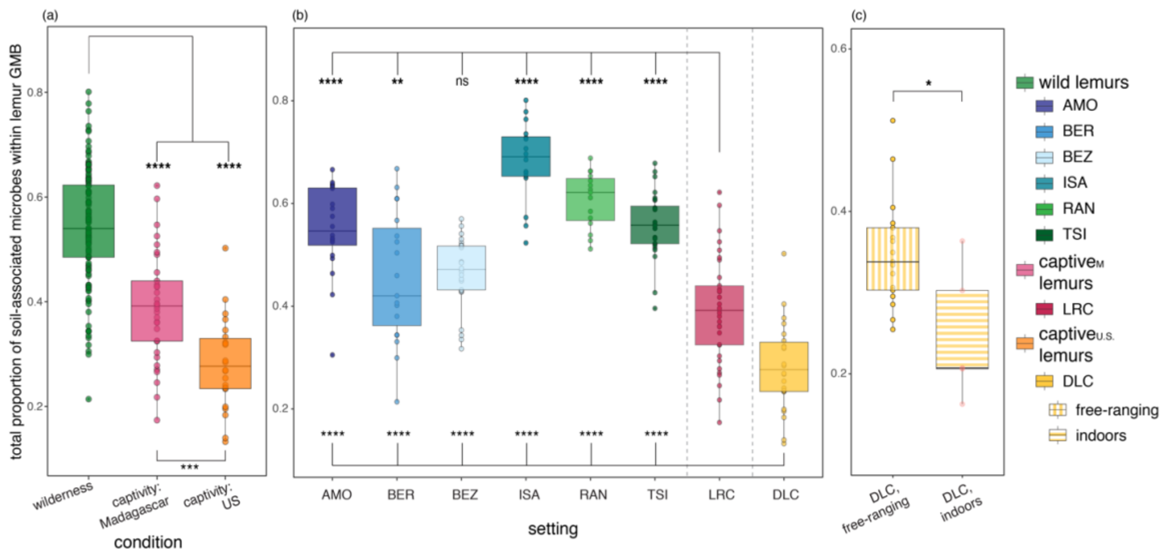

Figure 8. Mean proportion of total soil-associated microbes in the gut microbiota of lemurs (a) collapsed by environmental condition: wilderness (wild lemurs; green), captivity in Madagascar (captive$_M$ lemurs; pink), and captivity in the U.S. (captive$_{U.S.}$ lemurs; orange). Averaged soil-associated microbes across individuals for (b) each of the eight different settings (reprising the color codes of each condition, delineated by dashed vertical lines) and (c) at the Duke Lemur Center (DLC) that were semi-free-ranging in natural habitat enclosures or were housed indoors. Kruskal-Wallis test with pairwise comparisons and Benjamini-Hochberg correction; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ns = nonsignificant.

associated' microbes found in lemur gut microbiota varied significantly across conditions (Kruskal-Wallis test; $\chi^2 = 73.862$, $p < 0.001$; Figure 8a) and settings (Kruskal-Wallis test; $\chi^2 = 112.69$, $p < 0.001$; Figure 8b). Overall, the gut microbiota of wild lemurs had significantly greater proportions of soil-associated microbes compared to those of all captive lemurs (pairwise Wilcoxon test, $p < 0.001$; Figure 8). In addition, captive$_M$ lemurs had significantly greater proportions of soil-associated microbes in their gut microbiota compared to captive$_{U.S.}$ lemurs (pairwise Wilcoxon test; $p < 0.001$; Figure 8). For lemurs

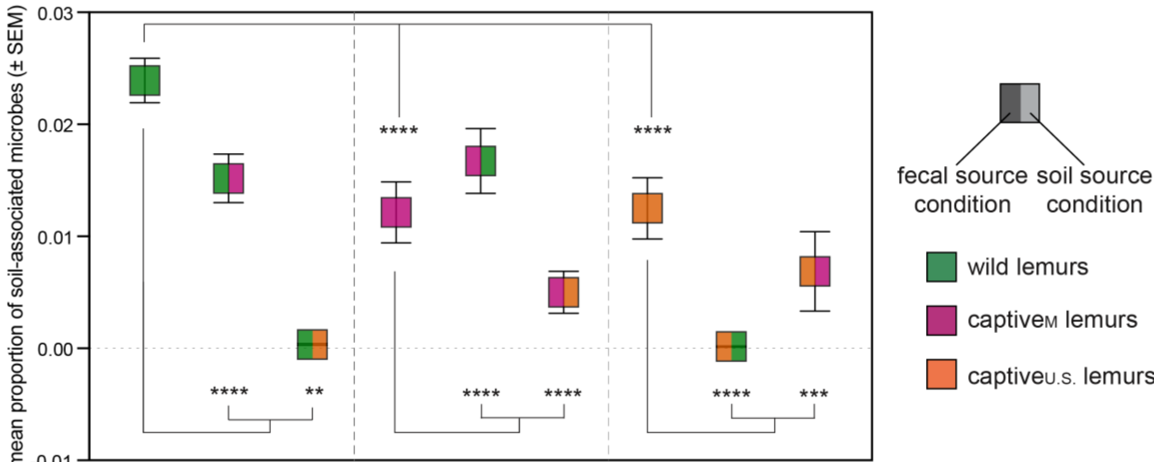

Figure 9. Mean proportion of soil-associated microbes in the gut microbiota of lemurs within (single color) and between (two colors) the three conditions: wilderness (wild lemurs; green), captivity in Madagascar ($captive_M$ lemurs; pink), and captivity in the U.S. ($captive_{U.S.}$ lemurs; orange) – that encompass eight setting. Within the gut microbiota of lemurs from a given condition (first color = fecal source condition), values show the proportion of soil associated microbes from a given condition (second color = soil source condition). Kruskal-Wallis test with pairwise comparisons and Benjamini-Hochberg correction; ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

housed at the DLC, those that semi-free-ranged in outdoor, natural habitat enclosures had significantly greater proportions of soil-associated microbes in their gut microbiota compared to lemurs that did not have access to forested enclosures (Kruskal-Wallis test; $\chi^2 = 4.641$, $p = 0.031$; Figure 8c).

Soil from within a lemur's setting accounted for, on average, significantly greater proportions of the lemur's gut microbiota than did soil communities from other settings (Figure 9; Appendix A.1.2; Table 9). Overall, the greatest proportion of soil-associated microbes within lemur gut microbiota occurred when the lemurs and soil were both

from the wilderness (Figure 9; Appendix A.1.2; Table 9). The proportion of soil-associated microbes in lemur gut microbiota dropped to near zero when comparing the lemur gut and soil microbiota between samples from the wilderness and those from captivity in the U.S. (Figure 9; Appendix A.1.2; Table 9).

Discussion

Through extensive fecal and soil sampling from multiple settings representing the ring-tailed lemurs' natural range in Madagascar and in captivity on two continents, we have highlighted (1) the wide and often underrepresented variety of gut microbiota present within a single host species, (2) the lack of a universal 'signal of captivity' that uniformly decreases microbial diversity, (3) aspects of microbiota membership and composition that differ markedly between wild and captive populations, and (4) covariance between lemur gut and soil microbiota, which points to a key role of environmental microbes. Researchers have reported host 'group signatures' in microbiota, often attributed to the social transmission of microbes (Leclaire, Nielsen, & Drea, 2014; Sarkar et al., 2020; Theis, Schmidt, & Holekamp, 2012; Tung et al., 2015; Vernier et al., 2020); our results expand this concept to 'population signatures' and draw attention to the potential role of environmental acquisition of microbes in mediating significant inter-population variation.

Across populations of wild lemurs, we first observed substantial variation in gut microbiota diversity, membership, and composition, indicating that there is not a single 'representative' gut community for wild ring-tailed lemurs, as is likely the case for most host species. Nonetheless, the pattern of natural variation observed did not always meet expectations. For example, lemurs living in what is considered a relatively 'pristine' site, IVO – a recently discovered humid forest patch that is relatively undisturbed by human activity – unexpectedly had the second-lowest diversity of gut microbes. To the extent that lack of disturbance is a proxy of habitat quality, this pattern would be inconsistent with previous reports that greater habitat quality promotes more diverse gut microbiota (Amato et al., 2013; Barelli et al., 2015). In prior studies, the gut microbiota of ring-tailed lemurs were relatively unaffected by habitat degradation (Bennett et al., 2016). Therefore, either pristine habitats can be of low quality or the ecological and dietary flexibility of this species may dampen the impact of variation in habitat quality and type, relative to more specialized primates (e.g., folivores; Barelli et al., 2020; Greene et al., 2019; Kohl, Skopec, & Dearing, 2014; Trosvik, Rueness, de Muinck, Moges, & Mekonnen, 2018). That we found significant, natural, inter-population variation in a relatively hardy and robust species (Cameron & Gould, 2013; Gabriel, 2013) suggests that hosts with greater sensitivity to environmental variation, including habitat quality and type, would likely show even greater variation than that described herein. If so, studies constrained

to single or few host populations are likely to underrepresent the wide-scale, natural variation in host gut microbiota.

We next observed gut microbiota to be compositionally distinct across populations of captive lemurs. Contrary to many previous studies (Chi et al., 2019; Clayton et al., 2018; Clayton et al., 2016; Hale et al., 2019), but consistent with others (Greene et al., 2021; Narat et al., 2020; Nelson, Rogers, Carlini, & Brown, 2013; Tsukayama et al., 2018), the gut microbiota of captive lemurs were not consistently less diverse than those of wild lemurs nor were they compositionally homogenized by the similar commercial diets provided to captive subjects (Clayton et al., 2016; Xiao et al., 2019). Heterogeneous gut microbiota could reflect slight differences in the diets provided (as the produce and browse available differ between captivity settings), but such minor dietary variation is unlikely to be the sole driver of such marked microbial differences, particularly in an omnivorous host. Non-dietary factors must have contributed to distinguishing the gut communities of captive lemurs. Indeed, the gut microbiota of captive lemurs in Madagascar were compositionally more similar to those of their wild counterparts than to those of captive lemurs in the U.S. Based on this observation, we suggest that the effect of a commercial diet is not necessarily the strongest differentiator of gut consortia and that the effects of captivity cannot be standardized across populations. Our results raise questions about the commonly held view that greater alpha diversity is both (a) a hallmark of wild individuals and (b) a

proxy for a healthier gut community (Borbón-García, Reyes, Vives-Flórez, & Caballero, 2017; Y. Cheng et al., 2015; Clayton et al., 2018; Fujimura, Slusher, Cabana, & Lynch, 2010; Ma et al., 2020). Although we did not assess gut health, pet lemurs fed diets of rice and fruit, living in close contact with people and domestic animals, often housed solitarily indoors, are prone to disease (Chomel, Belotto, & Meslin, 2007; LaFleur, Clarke, Reuter, & Schaefer, 2019; LaFleur et al., 2021); yet, their gut consortia were as diverse as those of wild lemurs living at the relatively pristine site, IVO. Moreover, captive lemurs from the DLC and NCZ in the U.S. had some of the most diverse gut consortia, equaling the greatest diversity seen in wild lemurs (e.g., in BEZ lemurs). These results add to the mounting evidence (Barelli et al., 2020; Tong et al., 2020; Watson et al., 2019) that alpha diversity alone, without the context of host ecology and other microbiome data, should not be used to extrapolate about the health state of gut consortia or the host's environment.

We also found that, between wild and captive lemurs, the membership and composition of gut microbiota was indicative of the environmental condition. There was little evidence of a diverse 'core' microbiome, as only four taxa were found to be abundant across all lemur populations. Two of those core taxa, *Erysipelotrichaceae UCG-004* and *Treponema 2*, were differentially abundant between the three conditions. Despite links between members of *Erysipelotrichaceae* and high-fat, commercial diets in humans (Kaakoush, 2015), *Erysipelotrichaceae* microbes were reported to be enriched in wild

compared to captive chimpanzees (Campbell et al., 2020), mirroring our findings in lemurs. Furthermore, the genus *Erysipelotrichaceae UCG-004* was more abundant in the gut microbiota of chimpanzees, relative to humans (Nishida & Ochman, 2019), and in folivorous woolly lemurs compared to other lemur species (Greene et al., 2020). The functionally diverse members of the *Treponema* genus were more abundant in the gut microbiota of captive vs. wild hosts in other species (Campbell et al., 2020; Sun et al., 2020). *Treponema* members break down pectin (Liu, Pu, Xie, Wang, & Liu, 2015; Liu et al., 2014), a complex plant polysaccharide enriched in ripe fruits, such as those commonly provided to captive ring-tailed lemurs (Dishman, Thomson, & Karnovsky, 2009; Mowry, Campbell, Mowry, & Campbell, 2001). Compositionally, the gut microbiota of wild lemurs were markedly less varied than those of lemurs in all captivity settings. These findings support the "Anna Karenina" principle (Ahmed, Herrera, Liew, & Aranda, 2019; Zaneveld, McMinds, & Thurber, 2017), which posits that perturbations of microbiota result in unstable communities and, thus, 'unperturbed' hosts have less variation in their microbiota than do 'disturbed' hosts. A single exception to the gut microbiota clustering according to the hosts' conditions was a pet lemur with gut microbiota that resembled that of wild lemurs. Although we can only speculate about this individual's history, if recently taken from their natural habitat, the gut microbiota could still reflect the wild origins of this animal, potentially indicative of gradual change

in response to environmental shifts (Kohl & Dearing, 2014; Martínez-Mota, Kohl, Orr, & Dearing, 2020).

Lastly, we observed that patterns in lemur gut microbiota were somewhat mirrored in the diversity and composition of soil microbiota, suggesting that environmental conditions other than diet, including exposure to external microbes in soils, may influence gut microbiomes (Grieneisen et al., 2019). Madagascar's geographical isolation for ~88 million years accounts for high levels of floral and faunal endemism (Ganzhorn et al., 2001; Goodman & Benstead, 2005). The same is true of microbes, as evidenced by the numerous, unique pathogenic microorganisms found on the island (Dietrich et al., 2014; Guiyoule et al., 1997; Jeffries et al., 2018; Larsen et al., 2016). Unsurprisingly, therefore, soil microbiota in Madagascar, whether originating in wilderness or captivity settings, were similar in composition and significantly divergent from soils in the U.S. (Bahram et al., 2018). Given the disparate geographic distributions of many wild vs. captive animals, environmental acquisition that reflects local microbial endemism may be particularly relevant for distinguishing gut microbiota between wild and captive conspecifics. For example, the natural ranges of most primates occur in the tropics (Mittermeier, 1988; Reed & Fleagle, 1995), yet most accredited zoos and captive facilities that house primates are found outside of tropical regions (in e.g., Europe and North America; Melfi, 2005; Primates et al., 2019); the distinct environmental consortia

surrounding wild and captive conspecifics should reflect their geographic or continental divides.

Regarding the exposure to environmental microbes, soil-associated microbes were more prevalent in lemurs that had greater exposure to natural environments and the acquired soil microbes were specific to the lemurs' environment, reflecting active environmental acquisition. This observation expands on findings that abiotic soil properties mediate primate gut microbiota (Grieneisen et al., 2019). Wild and captive ring-tailed lemurs perform geophagy (i.e., earth-eating), a behavior that is linked to nutrient and microbial supplementation (Johns & Duquette, 1991; Krishnamani & Mahaney, 2000) and is a potential vector for the incorporation of environmental microbes (Borruso et al., 2021). Similarly, dietary items may act as vessels of soil or environmental microbes (Li et al., 2016); dietary variation across wild and captive lemurs may influence gut microbiomes by simultaneously offering different nutrients and different microbes. Akin to most cross-sectional studies of microbiomes, we were unable to assess the persistence or viability of the soil-associated microbes in lemur gut communities. It is, therefore, possible that the soil-associated microbes in lemur guts were ephemeral or non-viable; however, our results indicate setting-specific, environmental acquisition, supporting that these patterns are not random and that the acquired microbes may be subject to filters that enable the incorporation of only specific microbes (Tout et al., 2017; Walke et al., 2014; Won et al., 2003). Furthermore, we

analyzed these data from the perspective that environmental consortia act as sources of microbes for host-associated communities, but we expect consistent, bidirectional transmission of microbes between hosts and their environments, a relationship that warrants further investigation.

While expanding our understanding of the factors that shape host-microbe relationships, these results also have significant potential to inform animal care and conservation strategies. Perturbed microbiota are increasingly recognized as culprits of obesity, gastrointestinal distress, and even associated mortality in captive animals (Caravaggi, Plowman, Wright, & Bishop, 2018; McKenney, Greene, Drea, & Yoder, 2017; McPherson, 2013; Clayton et al., 2018). Given that lemurs are among the most endangered mammals on the planet (Schwitzer et al., 2014), maintaining populations of healthy animals in captivity is an important 'safety net' that augments *in-vivo* conservation efforts (Tenhumberg, Tyre, Shea, & Possingham, 2004; Trevelline, Fontaine, Hartup, & Kohl, 2019). We suggest that environmental acquisition may be a key component of 'rewilding' or 'bioaugmenting' captive animal gut microbiota, a process by which gut consortia can be reshaped to better promote host-microbe symbiosis (Greene et al., 2019; Mills et al., 2017; Trevelline et al., 2019). Identifying what comprises healthy gut microbiota is a complex and ongoing area of research; nonetheless, we show that environmental acquisition is a potential driver of microbial communities and thus should be considered as a component of animal health.

Conclusions

Even in a relatively robust, omnivorous host, gut microbiota are distinct across populations. This variation reflects environmental variability that is underrepresented by a simple wild vs. captive dichotomy. Moreover, concurrent analysis of lemur gut and soil microbiota supports the premise that environmental acquisition contributes to shaping host-associated microbiota; hosts and their associated microbes are components of a larger landscape that includes interactions with environmental microbes. Together, these results expand our understanding of intraspecific host-microbe dynamics under varying environmental conditions and reinforce the value of broad-scale, comparative investigations of microbial variation within a single host species.

Methods

Study sites

Our research sites included 13 settings (one per 'population'), grouped under the following three environmental conditions: wilderness in Madagascar (8 settings), captivity in Madagascar (2 settings), and captivity in the U.S. (3 settings; Table 1). The wilderness settings occurred in protected areas (e.g., national parks, community-managed reserves) that varied in habitat type (Table 1). The captivity settings in Madagascar included the Lemur Rescue Center (LRC; Toliara, Madagascar), where the

animals were socially housed, and various townships that were home to individual pets. Lastly, the captivity settings in the U.S. included the North Carolina Zoo (NCZ; Asheboro, NC), the Duke Lemur Center (DLC; Durham, NC), and the National Zoological Park (NZP; Washington, DC). These facilities were comparable to one another, all with socially housed lemurs.

Subjects

Across all research sites, our subjects included 215 adult, ring-tailed lemurs (82 male, 81 female, 52 of unknown sex; Table 1). The wilderness sites were each occupied by multiple lemur troops, ranging in size from 5-24 individuals. Excluding the pets, all captive settings included groups of 2-7 lemurs that had access to indoor and outdoor enclosures, and were provided facility-standardized diets (i.e., fresh produce and commercial chow, freely available water). Certain animals at the LRC and the DLC also had access to natural habitat enclosures that, respectively, consisted of dry and spiny forest (LRC) or North American deciduous and pine hardwood forest (DLC). The pets were kept in human dwellings (i.e., houses or hotels) and were fed fruit, rice, and other foods intended for human consumption.

Sample collection

During a span of four years (2016-2020), we collected 'matched' fecal and soil samples from our subjects and study sites, respectively. Within 8 weeks of fecal or soil collection, the samples were transported to the U.S., where they were stored at -80 °C, until analysis.

For feces, we opportunistically collected fresh samples, upon the lemur's observed voiding. In Madagascar, collections occurred during the dry season (May-October) and, in the U.S., collections occurred end of summer through fall (August-November). To avoid soil contamination of the fecal sample, we removed the outer layer of each fecal pellet. We then placed the sample in an Omnigene tube that contained a stabilizing buffer that preserved microbial communities at room temperature for 8 weeks (Omnigene.Gut tube, DNAgenotek, Ontario, Canada (Choo, Leong, & Rogers, 2015; Song et al., 2016)). All settings were represented by fecal samples from minimally two lemurs (the maximum number of individuals represented was 33).

When collecting soil in nature, we avoided high-defecation areas (e.g., under sleeping trees) while identifying core areas where lemurs most commonly spent time on the ground. Within these core areas, we demarcated a 2-3 m^2 area and collected soil from each of five evenly spaced locations, using a clean, individually wrapped, sterile plastic spatula. For each area, the five aliquots of topsoil (top 2-3 cm of soil) were pooled in a single Omnigene tube to create a representative soil sample. Because multiple lemur

troops inhabited each of the wilderness settings, in some cases with overlapping core areas, we prioritized collecting soil samples from areas of maximal use. In some cases, we were unable to collect soil samples for every troop that provided fecal samples. At the LRC and DLC, we used the same collection methods to collect soil samples from areas in the natural habitat enclosures where lemurs semi-free-ranged. Because it is illegal to own pet lemurs in Madagascar, we minimized owner concern by collecting only fecal samples for this group. Because of other logistical and analytical constraints (see below), only five of the 13 settings were represented by usable, pooled soil samples.

Microbial DNA extraction and sequencing

Following the manufacturer's protocols for the DNeasy Powersoil kit (QIAGAN, Frederick, MD), we extracted bacterial genomic DNA from fecal and soil samples. We quantified DNA using a Fluorometer (broad-spectrum kit, Qubit 4, Thermo Fisher Scientific, Waltham, MA). Aliquots of extracted DNA were sent to Argonne National Laboratory's Environmental Sequencing facility (Lemont, IL) for library preparation and amplicon sequencing of the 16S rRNA gene. After amplification of the V4 region with region-specific primers and sample-specific 12-base barcodes, samples were pooled and amplicon libraries were cleaned using AMPure XP Beads. Amplicons were then sequenced on a 151 x 151 base pair Illumina MiSeq run (Caporaso et al., 2012).

Bioinformatics and statistics

We processed the raw sequence data using a previously published bioinformatics pipeline generated in QIIME2 (Bornbusch, Grebe, et al., 2020). In brief, we used the pipeline to join forward and reverse reads, demultiplex and quality filter the joined reads (DADA2), generate a phylogenetic tree, and assign taxonomy based on 99% sequence similarity (SILVA database, ver. 138.1; Quast et al., 2012; Yarza et al., 2014)). After quality filtering, samples with fewer than 10,000 sequences were removed from downstream analyses, resulting in 209 fecal samples and 25 soil samples with over 11 million combined reads and an average of ~50,000 reads per sample. To visually represent rare taxa that had relative abundances < 1% of the total sequences, we combined them into the conglomerate "Other" category (Figures 1 and 6). Using tables of amplicon sequence variants (ASVs), we calculated metrics of alpha diversity (Shannon and Faith's Phylogenetic diversity metric) and beta diversity (UniFrac distances).

To test for differences in alpha diversity between the gut microbiota of lemurs under the three environmental conditions and in the 13 settings, we first used generalized linear models (GLMs; glm in R, ver. 4.0.2) with condition or setting and sex as fixed effects. To further test for variation in lemur gut microbiota and soil microbiota alpha diversity, we used nonparametric statistics (e.g., Kruskal-Wallis tests, and pairwise Wilcoxon rank sum tests with Benjamini-Hochberg adjustment) to perform

pairwise comparisons between the various conditions and settings. To identify and test for effects of condition or setting on beta diversity (unweighted UniFrac distances) in lemur fecal and soil microbiota, we used principal coordinate analysis (i.e., to visualize clustering of microbiota composition) and Permutational Multivariate Analysis of Variance (PERMANOVA) in QIIME2. We then performed Random Forest Analysis (Breiman, 2001), which is a supervised learning technique that uses decision trees to classify data to specific categories and provides an overall model error rate (out of the bag error or OOB error). To identify microbes enriched in specific groups of samples, we used differential abundance analyses via Analysis of Compositions of Microbiomes (ANCOM) and songbird software (Morton et al., 2019) in QIIME2, paired with visualization through Qurro (Fedarko et al., 2020).

For the eight settings where we obtained matched fecal and soil samples (Table 1), we analyzed covariation between lemur gut microbiota and the associated soil communities by performing a Mantel test on microbial abundance matrices of lemur gut and soil microbiota. Because multiple lemur fecal samples were associated with each soil sample, we created comparable matrices for the Mantel test by averaging the microbial abundances across the fecal samples of lemurs directly associated with a given soil sample, resulting in a single, mean lemur gut community associated with each soil community. For this process, we omitted fecal samples from troops not represented by a soil sample or for which troop identity was unknown.

To test if soil-associated microbes were present in lemur gut microbiota, we used FEAST, a tool for fast expectation-maximization microbial source tracking (Shenhav et al., 2019). FEAST assumes each 'sink' sample is a convex combination of known and unknown 'sources' and uses multinomial distributions and machine-learning classification to model the microbial source-sink data (Shenhav et al., 2019). For this analysis, we used the matched lemur gut and soil samples; all soil samples collected in a given setting were used to represent the potential exposure to environmental microbes experienced by all sampled lemurs in that same setting, regardless of troop identity. Because we were testing whether environmental acquisition influences lemur gut microbiota, and because this analysis requires an assumption of directionality (i.e., from a source to a sink), we categorized soil samples as 'sources' and lemur fecal samples as 'sinks'; however, we acknowledge and discuss the potential for bi-directional transmission of microbes between lemurs and soil. For each lemur fecal sample, we calculated the proportions of microbes that were identified as stemming from each soil community and from a default 'unknown source' that accounts for microbes not relevant to soil microbiota. Lastly, we used FEAST to test for differences in the proportion of soil microbes in the gut microbiota of lemurs at the DLC that were either semi-free-ranging or sequestered to indoor enclosures.

Chapter 3. 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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Introduction

Various microbial communities occupy the epithelial surfaces of vertebrate hosts and interact with their physiological systems to mediate host health (Belkaid & Segre, 2014; Benavidez, Iruri-Tucker, Steiniche, & Wasserman, 2019; Gilbert et al., 2018; Sharon, Sampson, Geschwind, & Mazmanian, 2016) and reproductive success (Power, Quaglieri, & Schulkin, 2017; Reid et al., 2015). 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, & Grice, 2014) and through the manufacture of bodily odors used to chemically communicate mate quality (Ezenwa & Williams, 2014; Sharon et al., 2010). The range and specificity of functions served by microbes owe to differences in their community structure across body sites (Huttenhower et al., 2012; Schommer & Gallo, 2013). Over evolutionary time, host phylogeny largely predicts microbiome structure (Groussin et al., 2017; Ley et al., 2008; Nishida & Ochman, 2018), whereas over shallower time scales, microbiome structure can reflect more transient characteristics, such as the host's physiology or environmental condition (Greene et al., 2019; Nieuwdorp, Gilijamse, Pai, & Kaplan, 2014; Phillips et al., 2012). 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 (Council et al., 2016; Phillips et al., 2012). 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, Müller, Weese, & Neufeld, 2018). Thus, well-established patterns in humans (Grice et al., 2009; Huttenhower et al., 2012; Peterson et al., 2009) may not generalize to other primates (Davenport et al., 2017; Yildirim et al., 2014). 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 (Albone & Perry, 1976; Gorman, Nedwell, & Smith, 1974). 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, Ranta, Ruxton, & Lundberg, 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) follow 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 (Nunn et al., 2014; Thrall, Antonovics, & Dobson, 2000).

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 & Loesche, 1982; Nakagawa, Fujii, Machida, & Okuda, 1994). In women, changes in vaginal microbiota accompany variation in estradiol (E_2) concentrations during ovarian cycles (Mishell Jr et al., 1971), pregnancy

(Loriaux, Ruder, Knab, & Lipsett, 1972), and menopause (Rothman et al., 2011; Sherman, West, & Korenman, 1976). Indeed, the dominance of *Lactobacillus*, which mediates susceptibility to bacterial vaginosis and HIV, is regulated, in part, by E_2 (Miller, Beasley, Dunn, & Archie, 2016; Redondo-Lopez, Cook, & Sobel, 1990). Although vaginal microbiota vary across the ovarian cycles of certain anthropoids (Miller, Livermore, Alberts, Tung, & Archie, 2017; Narushima et al., 1997), 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 (Council et al., 2016; Hyde et al., 2016; Lax et al., 2014; Walke et al., 2014). Although the transmission of pathogenic, environmental microbes can negatively impact host health (Finley et al., 2013; Jones et al., 2008), 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 (Drea, 2019; Martin, 1972), 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 (delBarco-Trillo, Burkert, Goodwin, & Drea, 2011; Greene & Drea, 2014; Hayes, Morelli, & Wright, 2004; Scordato & Drea, 2007). 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 (Bogart, Kumamoto, & Lasley, 1977; Evans & Goy, 1968;

Van Horn & 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 (Greene et al., 2019; Starling, Charpentier, Fitzpatrick, Scordato, & Drea, 2010). 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 X 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 14 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, Weinberg, Wilcox, McConnaughey, & Musey, 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, Misawa, Kuma, & Miyata, 2002) and fasttree2 (Price, Dehal, & 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 2), 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, Norori, Zozaya-Hinchliffe, & Martin, 2007; Jakobsson & Forsum, 2007; Miller et al., 2016; Mirmonsef et al., 2014); *Streptococcus* (Bezirtzoglou et al., 2008; Cowley & Heiss, 1991; Noguchi, Tsukumi, Udono, & Urano, 2004); *Bacteroides* (Bezirtzoglou et al., 2008; Eschenbach et al., 2000; Kornman & Loesche, 1982; B. Larsen, Markovetz, & Galask, 1977); *Prevotella*, (Kumar, 2013; Miller et al., 2017; Nakagawa et al., 1994); and Family XI (Miller et al., 2017). For this group, we ran a third set of LMMs (LMM3; Appendix A.2.1;

Table 10) 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; Table 11) 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 & 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. Sequencing reads are also available on the National Center for Biotechnology Information's Sequence Read Archive (BioProject #1861841).

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,

Table 2. 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	**5.716**	**<0.001**
Free-ranging status	0.482	0.527
$\ln(E_2/P_4)$	**0.873**	**0.011**
Species × Body Site	**7.705**	**<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**

Ternericutes, and Verrucomicrobia phyla (Figure 10). 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 2). 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 10). 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 11; $p = 0.009$). Beta diversity also differed significantly between the two species (Figure 12; visualized in PCoA plot, pairwise adonis, $R^2 = 0.38$, $p < 0.05$). In both species, inter-individual variation in vaginal

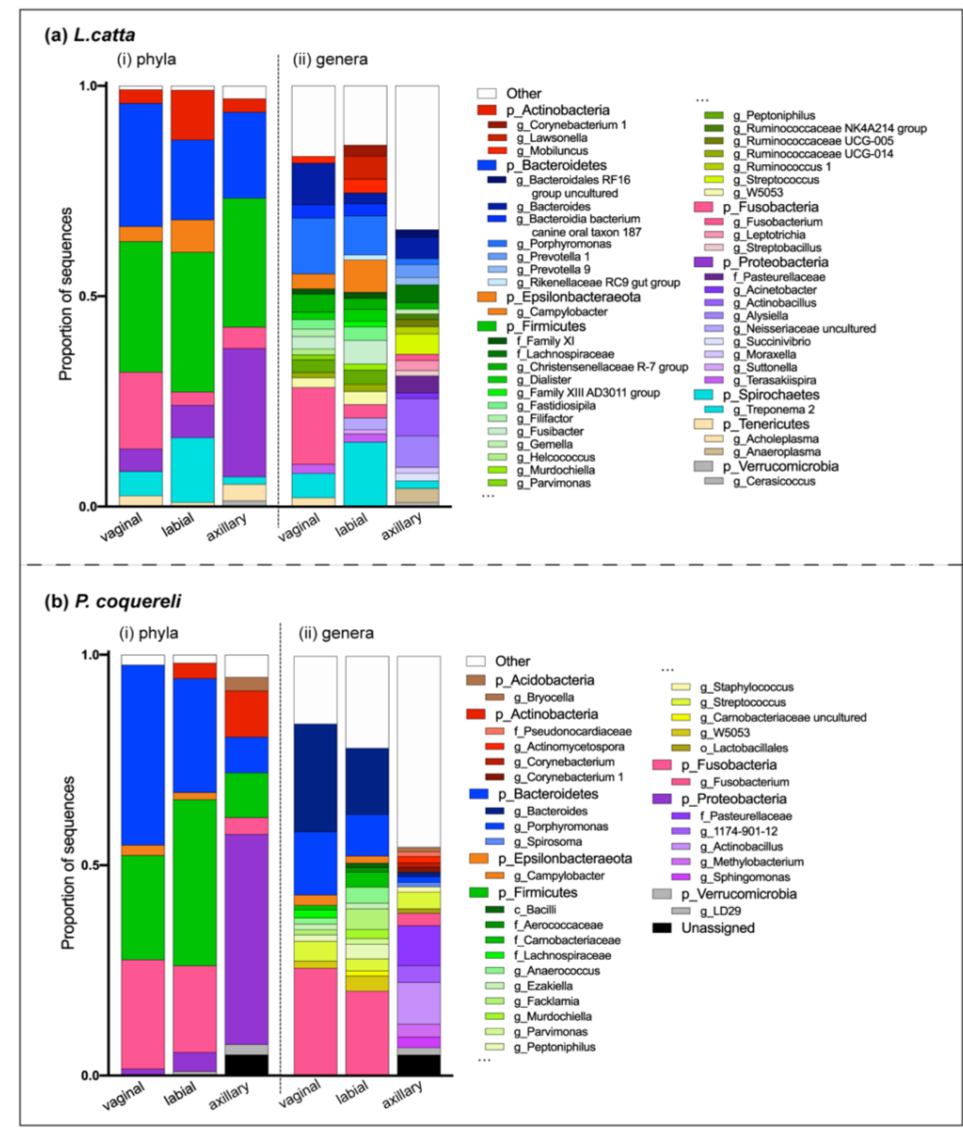

Figure 10. The relative abundances of bacterial taxa in the vaginal, labial, and axillary microbiomes of (a) ring-tailed lemurs (*L. catta*) and (b) sifakas (*P. coquereli*). For each microbiomes, bacterial (i) phyla and (ii) genera representing less than 1% of the microbiomes we combined into "Other".

microbiota composition was significantly less than in the other two body sites (Figure 13; 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 10). The labial communities of ring-tailed lemurs included a large proportion of *Treponema*, a genus of anaerobic spirochetes often associated with infection or disease (Radolf & Lukehart, 2006; Simonson, Goodman, Bial, & Morton, 1988), 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 11; 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, $R^2 = 0.09$, $p < 0.05$).

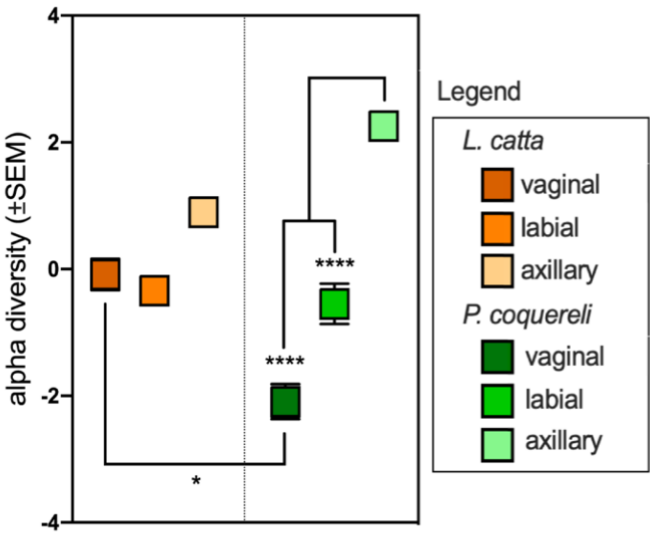

Figure 11. Alpha diversity with standard error means (SEM) across the three body sites in both lemur species. Kruskal-Wallis test with Dunn's multiple comparison corrections; p < 0.05*, p <0.0001****.

Within each species, interindividual variation in labial microbiome composition was significantly greater than that in the vaginal microbiome (Figure 13, see above), but was similar to that in the axillary microbiome (Figure 13; *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 10).

Alpha diversity of the axillary microbiomes was not significantly different between species ($p = 0.136$; Figure 11). Across species, axillary microbiomes had the greatest alpha diversity of the three body sites, but differences between sites were much stronger in sifakas (Figure 11; 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 10). 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 $R^2 = 0.26$, $p < 0.05$).

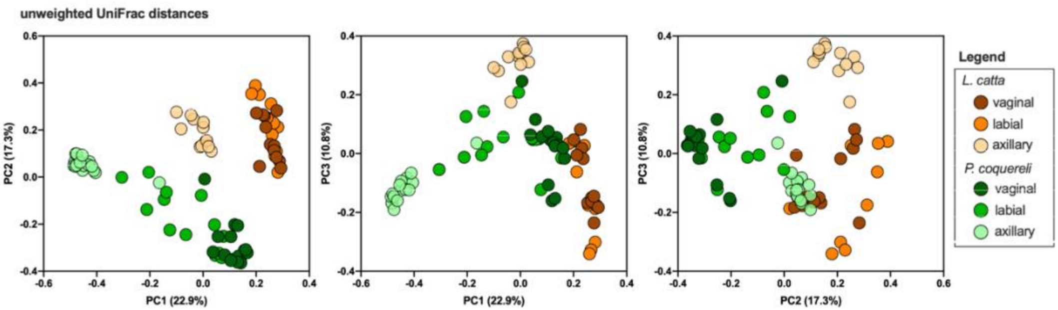

Figure 12. Beta diversity (principal components analysis) of unweighted UniFrac distances showing coordinate spaces of principal components 1, 2, and 3.

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 14a; 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 15; LMM1, $t = -2.18$, $p = 0.033$; Table 2), but significantly and positively related both to E_2 concentrations (Figure 15; LMM1, t = 2.83, $p = 0.018$) and to $\ln(E_2/P_4)$ (Figure 15; LMM2, t = 2.62, $p = 0.011$). Neither P_4 nor E_2

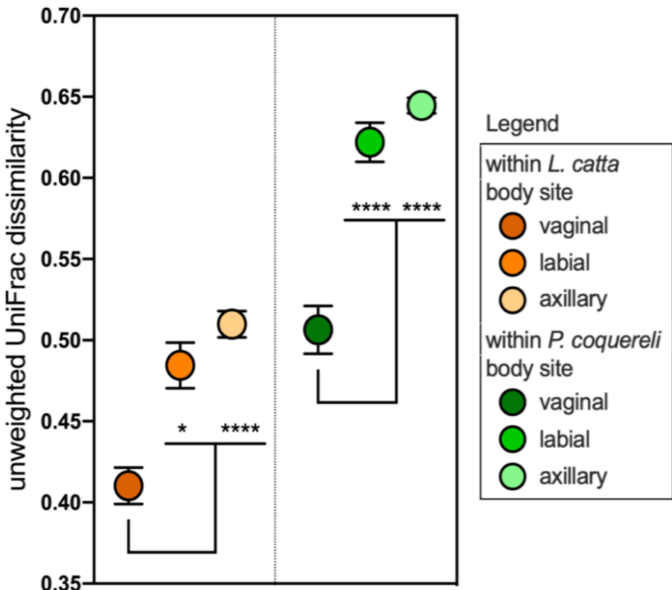

Figure 13. Variation in unweighted UniFrac distances between all samples within a given microbiome. Greater values indicate greater dissimilarity and greater interindividual variation in microbiome composition. Significance determined using *t*-tests with Bonferroni corrections.

were significantly related to beta diversity (PERMANOVA, *p*s = 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

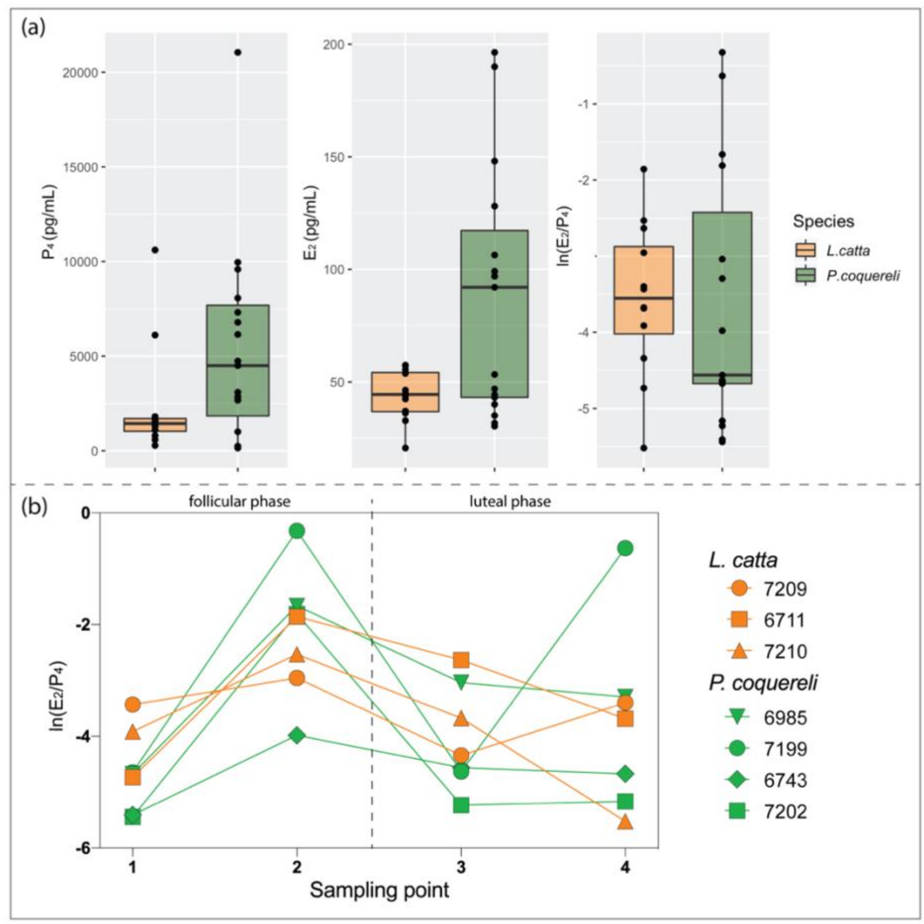

Figure 14. Variation in ovarian hormones in both lemur species. (a) E_2 and P_4 concentrations and lnE_2/P_4 in both lemur species and (b) variation in each lemur's lnE_2/P_4 across the four sampling points with inferred cycle phase.

of *Streptococcus* in the vaginal microbiomes (LMM3; $t = 2.54$, $p = 0.018$; Appendix A.2.1; Table 10) and of Lactobacillales (i.e., the order of lactic-acid producing bacteria that includes *Lactobacillus*) in the vaginal and labial microbiota (Figure 16b; LMM3; vaginal: $t = 3.94$, $p < 0.001$; labial, $t = 2.50$, $p = 0.046$; Appendix A.2.1; Table 10) of both host species. The relationship between microbial composition and E_2 concentrations varied between

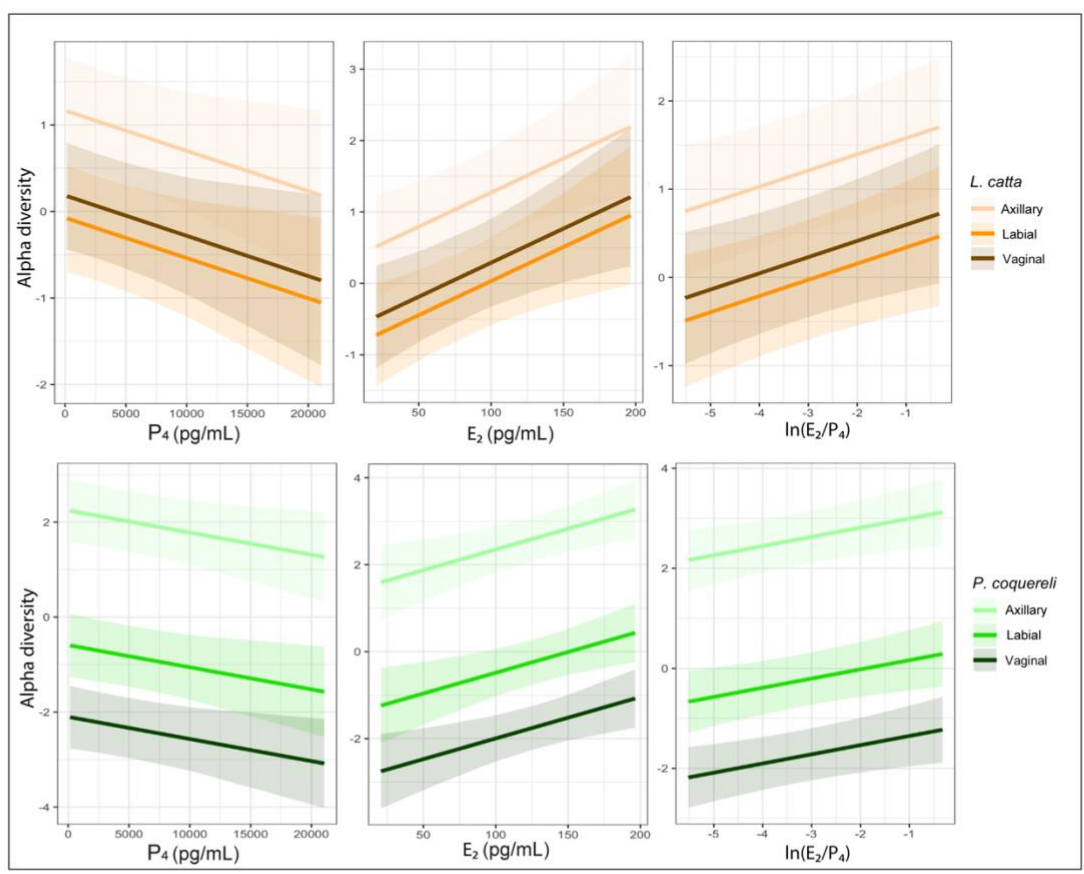

Figure 15. Model-based predictions of alpha diversity from P_4 and E_2 independently (GAM 1), and from ln(E2/P4) (GAM 2).

body sites, but the predicted patterns of correlation were similar between host species (Figure 16a).

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; $t = 6.013$, $p < 0.001$; Table 11).

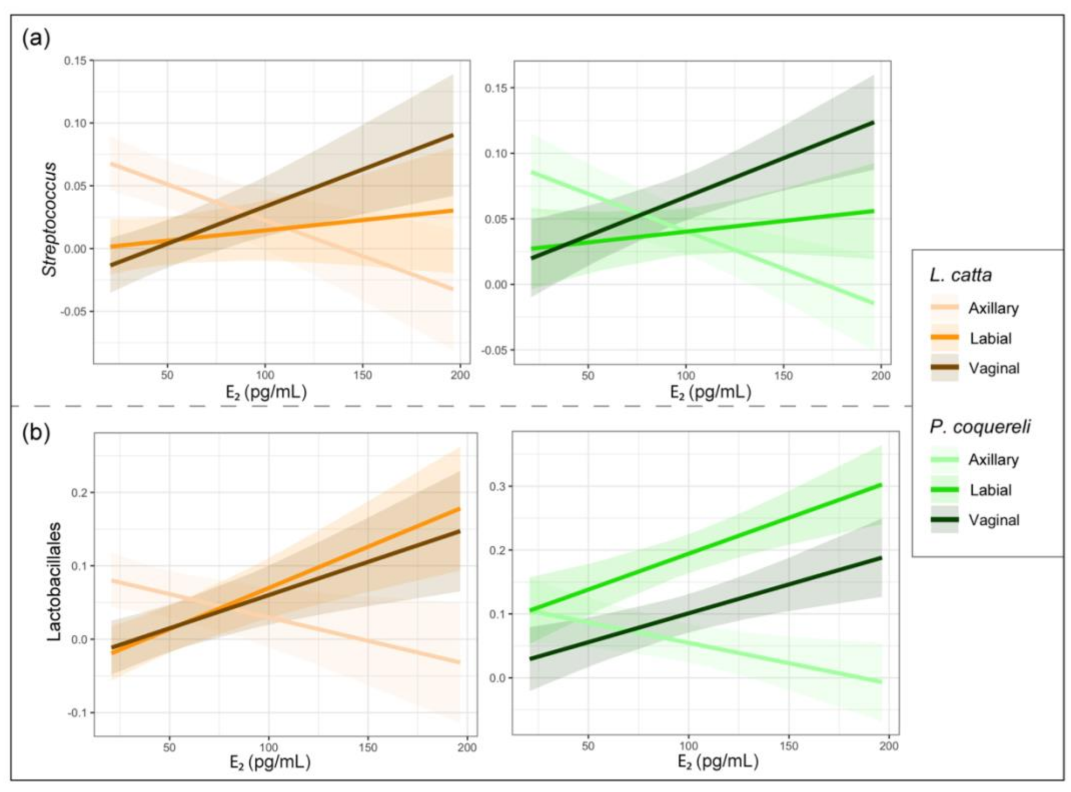

Figure 16. Model-based predictions of (a) *Streptococcus* and (b) Lactobacillales abundances from E_2 concentrations.

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 (Vo, Tsai, Maxwell, & Carbonero, 2017; Wegner & Liesack, 2016). 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 (Abdullah et al., 2010; Fournier & Lechat, 2016; Moore & Korf, 1963; Seaver, 1909; Tribe, Thines, & Weber, 2006; 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 2), 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 (Council et al., 2016; Stumpf et al., 2010), 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 of 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 & 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 (Greene et al., 2019; Yildirim et al., 2014). These diverse genera are often considered pathogenic in humans (Darveau, Hajishengallis, & Curtis, 2012; Genco, Van Dyke, & Amar, 1998; Kostic et al., 2012; Signat, Roques, Poulet, & Duffaut, 2011; Slots & Listgarten, 1988); 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 (Greene et al., 2019; Leclaire, Jacob, Greene, Dubay, & Drea, 2017; D. Li et al., 2016; Theis, Venkataraman, Wagner, Holekamp, & Schmidt, 2016; 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 (Aylıkcı & Çolak, 2013; Donaldson

et al., 2005; Porter & Scully, 2006). These VOCs include dodecanoic, tetradeconoic, and other long-chain, fatty acids that also occur in the labial secretions of ring-tailed lemurs (Scordato, Dubay, & Drea, 2007). In this species, despite the presence of specialized glandular tissue in the labia (Drea & 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 provide 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, Gettie, Miller, & Marx, 1998; Vishwanathan

et al., 2011; Wira & Fahey, 2008; Wira, Rodriguez-Garcia, & 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, & 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, Cone, Whaley, & Moench, 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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Chapter 4. Antibiotics and fecal transfaunation differentially affect microbiota recovery, associations, and antibiotic resistance in lemur guts

The content of this chapter is under review in *Animal Microbiome.* The full reference is as follows:

Bornbusch S. L., Harris R. L., Grebe N. M., Roche K., Dimac-Stohl K., & Drea C. M. *in review.* Antibiotics and fecal transfaunation differentially affect microbiota recovery, associations, and antibiotic resistance in lemur guts.

A preprint of the manuscript is also available on *bioRxiv* under a different title and reference:

Bornbusch S. L., Harris R. L., Grebe N. M., Roche K., Dimac-Stohl K., & Drea C. M. (2020). Longitudinal effects of antibiotics and fecal transplant on lemur gut microbiota structure, associations, and resistomes. *bioRxiv*.

Introduction

The long, co-evolutionary history between vertebrates and their microbes underpins the complex web of interactions linking commensal microbiota to host function (Koskella & Bergelson, 2020; Lynch & Hsiao, 2019). Because perturbations to these communities can have both short- and long-term negative consequences (Brown, DeCoffe, Molcan, & Gibson, 2012; Jing Li et al., 2017; Moloney, Desbonnet, Clarke, Dinan, & Cryan, 2014), we increasingly recognize the benefits provided by our endogenous microbiota and have come to view them as 'old friends' (Rook, 2010; Graham A W Rook, Martinelli, & Brunet, 2003). To exemplify, while antibiotic treatment effectively combats immediate bacterial infections, it can also lead to prolonged and severe, negative side-effects, such as the elimination of beneficial microbes, increased susceptibility to harmful pathogens (Buffie et al., 2012; Theriot et al., 2014), and deterioration of microbiome function (Francino, 2015; Langdon, Crook, & Dantas, 2016). Moreover, antibiotics also promote changes in microbial genomes; the ubiquitous use of antibiotics has spurred the spread of genes encoding antibiotic resistance (ABR), which can have potentially catastrophic consequences (Ventola, 2015a). Microbial therapies, such as fecal transfaunation, can mitigate the detrimental side-effects of antibiotics (Taur et al., 2018); however, because antibiotics are often studied in the context of preexisting illness or injury (which independently influences microbial communities), the severity, duration, and recovery from dysbiosis owing purely to antibiotics remain unclear. Here,

we apply an ecological framework in healthy animals to better understand the trajectory and processes governing recovery of or return to a stable, gut microbial community following antibiotic-induced disruption. Because nonhuman primates are increasingly utilized as models in which to probe microbial dynamics and the development of ABR in response to antibiotic treatment, we experimentally administered a broad-spectrum antibiotic to male ring-tailed lemurs (*Lemur catta*) and used a longitudinal approach to track impacts on the composition and resistomes of their gut microbiota. We further tested the effects of fecal transfaunation as an intervention to promote the recovery of microbial composition and to potentially mitigate the development and persistence of ABR.

Antibiotics and ABR genes have ancient origins as natural compounds or genetic defenses, respectively, used by microbes to compete and survive in densely populated communities, whether within or outside a host (D'Costa et al., 2011; Davies, 1997). The ability of bacteria to rapidly undergo mutation (Denamur & Matic, 2006; Suez et al., 2018) and share advantageous genes via lateral gene transfer (Ochman, Lawrence, & Groisman, 2000; Thomas & Nielsen, 2005) has resulted in myriad, naturally occurring ABR genes (Allen et al., 2010; Aminov, 2009). The response of a microbial community to natural antibiotics is largely dictated by the interactions between microbial taxa, which vary over time and across environments. The efficacy and ubiquity of man-made antibiotics have severely perturbed microbial communities via targeted (e.g. narrow

spectrum) or indiscriminate (e.g. broad spectrum) elimination of bacterial groups (Ferrer, Méndez-García, Rojo, Barbas, & Moya, 2017), thereby altering the composition and, ultimately, functional potential of microbiomes (Cho et al., 2012; Zarrinpar et al., 2018). In addition, these antibiotics have magnified selective pressure on bacterial communities, making ABR genes advantageous and instigating their proliferation (Kolář, Urbánek, & Látal, 2001; Witte, 2000), thereby altering the microbiota's genomic make-up. Within host-associated microbiomes, the propagation of ABR can result in virulent, resistant pathogens (Stokes & Gillings, 2011; Weber, Raasch, & Rutala, 1999) that reduce the diversity of native or beneficial microbes (Britton & Young, 2012; Stecher, Maier, & Hardt, 2013) and diminish immune capacity of the host. Our understanding of these phenomena primarily derives from studies that characterize the effects of antibiotics on the elimination or development of ABR within specific bacterial pathogens (Chambers & DeLeo, 2009; Petty et al., 2014). We know comparatively less about how man-made antibiotics influence the aggregate interactions within presumed healthy, host-associated communities and how those dynamics influence the recovery of microbiota.

Recognizing that commensal consortia are vital to the host has spurred increased research into microbial therapies to mitigate the negative consequences of dysbiosis. In fecal transfaunation, for example, a 'healthy' or 'native' community of microbes sourced from feces is transferred into a dysbiotic community to combat pathogens and promote

the growth of beneficial microbes (Aroniadis & Brandt, 2013; Grehan et al., 2010). Because coprophagy (the ingestion of fecal material either directly or via prey consumption) bolsters gut microbiota during development or illness (Bo et al., 2020; Osawa, Blanshard, & Ocallaghan, 1993), medical practitioners have examined the use of fecal transfaunations to treat gastrointestinal distress in a wide range of host taxa (Chaitman et al., 2016; Niederwerder, 2018). As in studies of antibiotics, however, the effects of fecal transfaunation are best understood in the context of infection (with e.g., *Clostridium difficile* (Bakken et al., 2011; Brandt et al., 2012)). Whether or not fecal transfaunation alters the trajectory of microbiome recovery more broadly remains unclear.

Understudied compared to anthropoid primates, lemurs underwent an unique evolutionary trajectory that makes them particularly diverse and interesting models in which to study the dynamics between hosts and their co-evolved microbes (Amato et al., 2019; Clayton, et al., 2018; Greene et al., 2019; Greene et al., 2019). Endemic to Madagascar, the ring-tailed lemur is a diurnal/cathemeral, primarily terrestrial species that lives in multimale-multifemale social groups and shows strict seasonal breeding (Gould, 2006). Ecologically flexible (Gould, 2006; Jolly, Sussman, et al., 2006b), owing in part to a highly omnivorous diet, it is one of the few lemur species to thrive in captivity. This flexibility is reflected in their resilient gut microbiota that seem relatively unperturbed by aspects of captivity (Greene et al., 2019). Ring-tailed lemurs also seem

robust to health concerns, such as gastrointestinal problems, that affect the microbiota and welfare of other captive strepsirrhines (McKenney et al., 2017).

Here, we apply classic ecological principles to gut microbial communities to investigate two non-exclusive hypotheses regarding post-perturbation recovery. We use experimental manipulations (antibiotic treatment with or without fecal transfaunation), paired with longitudinal follow-up, to examine patterns in microbiota structure (e.g., alpha and beta diversity via 16S rRNA amplicon sequencing), bacterial associations (via Bayesian models of covariation), and ABR gene profiles (via shotgun metagenomic sequencing). The first hypothesis is that diversity increases functional redundancy within a community and thus improves stability (Konopka, 2009; Moya & Ferrer, 2016; Wohl et al., 2004). Under this 'diversity begets stability' hypothesis, as applied to a dysbiotic microbiome, recovery of alpha diversity, regardless of microbial identity, should be vital and sufficient to achieve a stable microbiome (Johnson, Vogt, Clark, Schmitz, & Vogt, 1996; K. S. McCann, 2000; McNaughton, 1977). Accordingly, after antibiotic treatment, we would expect to see an increase in microbial richness (e.g., alpha diversity), independent of fecal transfaunation. The resulting stable communities of the two treatment groups could thus have similar richness, but different compositions. The second hypothesis is that certain community members (i.e., keystone species or specific 'old friends') are foundational to community function (Banerjee et al., 2018; Berry & Widder, 2014; Fisher & Mehta, 2014; Gibbons, 2020), such that recovery of a stable

microbiome requires specific community composition (e.g., beta diversity). Under this 'keystone species' hypothesis, we predict that there should be recovery of the same community composition following antibiotic-mediated disturbance, with fecal transfaunation accelerating the recovery rate. Accordingly, the resulting stable communities of both treatment groups would have similar compositions.

These two hypotheses could be alternatives or could work in concert, but along different schedules, with potentially more rapid recovery of richness, but slower and more variable recovery of composition. Notably, the complexity of the dynamics between specific community members (i.e., cooperation and competition) could create long-term fluctuations in community composition that would be highlighted by bacterial covariations between key members of the community. Furthermore, the presence of ABR within the microbiomes could exert a distinct force in driving community composition during the treatment and recovery phases. By tracking ABR prevalence and type, coupled with bacterial covariation, we can make inferences about which microbes may be harboring and expressing ABR genes.

Results

Baseline and control bacterial communities

In the baseline or pretreatment phase, neither alpha nor beta diversity varied significantly between the three experimental groups, i.e., control (CON), antibiotic-

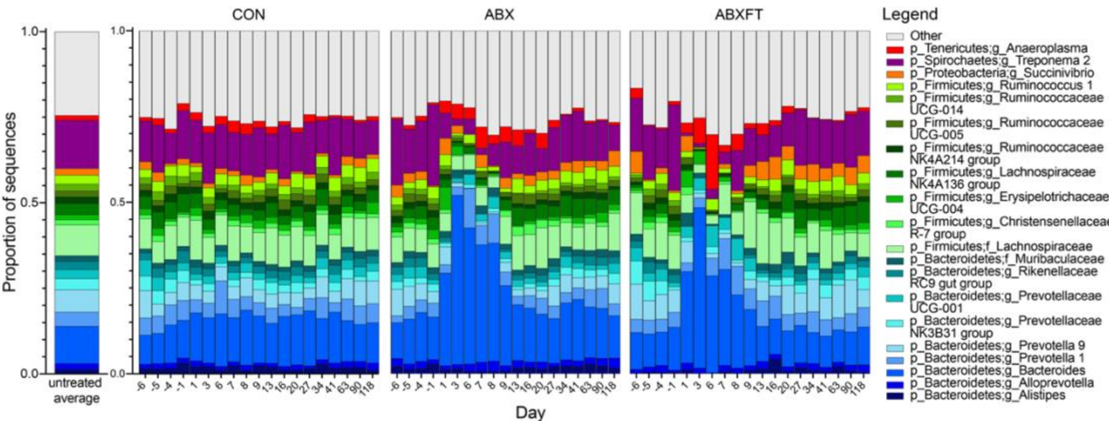

Figure 17. Mean relative abundances of bacterial genera over time in the gut microbiomes of three experimental groups of male ring-tailed lemurs (*Lemur catta*). Shown are values for healthy animals that received no treatment (CON), antibiotics only (ABX), or antibiotics plus fecal transfaunation (ABXFT). Genera are identified by color; those representing < 1% of the microbiomes were combined into the category "Other". The x axis shows day relative to three phases of study: pretreatment (days -6 to -1), treatment (days 0-6/7), and recovery (days 7/8-118).

treated (ABX), and antibiotic-treated subsequently receiving fecal transfaunation (ABXFT) (alpha diversity: Kruskal-Wallis test, H = 2.478, p = 0.289; beta diversity: H = 2.658, p = 0.264). Across all control samples (across all three phases of study, including pretreatment, treatment, and recovery; n = 184), the dominant bacterial taxa (Figure 17), as well as the alpha (Figure 18) and beta (Figure 19) diversities of CON animals, remained relatively stable over each of the two years' four-month study period, showing consistency across the breeding season. Adding the pretreatment phase of the other two groups (baseline samples; n = 43) to the control group (see 'untreated average' in Figure 1), the bacterial gut microbiota of healthy male ring-tailed lemurs, in captivity, were

dominated by taxa in the Bacteroidetes and Firmicutes phyla, with lesser contributions from Proteobacteria, Spirochaetes, and Tenericutes. Within these five phyla, 20 genera accounted for minimally 1% of the total sequences (Figure 17).

Response to and recovery from antibiotic treatment: alpha diversity and microbial membership

Across all three phases (days -6 – 120), we found a significant effect of experimental group on alpha diversity: relative to CON animals, ABX and ABXFT animals had significantly lower scores (HGAM: CON vs. ABX, $t = -3.535$, $p < 0.001$; CON vs. ABXFT, $t = -4.007$, $p < 0.001$; Figure 18). Neither year nor the interaction between year and experimental condition related to bacterial alpha diversity (HGAM1: year, $F = 0.001$, $p = 0.990$; year*experimental condition, $F = 0.942$, $p = 0.391$), showing that there was consistency of effects across both years.

As expected for the treatment phase, antibiotic-treated (ABX and ABXFT) animals showed a dramatic reduction in alpha diversity relative to CON animals (days 0-6; HGAM: CON vs. ABX, $t = -4.534$, $p < 0.001$; CON vs. ABXFT, $t = -3.754$, $p < 0.001$; see shaded bar in Figure 18). Consistent with the broad effects of amoxicillin, and based on qualitative assessments of relative and log-ratio abundances, antibiotic treatment in healthy lemurs was associated with dramatically reduced representation across a wide range of taxa, including numerous taxa in the Firmicutes phylum, such as members of the Clostridiales class (e.g., Ruminococcaceae and Lachnospiraceae families). Certain

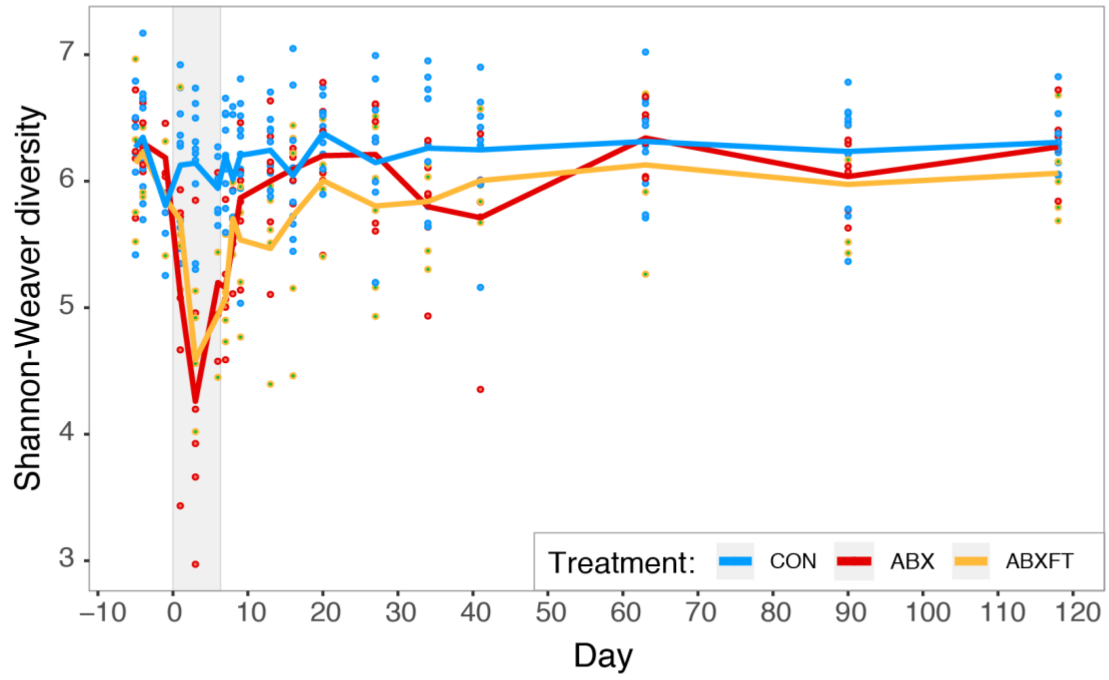

Figure 18. Shannon-Weaver alpha diversity over time in three experimental groups of male ring-tailed lemurs (*Lemur catta*). Shown are values for healthy animals that received no treatment (CON), antibiotics only (ABX), or antibiotics plus fecal transfaunation (ABXFT). Dots represent individual data points and lines connect the mean values of alpha diversity across individuals at each time point. The shaded window represents the period of antibiotic treatment (days 0-6), with fecal transfaunation administered on day 7; all values prior to the onset of treatment represent baseline values and all values post-treatment represent the period of recovery.

taxa, however, were markedly unaffected by antibiotic treatment, including the *Bacteroides* genus and other members of the Bacteroidales family, whose log-ratio abundances increased during treatment.

In the recovery phase only, we found that the differences in alpha diversity between CON and antibiotic-treated animals persisted over the nearly four-month, post-treatment period, suggesting long-lasting imbalance. Compared to CON animals,

antibiotic-treated groups maintained significantly lower alpha diversity (HGAM: CON vs. ABX, $t = -2.256$, $p < 0.025$; CON vs. ABXFT, $t = -3.036$, $p < 0.002$); however, there were no significant differences between the alpha diversities of ABX and ABXFT animals during recovery (HGAM: ABX vs. ABXFT, $t = 0.931$, $p = 0.354$; Figure 18), inconsistent with the notion that fecal transfaunation may benefit recovery of alpha diversity. Unexpectedly, however, we observed an initial, rapid increase in alpha diversity in both experimental groups, consistent with the 'diversity begets stability' hypothesis (Figure 18).

Response to and recovery from antibiotic treatment: beta diversity

Across all three study phases, we also found experimental condition to be a significant predictor of beta diversity (HGAM2: $F = 5.625$, $p = 0.004$; Figure 19), but in a manner that differed from the findings on alpha diversity. Notably, when analyzing beta diversity trajectories across the entire study period, we found that, compared to CON animals, ABX, but not ABXFT animals, showed significantly greater distances from their baseline communities (HGAM: CON vs. ABX, $t = 3.434$, $p < 0.001$; CON vs. ABXFT, $t = 1.726$, $p = 0.085$); however, when comparing ABX and ABXFT animals across the entire study period, we found no significant difference in their beta diversity trajectories (HGAM: ABX vs. ABXFT, $t = -1.607$, $p = 0.109$). During the treatment phase, specifically, and compared to CON animals, both groups of antibiotic-treated animals showed

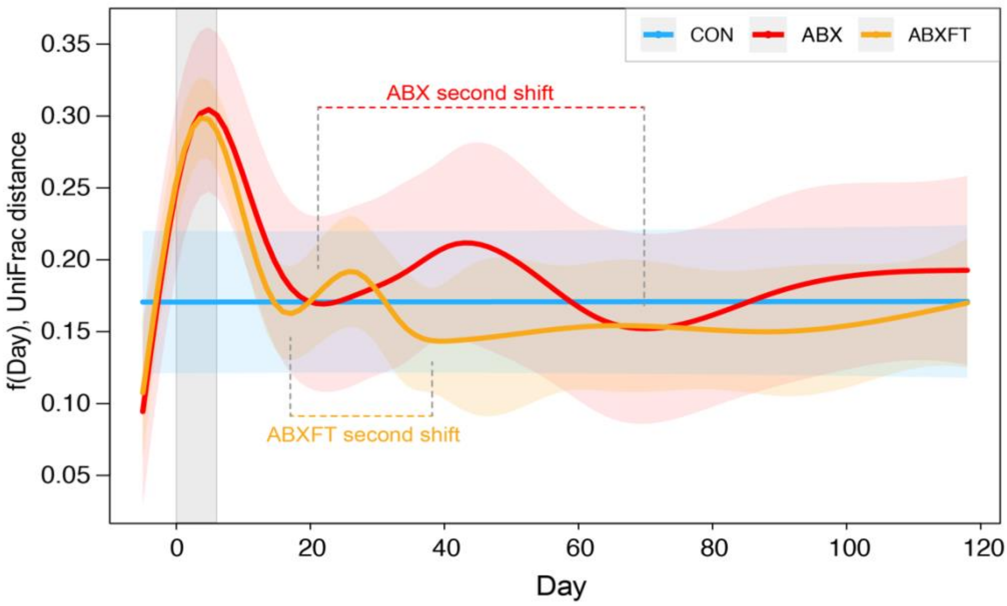

Figure 19. Model-predicted beta diversity for three experimental groups of male ring-tailed lemurs (*Lemur catta*). Beta diversity (Unweighted UniFrac distances) for three experimental groups of male ring-tailed lemurs (*Lemur catta*), represented as model-predicted distances from baseline, with 95% confidence intervals. Shown are values for healthy animals that received no treatment (CON), antibiotics only (ABX), or antibiotics plus fecal transfaunation (ABXFT). Trajectories represent predicted responses with smoothing splines that reduce minor variation and noise (e.g., CON animals shows minor variation over time in the raw beta diversity data (Appendix A.3.2; Figure 32) but the model-predicted values are shown as a straight line). The gray shaded window represents the period of antibiotic treatment, with the prior period representing baseline and the subsequent period representing recovery. The second shifts away from baseline are identified and labelled for ABX and ABXFT animals.

significantly greater distances from their baseline communities (HGAM: CON vs. ABX, $t = -3.847$, $p < 0.001$; CON vs. ABXFT, $t = -3.761$, $p < 0.001$). Furthermore, during the treatment period, the two antibiotic treatment groups did not differ in beta diversity (HGAM: ABX vs. ABXFT, $t = 0.081$, $p = 0.935$).

During the recovery phase, CON animals were significantly less distant from baseline when compared to ABX animals, but not when compared to ABXFT animals (HGAM: CON vs. ABX, $t = 2.790$, $p = 0.005$; CON vs. ABXFT, $t = 0.599$, $p = 0.549$), consistent with the 'keystone species' hypothesis. Furthermore, unlike during the treatment period, when comparing the recoveries of the two antibiotic-treated groups, ABX animals showed significantly greater distance from baseline compared to ABXFT animals (HGAM: $t = 2.115$, $p = 0.036$; Figure 19). The bacterial composition of ABX animals continued to oscillate throughout the course of the experiment, whereas in ABXFT animals, bacterial composition became relatively stable approximately 2 weeks after the treatment phase (Figure 19), consistent with the statistical results reported above. Specifically, after the first compositional shift during the treatment period, the bacterial composition of both ABX and ABXFT animals underwent a second shift away from baseline during the recovery period; however, the magnitude and span of these secondary shifts differed between the ABX and ABFT groups (Figure 19).

Bacterial associations

To characterize the bacterial covariations in the lemurs' gut, we used pairwise covariation analyses that detected several strong covariations ($\rho > 0.5$ or $\rho < -0.5$; hereafter 'associations') between pairs of microbial taxa within the lemurs' gut

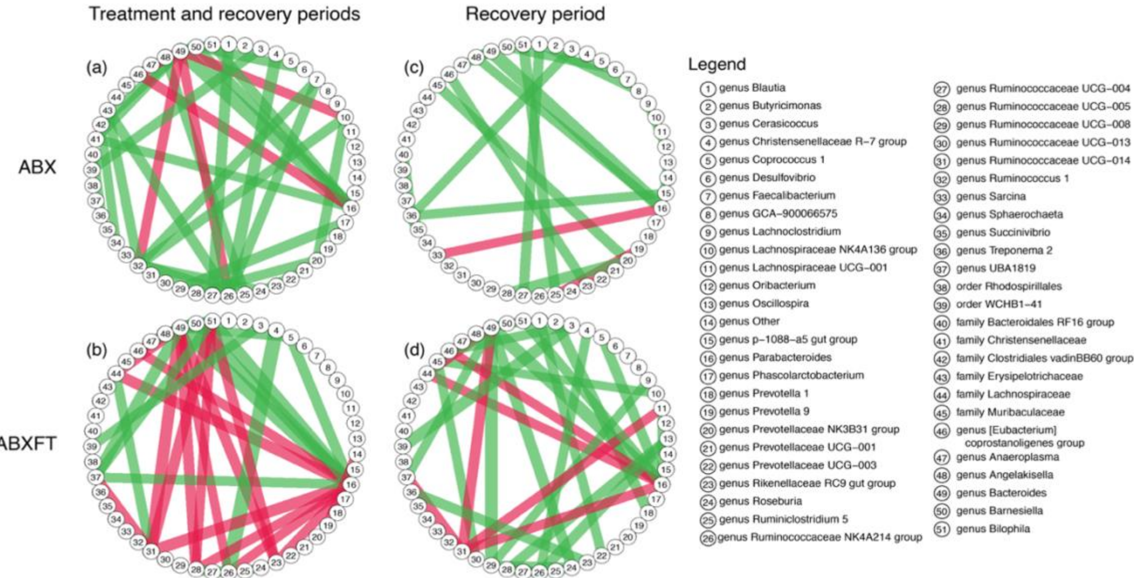

Figure 20. Bacterial associations for healthy, male ring-tailed lemurs (*Lemur catta*) either treated with antibiotics only or with antibiotics plus a fecal transfaunation. Line colors represent the direction of the correlation (green = positive, red = negative); line width is scaled to the magnitude of the correlation.

microbiomes. We investigated these associations under all three experimental conditions in two stages: across all three phases and during the recovery phase, specifically.

Minimal variation within the microbiota of CON animals limited the detectability of normal bacterial associations; nevertheless, two strong associations emerged. The first was between the genus *Cerasicoccus* and the order WCHB1-41 and was evident across all study phases; the second was between the genus *Cerasicoccus* and the order Rhodospirillales, and was evident during the recovery phase. These two relationships reflect the small-scale, yet ever-present, microbial dynamics that occur in healthy, unperturbed microbiomes.

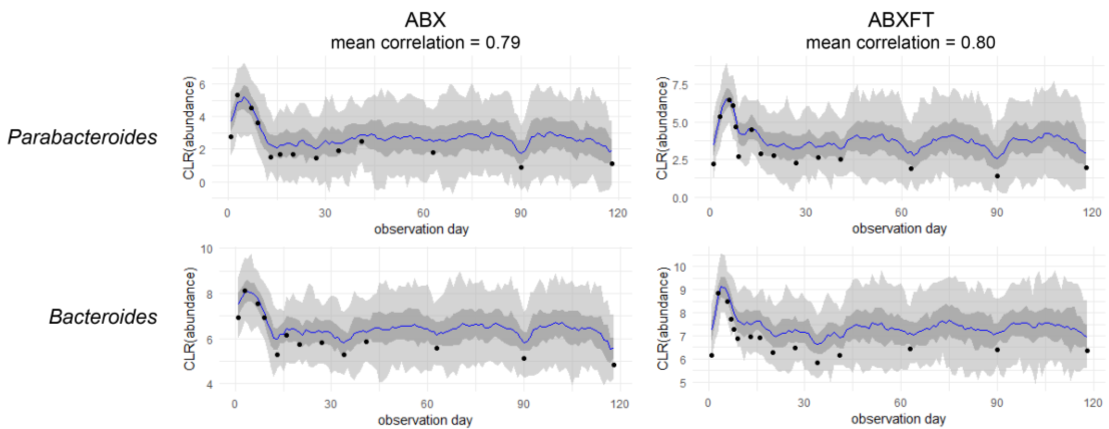

Figure 21. Associations between bacterial taxa in lemurs either treated with antibiotics only (ABX) or with antibiotics plus fecal transfaunation (ABXFT). Representative correlation plots for the association between the Centered Log Ratio (CLR) of Bacteroides and Parabacteroides abundances in healthy, male ring-tailed lemurs (*Lemur catta*) either treated with antibiotics only (ABX) or with antibiotics plus fecal transfaunation (ABXFT). The antibiotic treatment period spans days 0-6, fecal transfaunation was administered on day 7, and all days thereafter constitute the period of recovery.

Within the more variable gut microbiota of ABX and ABXFT lemurs (Figure 19), and across all three study phases, there were 35 and 31 strong associations, respectively (Figures 20a,b). In ABX animals, these associations were predominately positive, with only six negative associations, whereas in ABXFT lemurs, positive and negative associations were equally represented (15 and 16, respectively). Shared across ABX and ABXFT animals were 10 strong associations, eight positive and two negative. Within these shared associations, nine involved either *Parabacteroides* or *Bacteroides* (genus 12 and 39, respectively, in Figures 20a,b). The positive association between these two taxa was the strongest association for both ABX and ABXFT animals. Moreover, in ABX and

ABXFT animals, the log ratios of *Parabacteroides* and *Bacteroides* abundances both showed increases during the treatment phase. Because our analyses of log-ratios represent the relationships between abundances of specific taxa relative to the mean abundances of all other taxa, patterns of increasing log-ratios could reflect three possibilities: the abundances of *Parabacteroides* and *Bacteroides* were (1) truly increasing, (2) remaining stable while the mean decreased, or (3) decreasing in abundance less dramatically than the mean. Regardless of which pattern they were showing, it would suggest that these two taxa were relatively unaffected by antibiotic treatment (Figure 21). The majority of strong pairwise associations with *Parabacteroides* or *Bacteroides* were positive, indicating that the associated taxa also withstood the effects of antibiotic treatment, potentially via shared ABR genes.

During the recovery phase, the gut microbiota of ABX animals retained only 20 (18 positive and 2 negative) of their original 35 strong associations (Figure 20c), likely reflecting the reduction of bacterial taxa that survived antibiotic treatment. By contrast, the gut microbiomes of ABXFT animals retained their same 31 (23 positive and 8 negative) strong associations (Figure 20d), likely reflecting the immediate reintroduction of baseline microbes via fecal transfaunation. Only two associations were shared between ABX and ABXFT animals during recovery: *Parabacteroides* and *Bacteroides,* and *Christensenellaceae R-7 group* and *Ruminococcaceae NK4A214 group,* both of which were

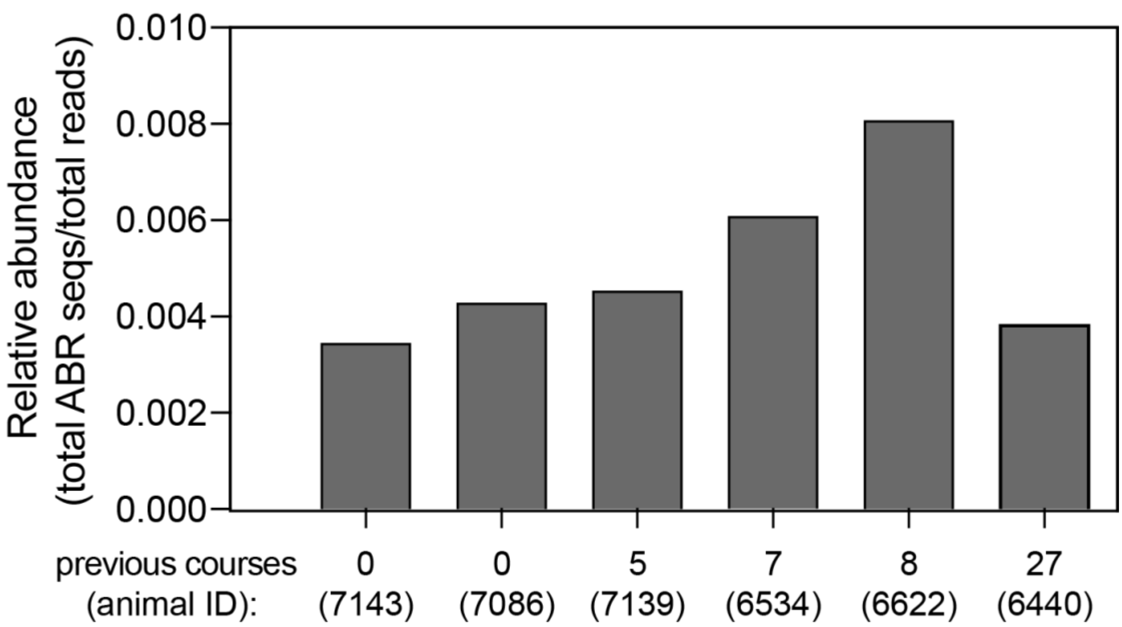

Figure 22. Relative abundance of antibiotic resistance (ABR) genes in six healthy, male ring-tailed lemurs (*Lemur catta*) that had received different numbers of treatment courses of antibiotics across their lifetime.

also shared during the entire study period. Despite variability across treatment groups and phases, some of the strongest associations persisted during recovery.

Cross-sectional and longitudinal ABR

Across the 30 fecal samples selected for shotgun sequencing (from a subset of six subjects), 3.2 million sequences were assigned to 83 known ABR genes. On average, the majority of the ABR genes detected belonged to four resistance gene families: Tetracycline (mean ± SEM; 51.4% ± 3.44%), Beta-lactam (29.5% ± 3.17%), Aminoglycoside (7.9% ± 2.25%), and Macrolide (1.2% ± 0.24%). There was also minor (>1%)

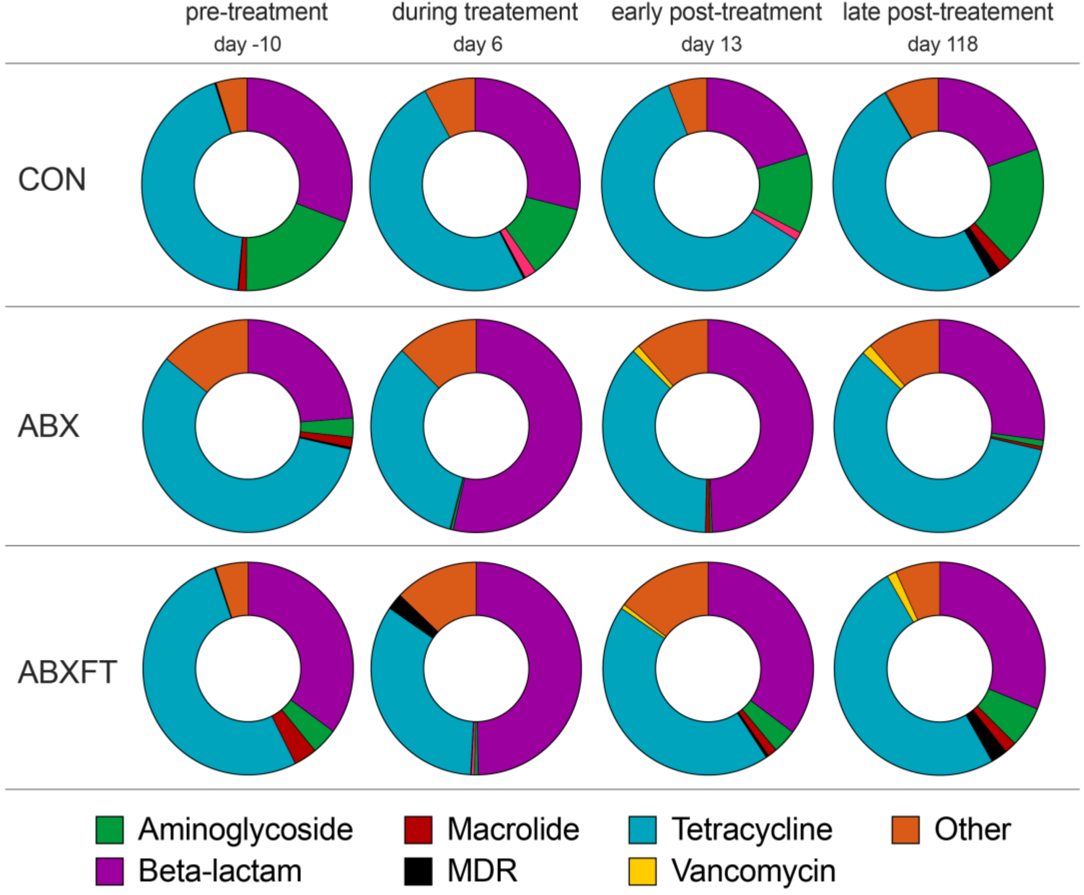

Figure 23. Proportions of antibiotic resistance (ABR) genes identified in healthy, male ring-tailed lemurs (*Lemur catta*) that received no treatment (CON), antibiotics only (ABX), or antibiotics plus fecal transfaunation (ABXFT). Shown are color-coded resistance gene families at four time points during the study, during which antibiotic treatment was administered on days 0-6 and fecal transfaunation was administered on day 7. MDR = Multi-Drug Resistant.

representation of genes in the Vancomycin, Multi-Drug Resistant, and Sulphonamide families.

The cross-sectional data on the six, focal animals revealed unexpected variation in ABR. Notably, the two animals (IDs 7143 and 7086) that had never been treated with

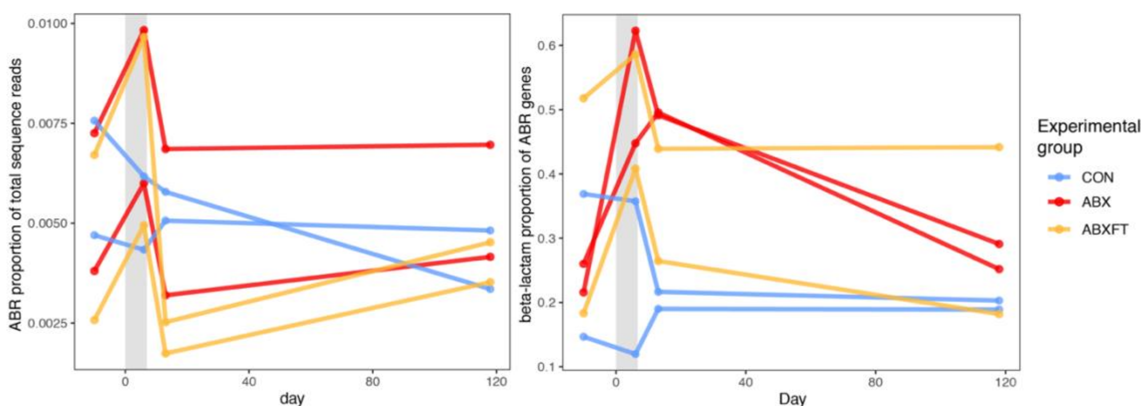

Figure 24. Patterns in antibiotic resistance across healthy, male ring-tailed lemurs (*Lemur catta*) that received no treatment (CON), antibiotics only (ABX), or antibiotics plus fecal transfaunation (ABXFT). Shown is variation over time in (a) the relative abundance of antibiotic resistance (ABR) genes and (b) the proportion of ABR genes assigned to the beta-lactam resistance gene family. The gray shaded window represents the period of antibiotic treatment, with the prior period representing baseline and the subsequent period representing recovery. SEM = standard error of the means.

antibiotics nevertheless harbored ABR at levels similar to those of animals that had previously received numerous courses of antibiotics (Figure 22). Additionally, the animal (ID 6440) with the most numerous antibiotic treatments (n = 27 courses), harbored the second lowest abundance of ABR genes, similar to that of the animals that had no previous treatment (Figure 22).

Longitudinally, the relative abundance of ABR genes varied across experimental groups (Figure 23). Whereas CON animals showed relatively little variation in mean relative abundance of ABR genes, in both ABX and ABXFT animals, ABR abundance increased between the pre-treatment and treatment phases, followed by a decrease during the recovery period. Similarly, compared to CON animals, ABX and ABXFT

animals showed an increase in the proportions of beta-lactam resistance genes during treatment, reflecting the impact of treatment with a beta-lactam antibiotic (amoxicillin; Figure 23 & 24). The proportion of beta-lactam resistance genes during early post-treatment tended to be greater in ABX animals compared to ABXFT animals (Figure 24), which may hint at a potential mitigating effect of fecal transfaunation on the persistence of ABR in lemur gut microbiomes. Although these results are qualitative, they provide preliminary insights into the acute development and persistence of ABR in relation to antibiotic treatment.

Discussion

Using a longitudinal, experimental approach in healthy nonhuman primates, we provide support for both the 'diversity begets stability' and 'keystone species' hypotheses in our ecological framework for interpreting the dynamics of host-associated, gut microbiomes following antibiotic-mediated disruption. This approach allowed us to distinguish different schedules of action associated with achieving diversity versus recovering keystone species: microbial alpha diversity rebounded quickly (albeit incompletely) in treated animals, whereas beta diversity reflected a trajectory of long-term, microbial instability in animals that received antibiotics alone. The effects of fecal transfaunation on recovery of alpha diversity were negligeable, but for beta diversity, this procedure hastened and stabilized the recovery of community

composition, supporting fecal transfaunation as an effective tool for treating microbial perturbation. The bacterial associations varied between experimental conditions, reflecting differential sensitivity to antibiotic treatment across bacterial groups and suggesting that microbial dynamics may have contributed to the differential effects of ABX and ABXFT treatments, including through the potential presence of ABR genes. Our cross-sectional analysis of ABR showed that the prevalence of ABR genes in a host is not necessarily correlated with that host's previous exposure to antibiotics; ABR can be acquired and maintained in the gut microbiomes of lemurs that had no previous antibiotic treatment. Longitudinally, ABR gene profiles reflected the type of antibiotic used. As expected, the proportion of ABR genes that confer resistance to beta-lactamase antibiotics increased during treatment with amoxicillin. Lastly, fecal transfaunation may mitigate the persistence of ABR during recovery from antibiotic treatment. Using a holistic and longitudinal approach, across scales, allowed elucidating microbial dynamics that otherwise would have been imperceptible.

Consistent with previous findings (Palleja et al., 2018; Vlčková et al., 2016), and with the known efficacy of amoxicillin as a broad-spectrum antibiotic (Kaur, Rao, & Nanda, 2011), we found that animals receiving antibiotics showed a concurrent and drastic decrease in alpha diversity. Contrary to expectations, however, lemurs post treatment showed a rapid rebound in alpha diversity, with ABX and ABFT animals showing no significant difference in their recovery trajectories. The rapidity of these

patterns may owe to the healthy status of the hosts and to the relatively short period of antibiotic treatment. The ability to recolonize or re-diversify a microbiome after a perturbation can be severely dampened by injury or disease (Krezalek & Alverdy, 2016; Nicholson et al., 2018) and by recurrent antibiotic treatment (Korpela et al., 2016; McDonald, 2017). Or, as omnivores that have a broad dietary range, ring-tailed lemurs may have shown more rapid recovery than would be observed by dietary specialists (Greene et al., 2019). Beyond external influences (e.g., from diet, the physical environment or social interaction), alpha diversity can also increase from within. Indeed, even if antibiotic treatment causes local bacterial extirpations, certain taxa can persist either by expressing ABR genes (Francino, 2015; Van Schaik, 2015) or by sequestering in areas of the gastrointestinal tract that are less affected by antibiotics (i.e., the appendix or cecum (Bollinger, Barbas, Bush, Lin, & Parker, 2007; Laurin, Everett, & Parker, 2011)), allowing for in-kind recolonization after disruption. Nevertheless, as evidenced by the results on beta diversity, early recovery of alpha diversity did not entail strictly in-kind recolonization of lemur gut microbiota. The rapidity in these patterns could thus lend support to the 'diversity begets stability' hypothesis, in that the key first step in community restoration may be to regain diversity, regardless of microbial composition.

Patterns of beta diversity elucidated longer-term effects of antibiotics on gut microbiome community composition. At no point during the four-month recovery period (collapsed across years) did the microbial community of ABX animals return to

baseline composition or even reach an alternative stable state. Therefore, recouping key microbial members may be more elusive (and perhaps more critical to stability) than recouping sheer numbers of taxa. This interpretation is consistent with the 'keystone species' hypothesis and with previous evidence (Gibbons, 2020; Theriot et al., 2014). The prolonged absence of key microbial taxa may have significant consequences to the host. Although it was beyond the scope of this study to assess changes in host condition or microbial function, it is well established that antibiotic-induced imbalances in the gut microbiota of healthy animal models can cause increased susceptibility to enteric pathogens (Buffie et al., 2012; Theriot et al., 2014) and altered or diminished immune function (Ekmekciu et al., 2017). Likewise, even natural variation in microbial community composition (e.g., between seasons or host populations) are associated with changes in microbiome function (Baniel et al., 2021; Greene et al., 2021; Hicks et al., 2018; Orkin et al., 2019).

Patterns of beta diversity also revealed the stabilizing effect on community composition of fecal transfaunation, again consistent with the 'keystone species' hypothesis. In our study, hosts were administered their own baseline feces, but similar findings were obtained in a previous study in which antibiotic-treated mice consumed feces from healthy cagemates (Antonopoulos et al., 2009). The bacterial associations that characterized the recovery phase in ABXFT lemurs were more numerous and almost wholly different from those in ABX lemurs, indicating that microbial interactions may

underpin some of the effects of fecal transfaunation. These findings are consistent with the concept of competitive exclusion, whereby the diverse group of reintroduced, native bacteria outcompete pathogenic or opportunistic microbes (Willing & Jansson, 2010; Zeng et al., 2019). Although there is much to learn about the modes of action in successful transfaunation, we contribute evidence, unconfounded by host health status, for this promising tool to hasten recovery from antibiotic exposure (Jin Song et al., 2019; Schmidt et al., 2020; Wei et al., 2015).

Of the bacterial associations present in the two treatment conditions, *Parabacteroides* and *Bacteroides* – two, closely related taxa with similar functional potential (Karlsson, Ussery, Nielsen, & Nookaew, 2011) – dominated the observed relationships. Notably, increases in the log ratio of *Bacteroides* during antibiotic treatment indicated that, while other taxa were eliminated, abundances of *Bacteroides* members were increasing, remaining stable, or decreasing less dramatically relative to the mean abundances of the other taxa. Indeed, the *Bacteroides* genus is notorious for showing ABR. The diversity of its resistance mechanisms (Macrina, Mays, Smith, & Welch, 1981; Whittle, Shoemaker, & Salyers, 2002), coupled with extensive lateral gene transfer within members of the genus and with non-*Bacteroides* taxa (Privitera, Dublanchet, & Sebald, 1979; Shoemaker, Vlamakis, Hayes, & Salyers, 2001), contributed to certain *Bacteroides spp*. having one of the highest resistance rates among known anaerobic pathogens (Wexler, 2007). Furthermore, certain *Bacteroides* species can harbor

an unknown or potentially diet-mediated mechanism that confers resistance specifically to amoxicillin (Cabral et al., 2019; Veloo, Baas, Haan, Coco, & Rossen, 2019). Certain *Bacteroides* strains can even shield other taxa from the effects of beta-lactam antibiotics (Stiefel, Tima, & Nerandzic, 2015). Combined with this evidence, our results suggest that *Bacteroides* in the lemur gut microbiome likely have amoxicillin resistance mechanisms and that bacterial taxa with log-ratios that similarly increased during treatment may share similar resistance to amoxicillin treatment.

ABR genes, including some that are considered clinically relevant (Zhang et al., 2019), were present within the gut microbiome of all lemurs. Somewhat surprisingly, lemurs that had never received antibiotic treatment showed abundances of ABR similar to those of lemurs previously treated with antibiotics. Researchers have shown that bacteria and their genes can be shared between hosts that cohabitate (Finnicum et al., 2019; Song et al., 2013) or share social partners (Archie & Tung, 2015). Furthermore, ABR genes often reside on mobile genetic units and are prone to rapid transfer between microbes (Bennett, 2008). Indeed, the 'resistance crisis' is perpetuated by the ubiquitous spread of ABR genes around the world(Van Puyvelde, Deborggraeve, & Jacobs, 2018; Ventola, 2015b, 2015a). Here, we find that lemurs are not exempt from these phenomena and, for captive animals especially, ABR could pose a severe threat to animal health(Bengtsson & Greko, 2014; Cabello, 2006). Methods to mitigate the development and spread of ABR among animal populations, including perhaps via fecal

transfaunation, may prove to be a critical facet of combatting the resistance crisis(Perry, McClean, Simonet, Woolhouse, & McNally, 2018; Ventola, 2015b).

Conclusions

Collectively, these results further our understanding of host-microbe relationships in the Anthropocene era(Austvoll, Gallo, & Montag, 2020; Gillings & Paulsen, 2014). Because antibiotics are an unavoidable component of animal care, understanding their impact on host-associated communities will provide context for studying the biology of animals under human care and strengthen protocols for animal well-being. Ultimately, shedding light on how 'old friends' react to aspects of the 'new world' is relevant both to our understanding of the evolution of symbiosis and to its implications for animal welfare and conservation.

Methods

Study subjects and housing

Our study subjects were 11 healthy, reproductively intact, adult (4-16 yrs old), male ring-tailed lemurs housed in 10 conspecific, mixed-sex groups at the Duke Lemur Center (DLC; Durham, NC, USA). Within a two-year period, 10 subjects underwent a control round with no treatment, but all 11 underwent one round of antibiotic treatment (see below), all while living in their same social groups. During inclement weather (< 5

°C), the groups would be sequestered in temperature-controlled indoor enclosures, otherwise, they all had access to indoor and outdoor enclosures (approximately 146 m^2/animal). Some of the groups additionally had access to large, forest enclosures where they semi-free-ranged with heterospecific lemurs. The animals received a diet of produce and commercial primate chow and, while semi-free-ranging, had access to natural foods foraged from the forest. Additional information on the lemurs' diet, foraging, and social behavior have been reported elsewhere(Starling et al., 2010). 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 and sample collection

To allow for a partial cross-over design, we conducted the study during two matched periods (October-February) in consecutive years, during the subjects' breeding season in the Northern Hemisphere(Drea, 2007): 2016-2017 (Y1, n = 10 subjects) and 2017-2018 (Y2, n = 11 subjects). In each year, we assigned the subjects to one of three experimental groups: control animals (CON: Y1, n = 4; Y2, n = 6), antibiotic-treated animals (ABX: Y1, n = 3; Y2, n = 3), and antibiotic-treated animals receiving a fecal transfaunation (ABXFT: Y1, n = 3; Y2, n = 2). To avoid administering antibiotics twice to any animal, each animal was assigned to the CON group in one of the two years.

Each year of study involved three phases, lasting a total of ~125 days: a pretreatment or baseline phase (lasting ~ 6 days; i.e., days -6 to -1), a treatment phase (lasting 7-8 days; i.e., days 0 to 6/7), and a recovery phase (lasting ~110 days). In the treatment phase, all treated animals (ABX and ABXFT; n = 11) received a 7-day course of the broad-spectrum, beta-lactam antibiotic, amoxicillin (10 mg/kg body weight, received orally, twice daily). Approximately 24 hrs after completion of the full antibiotic regimen, ABXFT subjects received a fecal transfaunation consisting of their own feces collected pretreatment (4 days prior to the onset of treatment for all animals): 2-3 fecal pellets were mixed with water and administered orally via syringe or feeding tube, according to routine procedures that have been adopted by the DLC since the mid 1980s to treat outbreaks of gastrointestinal diseases(Charles-Smith, Cowen, & Schopler, 2010; da Silva et al., 2003; McKenney et al., 2017).

The study phases were additionally differentiated by the frequency with which we collected fecal samples: We collected samples every 1-3 days before, during, and immediately after the treatment phase, after which sampling occurred every 5-28 days. Typically, upon the subject's morning voiding, between 7:00 am and 11:30 am, we opportunistically collected fresh fecal samples. On occasion, we collected samples from awake, gently restrained animals that were habituated to capture and collection procedures. At each time point, we sampled all subjects and we maintained analogous sampling regimes across years. We collected all samples in sterile, 15-ml falcon tubes,

immediately placed them on ice, and then stored them in a -80 °C freezer within 2 hours, until analysis.

Microbial DNA extraction, sequencing and bioinformatics

Using the DNeasy Powersoil kit (QIAGAN, Frederick, MD), we extracted microbial gDNA from fecal samples and from four blank controls, to control for possible contamination. We quantified the extracted gDNA using a Fluorometer (broad-spectrum kit, Qubit 4, Thermo Fisher Scientific, Waltham, MA). These extractions were used for bacterial identification (via 16S rRNA amplicon sequencing) and ABR gene identification (via shotgun sequencing), as described below.

Bacterial identification

We shipped aliquots of extracted gDNA to the Argonne National Laboratory's Environmental Sequencing facility (Lemont, IL) for library preparation and sequencing of the 16S rRNA gene. There, the V4 region of the 16S rRNA gene (515F-806R) was amplified with region-specific primers adapted for the Illumina MiSeq platform(Caporaso et al., 2012). Forward primers contained a 12-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 2 x 151 bp Illumina

MiSeq run(Caporaso et al., 2012). Sequencing reads are available on the National Center for Biotechnology Information's Sequence Read Archive (BioProject ID #TBD, BioSample accession #s TBD).

In collaboration with Duke University's Genomic Analysis and Bioinformatics Shared Resource, 16S raw sequence data were analyzed using a bioinformatics pipeline generated in QIIME2 (ver 2018.11)(Hall & Beiko, 2018). The pipeline included steps to join, demultiplex, and quality-filter sequence reads. The DADA2 plugin (q2-dada2)(Callahan et al., 2016) was used to denoise, quality-filter, and remove phiX and chimeric sequences from the demultiplexed reads. Using the resulting sequences, we discarded samples with < 10,000 reads. To determine taxonomic classification, we used a pre-trained Naive Bayes classifier at 99% sequence identity (SILVA-132 database)(Quast et al., 2012; Yarza et al., 2014). After bioinformatic processing, a total of 344 fecal samples (from all subjects across all study phases) yielded over 23.4 million 16S sequences (mean per sample = 59,766).

To calculate metrics of alpha and beta diversity, we first subsampled our data to a depth of 15,000 reads per sample. We then used the feature tables and taxonomies of bacterial members to calculate Shannon-Weaver diversity (i.e., alpha diversity). To assess microbial composition (i.e., beta diversity), we calculated multiple metrics of UniFrac distances. Both UniFrac metrics showed similar patterns of microbial composition (Appendix A.3.2: Figures 33 & 34), and so, in our results, we report only

unweighted UniFrac, which considers the phylogenetic relationships between taxa and, importantly, gives equal weight to rare and abundant taxa. After calculating these diversity metrics, we combined features without assigned taxonomy below the Kingdom level into an "Unassigned" category. We also included the conglomerate "Other" to represent the rare taxa that had relative abundances < 1%.

ABR identification

To allow for cross-sectional and longitudinal analyses of ABR genes, while limiting the expense of metagenomic analyses, we performed shotgun sequencing on samples from six lemurs, two per experimental group, that ranged in their previous exposure to antibiotics (0-27 previous courses). For cross-sectional analysis, we included one sample from each subject's pretreatment phase in Y1. For longitudinal analysis (which we prioritized), we included four samples from each animal (days -10, 6, 13, and 118) in Y2. We shipped this subset of extractions (n = 30) for shotgun sequencing to CosmosID (Rockville, MD), where DNA libraries were prepared using the Illumina Nextera XT library preparation kit, with a modified protocol. Library quantity was assessed with Qubit (ThermoFisher). Libraries were then sequenced on an Illumina HiSeq platform 2 x 150 bp.

The samples selected for shotgun sequencing averaged 20.4 million sequences per sample. The resulting unassembled sequencing reads underwent multi-kingdom

microbiome analysis and profiling of antibiotic resistance genes using the CosmosID bioinformatics platform (CosmosID Inc., Rockville, MD), as described elsewhere(Hasan et al., 2014; Yan et al., 2019). The antibiotic resistance and virulence genes in the microbiome were identified by querying the unassembled sequence reads against the CosmosID-curated antibiotic resistance and virulence associated gene databases(Chekabab, Lawrence, Alvarado, Predicala, & Korber, 2020; Feehan & Garcia-Diaz, 2020).

Statistical analyses

To characterize variation in bacterial diversity and composition, we used Hierarchical General Additive Models (HGAM)(E. J. Pedersen, Miller, Simpson, & Ross, 2019), which have the flexibility to accommodate nonlinear trends (for full model syntax and model description, see Appendix A.3.1, *i. Descriptions of statistical models*). HGAMs use predictor and response variables to predict smooth functional relationships that can vary by different groups (e.g., the three experimental groups). In our case, the three experimental groups were expected to have different response trajectories, so our HGAMs were structured to fit smoothing splines specific to the responses of each experimental group over time. We used this model to test for patterns in bacterial alpha and beta diversity. For analyses of beta diversity, we first used Principal Coordinates Analysis (PCoA) to visualize variation in bacterial composition (both unweighted and

weighted UniFrac distances) in coordinate space (Appendix A.3.2; Figures 33 and 34, respectively). Subsequently, we used distance metrics to calculate change in bacterial composition relative to a pretreatment, baseline community (these were the samples used for FT, collected 4 days before the onset of treatment for all animals; QIIME2). We tested for variation in these calculated distance measures using our HGAM. To assess the response to and recovery from treatment, we first used our models to test for variation across the entire dataset and, then, using subsets of the alpha and beta diversity data, we focused our model on the treatment and recovery phases.

To better understand the short- and longer-term process of recovery, we tested for associations between bacterial taxa over time and evaluated how these associations may have differed between treatment groups. To exclude spurious associations, we first removed the pretreatment samples from each animal's series, further allowing us to focus on associations during the treatment and recovery phases. To reduce sparsity in the dataset and ease the computational burden, we removed rare taxa present in less than five samples across the full dataset, clustered taxa at the genus level, and grouped as 'Other' all low-abundance genera with than 0.01% of total counts. This filtering removed less than 1% of total sequence counts.

To naturally model the irregular temporal spacing in the observations and manage autocorrelation between samples, we fitted a Bayesian multivariate Gaussian process to each of multiple, synthetic replicates of the dataset (see resampling procedure

below and a detailed description of this procedure in Appendix A.3.2, *i. Descriptions of statistical models*). We then inferred a distribution over the covariance between microbes. The sample collection schedule motivated two key choices in noise modeling and data representation. First, because stochasticity exists in sample collection, processing, and sequencing, we used a resampling method similar to that of ALDEx2(Fernandes et al., 2014; Gloor, Macklaim, & Fernandes, 2016) to emulate the variation that would be expected from replicate measurements. Second, to account for the compositional nature of the sequence count data within our model(Gloor, Macklaim, Pawlowsky-Glahn, & Egozcue, 2017), we used log ratios as compositional representations of the data that reflect the relationship between the abundances of specific taxa and the mean behavior of all taxa. We converted the estimated covariance matrices to correlation matrices and thresholded all pairwise correlations between microbes to select as significant those with 95% credible intervals that excluded zero correlation (i.e., those with strong positive or negative associations). We then ranked associations by their median strength and selected those in excess of correlation $\rho > 0.5$ or $\rho < -0.5$ as strong associations.

Because both our cross-sectional and longitudinal ABR data had small sample sizes and minimal statistical power, we were limited to examining qualitative trends in the prevalence of total ABR genes and the types of ABR gene family to which they belonged.

Declarations

Ethics approval and consent to participate

This research was approved by Duke University's Institutional Animal Care and Use Committee (protocol #A111-16-05).

Consent for publication

Not applicable. This study does not contain any individual person's data in any form.

Availability of data and material

Sequencing reads are available in the National Center for Biotechnology Information's Sequence Read Archive (BioProject ID #TBD, BioSample accession #s TBD). Additional datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Competing interests

We attest that no author has financial or non-financial competing interests.

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Authors' contributions

CMD conceived the study, with input from SLB, RLH, and NMG. SLB, RLH, NMG, KDS, and CMD collected samples. SLB and KR performed the bioinformatic and statistical analyses. SLB and CMD wrote the manuscript and all authors read and approved the submitted version.

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Chapter 5. Antibiotic resistance genes in lemur gut and soil microbiota along a gradient of anthropogenic disturbance

The content of this chapter has been submitted as an invited submission for publication in *Frontiers in Ecology and Evolution* under a special research topic “Impact of Anthropogenic Environmental Changes on Animal Microbiomes.” The full reference is as follows:

Bornbusch, S.L., & Drea, C.M. *Submitted*. Antibiotic resistance genes in lemur gut and soil microbiota along a gradient of anthropogenic disturbance.

Introduction

Although antibiotic resistance genes (ARGs) occur naturally and are evolutionarily ancient (Aminov & Mackie, 2007; D'Costa et al., 2011; Sengupta, Chattopadhyay, & Grossart, 2013), the pervasive use of antibiotics has accelerated the global propagation of ARGs and precipitated an antibiotic resistance crisis (Neu, 1992; Van Puyvelde et al., 2018; Ventola, 2015a). ARGs influence the function of bacterial communities (Kim et al., 2020), increase pathogen morbidity and mortality rates (Howard, Scott, Packard, & Jones, 2003; Lin et al., 2015), and diminish the efficacy of antibiotics (Martens & Demain, 2017; Rossolini, Arena, Pecile, & Pollini, 2014). ARGs are thus well-studied in systems that receive antibiotic treatment (e.g., clinical populations or agricultural animals and crops; W. Cheng, Chen, Su, & Yan, 2013; French, 2005; Ghosh & LaPara, 2007; Lerminiaux & Cameron, 2019). In the absence of antibiotic treatment, ARGs act as components of anthropogenic disturbance, contaminating natural microbial consortia and facilitating the transfer and incorporation of ARGs into microbiota (e.g., via contact with infected hosts or contaminated environments; Hiltunen, Virta, & Laine, 2017; Manaia, 2017). Despite the potential for ARGs to be acquired via multiple routes, the presence and abundance of ARGs in wildlife and non-model animals is relatively understudied (Dolejska & Literak, 2019; Ramey & Ahlstrom, 2020). Here, we use a site-comparative approach within a single host species, the endangered ring-tailed lemur (*Lemur catta*), to (a) characterize ARGs in the gut

microbiota of multiple wild and captive lemur populations, (b) determine if identified ARGs correlate with components of anthropogenic disturbance faced by these lemur populations, and (c) test for covariation between ARGs in wild lemurs and their environments (as proxied by ARGs in soil microbes).

ARGs evolved as mechanisms to protect microbes from both endogenously produced and external antibiotics (Baquero, Alvarez-Ortega, & Martinez, 2009; Martínez, 2008, 2012). A propensity to be exchanged via horizontal gene transfer has allowed ARGs to easily pass between and persist within host-associated and environmental microbial communities (Calero-Cáceres, Méndez, Martín-Díaz, & Muniesa, 2017; Forsberg et al., 2012; H. H. Wang et al., 2006). The associated arms race between antibiotic production and the evolution of protective ARGs has resulted in myriad, naturally occurring resistance mechanisms (Sengupta et al., 2013; Van Goethem et al., 2018; Versluis et al., 2015). Many ARGs confer resistance when present in a specific pathogen but serve non-resistance functions in their original bacterial hosts (Martinez, 2009; Versluis et al., 2015). Thus, natural ARGs are present in low-abundance, yet diverse, resistomes that can persist in microbial communities with no known human contamination (e.g., remote or extreme environments and relatively undisturbed wildlife; Jardine, Mavumengwana, & Ubomba-Jaswa, 2019; Segawa et al., 2013; Van Goethem et al., 2018). The increased selective pressure from human antibiotic use, coupled with wide-spread human encroachment into natural environments, is

expanding these ARG reservoirs in wildlife and their environments (Berglund, 2015; Gatica & Cytryn, 2013; Tripathi & Cytryn, 2017).

The most notable and well-studied route of ARG enrichment within a microbial community stems from treatment with man-made antibiotics. In response to such treatment, bacteria can rapidly evolve and/or transmit resistance to the specific drug encountered (Alanis, 2005), leading to approximately 2.8 million antibiotic-resistant infections and 35,000 human deaths per year in the U.S. alone (Centers for Disease Control and Prevention, 2019). This direct route of enrichment has greatly contributed to the spread of clinically relevant ARGs – those that confer resistance to multiple drug types or last resort antibiotics (Koch et al., 2014; Mühlberg et al., 2020; Osei Sekyere, 2016). Once resistant microbes are present in a microbial community, they can enrich the community resistome, even in the absence of antibiotic treatment, by persisting as community members and/or by transferring ARGs to native microbes (Chee-Sanford et al., 2009; Gao et al., 2018). For example, resistant microbes can be shared between humans and companion animals (Pomba et al., 2017) or acquired through consumption of contaminated dietary items (Nawaz et al., 2011; Van, Moutafis, Tran, & Coloe, 2007; H. H. Wang et al., 2006). Moreover, poor containment of human or agricultural waste that includes antibiotic residues or ARGs (Addison, 1984) has led to the contamination of urban and natural environments, expanding global ARG reservoirs (Anthony, Adekunle, & Thor 2018; Berglund, 2015). This kind of contamination is increasingly

investigated in agricultural environments (e.g., soil microbiomes; D'Costa, Griffiths, & Wright, 2007; Esiobu, Armenta, & Ike, 2002); however, presence and abundance of ARGs in wildlife remains largely unexplored (Dolejska & Literak, 2019).

ARGs are thus a newly recognized component of the Anthropocene, expanding the traditional definition of disturbance to include perturbations of microbial communities (Tan et al., 2018; Tripathi & Cytryn, 2017; Y.-G. Zhu et al., 2017) and the potential exacerbation of other consequences of human activity (such as habitat degradation negatively impacting the composition of wildlife microbiota (Amato et al., 2013; Bennett et al., 2016; Trevelline et al., 2019)). Although the consequences of ARG enrichment to wildlife health remain unknown, resistant bacteria can influence host-associated microbiota, transfer ARGs to pathogens, and alter host immune function (Bengtsson-Palme, Kristiansson, & Larsson, 2018; Brandl et al., 2008; Dafale, Srivastava, & Purohit, 2020; Nizet, 2006; Tollefson, Angulo, & Fedorka–Cray, 1998). Furthermore, ARG spillover into wild animals has the potential to subsequently re-infect exposed humans or other animals (Pomba et al., 2017). The risk of ARG enrichment in wildlife is linked to the potential for direct and indirect routes that relate to different aspects of anthropogenic disturbance: Relative to direct antibiotic administration, the risk of ARG enrichment in wildlife is expected to involve more indirect routes of exposure, including via human and agriculture presence or direct human contact. Increased study of ARGs and their transmission outside of clinical settings thus has the potential to provide a

more comprehensive understanding of naturally occurring resistance and highlight the antibiotic resistance crisis as a quintessential One Health concern with significant implications for humans and other animals, and for overall environmental health (Hernando-Amado, Coque, Baquero, & Martínez, 2019; Van Puyvelde et al., 2018). As a first step to determining the respective roles of these different enrichment routes, it is necessary to characterize ARGs in systems that portray varying types and severities of the disturbance components.

Madagascar's ongoing population boom and increasing agricultural expansion has increased the demand for antibiotics to treat human and agricultural diseases, resulting in the enrichment of ARGs in human and domestic animal populations (Gay et al., 2017; Padget et al., 2017; Frédérique Randrianirina et al., 2010). Furthermore, antibiotics are used to treat Madagascar's semi-regular plague outbreaks (caused by the bacterium *Yersinia pestis*; Andrianaivoarimanana et al., 2013; Boisier et al., 2002; Salam et al., 2020), which has further fueled the demand for easily accessible antibiotics (Rasamiravaka, 2020) and cultivated the misguided notion that antibiotics are a panacea. Coupled with a lack of infrastructure to contain human and agricultural waste, ubiquitous antibiotic use in Madagascar poses a significant risk of wide-spread ARG propagation to endemic wildlife, including lemurs.

Unlike many specialist lemur species, the ecologically flexible ring-tailed lemur can survive and reproduce in greatly disturbed habitats and in a wide range of captivity

settings (Gabriel, 2013; Jolly, Sussman, Koyama, & Rasamimanana, 2006a; Mason, 2010). Thus, across their natural range in Madagascar, ring-tailed lemurs live along a gradient of disturbance from near-pristine forests, that have minimal human activity, to degraded habitats with heavy human encroachment (including logging, agricultural land use, hunting). Similarly, captive ring-tailed lemurs live under conditions that range from naturalized settings (e.g., social housing in natural habitat enclosures) to artificial settings, some even living solitarily, as pets, in human dwellings with consistent human contact (LaFleur et al., 2019). These conditions should provide varying exposure to ARGs. Regarding direct routes of ARG enrichment, more specifically, lemurs in natural and captivity settings also have differential exposure to antibiotic treatment: whereas (a) most wild lemurs and lemurs kept as pets in Madagascar will have never experienced antibiotic treatment, (b) those populations at well-established field sites may have, on occasion, experienced antibiotic treatment during capture sessions or as part of a research protocol, and (c) lemurs at research or rescue facilities will have experienced routine veterinary care, including antibiotic treatment. Regarding indirect routes of ARG enrichment, animals are known to share microbes – and, presumably, microbial genes - with conspecifics, indicating a potential route via social transmission. Furthermore, wild and captive ring-tailed lemurs perform geophagy (i.e., earth-eating), a route by which lemurs could acquire ARGs from their environment.

Here, we first characterize ARGs in the gut microbiota of wild and captive ring-tailed lemur populations along these gradients of anthropogenic disturbance that reflect varying exposure to ARGs. We predict that the degree of overall anthropogenic disturbance will positively correlate with the abundance and diversity of ARGs across lemur populations. In addition, we predict that any clinically relevant ARGs in wild lemurs will reflect antibiotic use in human and agricultural settings in Madagascar. For lemurs in captivity settings, we expect overall ARG abundance to be greater than in wild lemurs; however, we expect the diversity and type of ARGs to reflect different routes of enrichment. For example, in captive lemurs at facilities that provide naturalized settings and routine veterinary care, we expect ARGs to reflect treatment with common veterinary antibiotics. By contrast, although pet lemurs are unlikely to receive antibiotic treatment, they have consistent and sustained direct contact with humans and domestic animals, and so we would expect pet lemurs to harbor specific ARGs that confer resistance to antibiotics commonly used by Malagasy people (e.g., antibiotics used to treat the plague).

Similar to the patterns predicted for lemur ARGs, we expect ARGs in soil microbiota to correlate with the degree of anthropogenic disturbance to the natural environment and reflect the types of antibiotics used in human and agricultural settings in Madagascar. In regard to ARG covariation between lemur and soil resistomes, we expect soil ARGs to mirror the patterns described for wild lemurs: soil from more

greatly disturbed sites should have greater abundance and diversity of ARGs relative to soil from more pristine habitats. Consistent with evidence that hosts acquire microbes from their environments (Adair & Douglas, 2017; Selway et al., 2020; C. C. Smith et al., 2015), if ARGs are shared between lemurs and soil, we would expect the lemur resistomes to be more similar to those of the soil from their local habitat compared to the soil from outside their home ranges.

Methods

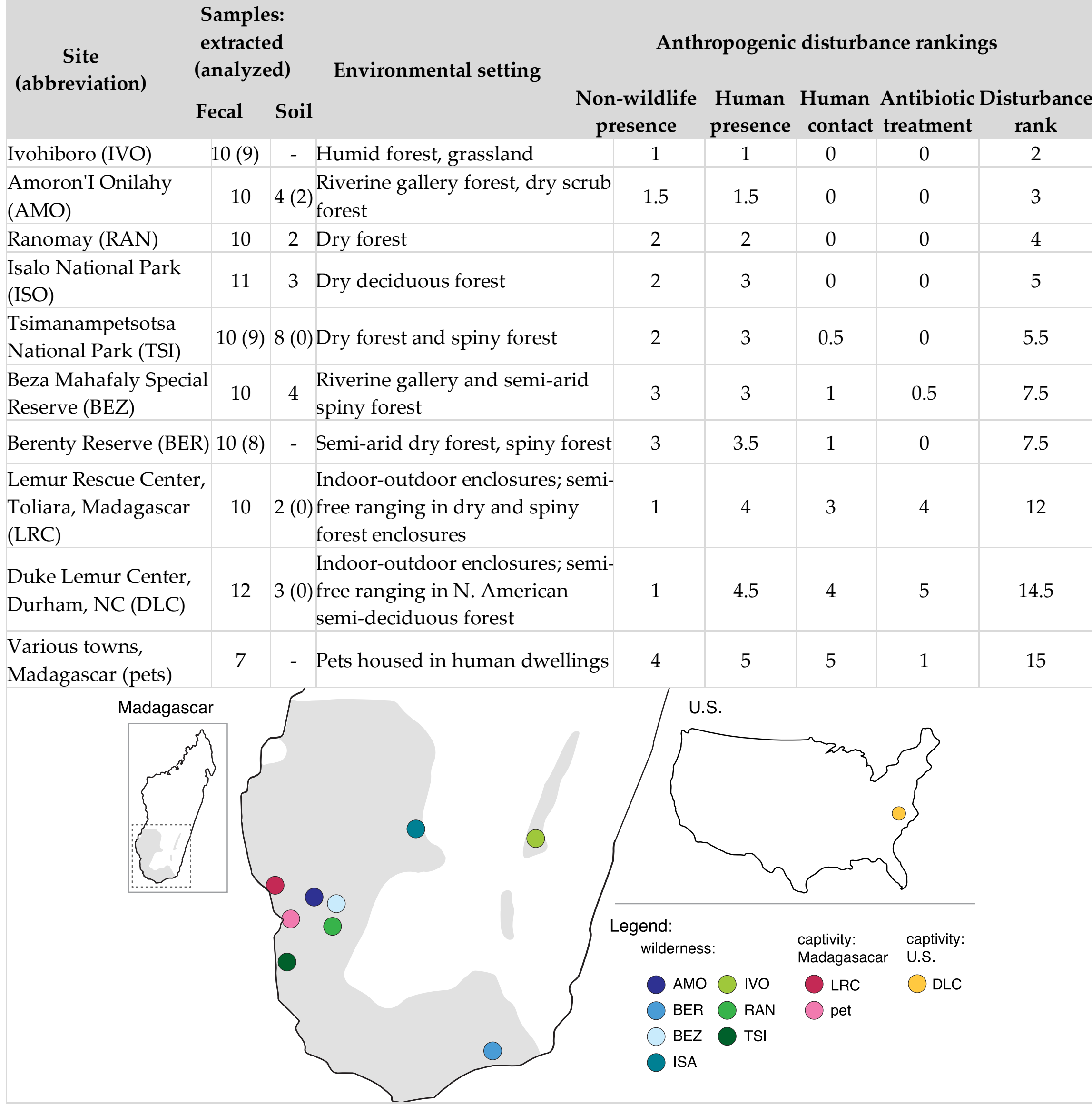
Study sites and subjects

We collected lemur fecal samples and soil samples from the following 10 habitats or settings, hereafter called 'sites' (Table 3): seven natural sites (e.g., national parks, community-managed reserves across the lemurs' natural range in Madagascar); two captivity sites in Madagascar (the Lemur Rescue Center or LRC and, collectively, households with pet lemurs), and; one captivity site in the U.S. (the Duke Lemur Center or DLC). We selected these sites based on the presence of ring-tailed lemurs, feasibility of sample collection, and a priori predictions that they would span a gradient of anthropogenic disturbance.

The lemurs at the seven natural sites (n = 71) free-ranged in groups of 5-24 individuals. Those at the LRC (n = 10) and DLC (n = 12) were socially housed in indoor-outdoor enclosures, and some had additional access to forested enclosures, where they

Table 3. Descriptions of sites, lemur fecal and soil samples, and disturbance ranks. Sites include seven natural habitats (IVO-BER), two captivity settings in Madagascar (LRC, pets) and one captivity setting in the U.S. (DLC). Some of the samples that were extracted were not included in the analyses owing to low-yield extractions or low-quality sequencing. Rankings for each of the four components of anthropogenic disturbance (scale of 0-5; 0 = no known observations, 5 = frequent observations) were based on communications with researchers who had visited a subset of the sites, combined with existing literature and personal observations. Disturbance rank is the sum of ranks across all four components. Maps show locations of each site; the gray shaded area shows the natural range of wild ring-tailed lemurs in Madagascar.

Site (abbreviation)	Samples: extracted (analyzed)		Environmental setting	Anthropogenic disturbance rankings				
	Fecal	Soil		Non-wildlife presence	Human presence	Human contact	Antibiotic treatment	Disturbance rank
Ivohiboro (IVO)	10 (9)	-	Humid forest, grassland	1	1	0	0	2
Amoron'I Onilahy (AMO)	10	4 (2)	Riverine gallery forest, dry scrub forest	1.5	1.5	0	0	3
Ranomay (RAN)	10	2	Dry forest	2	2	0	0	4
Isalo National Park (ISO)	11	3	Dry deciduous forest	2	3	0	0	5
Tsimanampetsotsa National Park (TSI)	10 (9)	8 (0)	Dry forest and spiny forest	2	3	0.5	0	5.5
Beza Mahafaly Special Reserve (BEZ)	10	4	Riverine gallery and semi-arid spiny forest	3	3	1	0.5	7.5
Berenty Reserve (BER)	10 (8)	-	Semi-arid dry forest, spiny forest	3	3.5	1	0	7.5
Lemur Rescue Center, Toliara, Madagascar (LRC)	10	2 (0)	Indoor-outdoor enclosures; semi-free ranging in dry and spiny forest enclosures	1	4	3	4	12
Duke Lemur Center, Durham, NC (DLC)	12	3 (0)	Indoor-outdoor enclosures; semi-free ranging in N. American semi-deciduous forest	1	4.5	4	5	14.5
Various towns, Madagascar (pets)	7	-	Pets housed in human dwellings	4	5	5	1	15



could semi-free range. The latter were provided facility-standardized diets (i.e., fresh produce and commercial chow73). The pet lemurs (n = 7) were kept solitarily in human dwellings, were fed fruit, rice, and other foods marketed for human consumption, and had frequent contact with humans and domestic animals (personal observations and communications).

Assessing anthropogenic disturbance

We identified four components of potential anthropogenic disturbance across our 10 sites that we expected might influence ARG prevalence: non-wildlife animal presence, human presence, direct human contact, and antibiotic treatment. For the purpose of ranking these threats, we weighted each component independently and equally (rankings in Table 3 are based on the criteria and information presented in Table 4). Each of five researchers, who had visited or performed research at a subset of the sites, provided descriptions of the sites and information on the presence or frequency of the four components (Table 4). We compiled this information, combined it with existing literature on the sites and disturbance components (included in Table 4), and ranked each component, individually, on a scale of 0-5 (0 = no known observations, 5 = frequent observations) for each of the 10 sites. We then tallied the rankings across the four components (Tables 3 and 4 list sites in ascending order of total estimated disturbance)

Table 4. Criteria used to define and characterize the four components of anthropogenic disturbance: (A) Non-wildlife animal presence, (B) human presence, (C) direct human contact, and (D) antibiotic treatment. Included information is based on existing literature, personal observations, and communications with researchers at each site.

A. Non-wildlife, animal presence. Lemurs are exposed to agricultural, domestic, and companion animals, and introduced or pest animals. Examples of exposure in natural habitats include via grazing or holding of cattle, goats, sheep, and poultry, as well as via free-roaming domestic or feral dogs and cats. In more developed areas, non-wildlife animals are kept in proximity to human dwellings and/or can roam in areas shared with humans (e.g., companion animals, free-ranging poultry, rats). For examples and reviews of ARGs in non-wildlife animals: (Bâtie et al., 2020; Dulo et al., 2015; Duquette & Nuttall, 2004; Epstein, Yam, Peiris, & Epstein, 2009; Gay et al., 2017; Iramiot, Kajumbula, Bazira, Kansiime, & Asiimwe, 2020; Ortega-Paredes et al., 2019; Rakotoharinome et al., 2014; Rousham, Unicomb, & Islam, 2018; Van den Honert, Gouws, & Hoffman, 2018; Worsley-Tonks et al., 2020)	
IVO	Minimal agricultural animal holding and grazing
AMO	Minimal agricultural animal holding and grazing
RAN	Agricultural animal holding and grazing
ISO	Agricultural animal holding and grazing
TSI	Agricultural animal holding and grazing
BEZ	Agricultural animal holding and grazing; few free-roaming domestic animals (e.g., cats or dogs)
BER	Agricultural animal holding and grazing; few free-roaming domestic animals (e.g., cats or dogs)
LRC	Minimal presence of agricultural and domestic species around facility.
DLC	Minimal non-wildlife animal presence within or around the facility; outdoor rats and other pest animals (e.g., racoons and squirrels).
pets	Cohabitate with agricultural (i.e., poultry) and companion animals; exposed to pest animals (e.g., rats)
B. Human presence. Lemurs are exposed to the local Malagasy and to foreigners (e.g., researchers and tourists), and to their waste (via e.g., defecation). Most common examples of human activity within natural habitats are (a) shepherding animals, (b) harvesting natural resources (e.g., logging, hunting), (c) agriculture (e.g., tending rice paddies), and (d) foot traffic. In captivity settings, human presence encompasses animal caretakers, veterinarians, visitors, and/or pet owners. For examples and reviews of the links between human presence and ARGs, see: (Gay et al., 2017; Lemos, Pedrinho, de Vasconcelos, Tsai, & Mendes, 2021; Pehrsson et al., 2016; Pruden, Pei, Storteboom, & Carlson, 2006; Rajgire, 2013; Frederique Randrianirina et al., 2014; Rousham et al., 2018; Tripathi & Cytryn, 2017; F. Wang et al., 2016)	
IVO	Limited shepherding of agricultural animals; resource harvesting (e.g., honey).
AMO	Limited shepherding of agricultural animals; light logging and resource harvesting (e.g., tamarind); foot traffic between villages.
RAN	Limited shepherding of agricultural animals; light logging and resource harvesting; foot traffic between villages.
ISO	Eco-tourists and researchers frequent certain areas; camping within park boundaries; agricultural land (e.g., rice paddies and sugar cane fields) on park boundaries.
TSI	Eco-tourists and researchers frequent certain areas; camping within park boundaries; light logging; occasional hunting or poaching.
BEZ	Researchers frequent established field site; infrequent ecotourists; commonly used trails; shepherding of agricultural animals; light logging.
BER	Eco-tourists and researchers frequent the small reserve; tourist accommodations and camping within the reserve; commonly used trails; surrounded by agricultural land (e.g., rice paddies)

<table>
<tr><td>LRC</td><td>Intermittent, daily presence of animal keepers and facility staff; sporadic researchers and visitors.</td></tr>
<tr><td>DLC</td><td>Intermittent, daily presence of animal keepers, facility staff, and researchers in indoor and outdoor enclosures; frequent tour groups.</td></tr>
<tr><td>pets</td><td>Consistent presence of household members, tourists, and villagers.</td></tr>
<tr><td colspan="2">C. Direct human contact. Most commonly, human contact with lemurs entails handling for research or veterinary purposes. Although it is rare for certain populations of wild lemurs to have human contact, some have been subjects of long-term research, including annual captures. Pet lemurs have frequent contact with their owners and other humans (e.g., for promoting tourism (Clarke, Reuter, LaFleur, & Schaefer, 2019; LaFleur et al., 2019; Reuter & Schaefer, 2016)). For examples and reviews of the links between human contact and ARGs, see: (Booton et al., 2021; De Souze Lopez et al., 2015; Iramiot et al., 2020; Frederique Randrianirina et al., 2014; Frédérique Randrianirina et al., 2010; Richards et al., 2019)</td></tr>
<tr><td>IVO</td><td>No animal handling</td></tr>
<tr><td>AMO</td><td>No animal handling</td></tr>
<tr><td>RAN</td><td>No animal handling</td></tr>
<tr><td>ISO</td><td>No handling for research; Animal handling by tourists is prohibited; first protected in 1927, managed by Madagascar National Park since 1996.</td></tr>
<tr><td>TSI</td><td>Handling of animals for research occurred recently, but rarely (twice in the 10 years before sampling); Animal handling by tourists is prohibited; first protected in 1927, managed by Madagascar National Park since 1996.</td></tr>
<tr><td>BEZ</td><td>Semi-annual handling of animals for research purposes; animal handling by tourists is prohibited; established in 1975 as a research field site (Sussman & Ratsirarson, 2006).</td></tr>
<tr><td>BER</td><td>Semi-annual handling of animals for research purposes; animal handling by tourists is prohibited; established reserve since 1936, first lemur research occurred in 1963 (Jolly, Koyama, Rasamimanana, Crowley, & Williams, 2006).</td></tr>
<tr><td>LRC</td><td>Annual (minimum frequency) to sporadic handling of animals for research and veterinary purposes; founded in 2011 as an animal rescue facility.</td></tr>
<tr><td>DLC</td><td>Yearly (minimum frequency) to bi-weekly (maximum frequency) handling of animals for research and veterinary purposes; founded in 1966 as a primate research center.</td></tr>
<tr><td>pets</td><td>Potentially daily handling by owners and tourists.</td></tr>
<tr><td colspan="2">D. Antibiotic treatment. Treatment of wild lemurs is only applicable at certain field sites (e.g., TSI, BEZ, BER) where antibiotics have been used during capture sessions for veterinary purposes. For captive animals, antibiotics are used for veterinary treatment and research purposes. For examples and reviews of the links between antibiotic treatment and ARGs, see: (Bornbusch et al., in prep.; Campbell et al., 2020; Gillings, 2013; Higuera-Llantén et al., 2018; Kang et al., 2021; Jun Li et al., 2019; Xiong et al., 2018)</td></tr>
<tr><td>IVO</td><td>N/A</td></tr>
<tr><td>AMO</td><td>N/A</td></tr>
<tr><td>RAN</td><td>N/A</td></tr>
<tr><td>ISO</td><td>N/A</td></tr>
<tr><td>TSI</td><td>N/A</td></tr>
<tr><td>BEZ</td><td>Rare cases of treatment during capture sessions; lemurs sampled for this study had not been treated.</td></tr>
<tr><td>BER</td><td>N/A</td></tr>
<tr><td>LRC</td><td>Antibiotics administered for veterinary purposes; drug availability can be limited.</td></tr>
<tr><td>DLC</td><td>Antibiotics administered for veterinary and research purposes; lifetime number of antibiotic courses range from 1-27 across our DLC subjects.</td></tr>
<tr><td>pets</td><td>Limited potential for veterinary treatment.</td></tr>
</table>

to estimate an overall disturbance at each site (Table 3). Although differing in severity, agriculture and human presence were estimated to influence lemurs at nearly all sites. By contrast, direct human contact and antibiotic treatment were largely limited to impacting lemurs in captivity settings, with the exceptions being TSI, BEZ, and BER where lemurs are infrequently handled for research purposes.

Sample collection

The protocols associated with sample collection are detailed in Chapter 2 (Bornbusch et al., *in prep*.). In brief, we opportunistically collected fresh fecal samples from lemurs at all 10 sites. We additionally collected soil samples from the lemurs' core areas at four of the natural habitat sites (Table 3). To avoid the confound of seasonality, we collected all samples during the dry season in Madagascar (May-October, 2016-2020) and a single fall season in the U.S. (October 2017). Each sample was placed in an Omnigene tube containing a stabilizing buffer that preserves microbial communities at room temperature for 8 weeks (Omnigene.Gut tube, DNAgenotek, Ontario, Canada75,76) and, within that 8-week period, the samples were transported to the U.S. and stored at -80 °C until analysis.

Microbial DNA extraction, sequencing, and bioinformatics

Following the manufacturer's protocols for the DNeasy Powersoil kit (QIAGAN, Frederick, MD), we extracted bacterial genomic DNA from fecal and soil samples. We quantified DNA using a Fluorometer (broad-spectrum kit, Qubit 4, Thermo Fisher Scientific, Waltham, MA). Extracted samples that did not yield sufficient, extracted DNA were excluded from shotgun sequencing. Aliquots of extracted DNA were sent to CosmosID Inc. (Rockville, MD) for shotgun metagenomic sequencing. DNA libraries were prepared using the Illumina Nextera XT library preparation kit, with a modified protocol (Hasan et al., 2014; Lax et al., 2014; Ottesen et al., 2016). Library quantity was assessed with Qubit (ThermoFisher). Libraries were then sequenced on an Illumina HiSeq platform 2 x 150 bp.

Unassembled sequencing reads were directly analyzed using CosmosID's bioinformatics platform for identifying and profiling ARGs (described in 77–79). Briefly, the system uses curated genome databases and a high-performance, data-mining algorithm that rapidly disambiguates millions of metagenomic sequence reads. ARGs in the microbiome were identified by querying the unassembled sequence reads against the CosmosID curated antibiotic resistance and virulence-associated gene databases. Outputs include the identity and family, percent gene coverage, and frequency counts of ARGs within each sample. We categorized clinically relevant ARGs (i.e., those that pose significant risk to human health) using existing literature on the global enrichment,

mobility, and pathogenicity of ARGs (Martínez, Coque, & Baquero, 2015; Zhang et al., 2019).

Statistical analyses

To calculate the relative abundance of ARGs within a fecal or soil sample, we divided the frequency count of all ARGs or specific gene families by the sample's total read count. To determine the diversity of ARGs, we calculated Shannon-Weaver and Simpson diversity indices (R studio, version 4.0.2). For fecal samples, we used linear mixed models (LMMs) of the relative abundance or diversity of ARGs to test for correlations with the disturbance ranks, while taking into account the research site as a random effect. To determine the composition of ARGs in lemur gut and soil microbiota, we calculated Bray-Curtis dissimilarities (Bray & Curtis, 1957). We used the resulting dissimilarity matrix to perform principal coordinate analyses, allowing us to visualize the clustering of ARG composition, and to compare ARG composition within and between sites (via Kruskal-Wallis tests with Benjamin-Hochberg adjustments).

To test if soil-associated ARGs were present in lemur fecal samples, we used FEAST, a tool for microbial source tracking that relies on fast expectation-maximization 83. For this analysis, we used the four natural sites for which we had matched fecal and soil samples. Because this analysis requires an assumption of directionality (i.e., from a source to a sink), we categorized soil samples as 'sources' of ARGs and lemur fecal

samples as 'sinks' (Shenhav et al., 2019); however, we acknowledge the potential for bi-directional transmission of ARGs between lemurs and soils. For each lemur fecal sample, we calculated the proportions of ARGs that were shared with each soil community or from a default, hypothetical "unknown source" that accounts for ARGs not found in the soil samples.

Results

Ring-tailed lemur resistomes

We identified ARGs in the fecal samples of 89% (i.e., all but 11) of the ring-tailed lemurs in this study. Their relative abundances (i.e., proportion of all metagenomic sequence reads assigned to known ARGs) ranged from ~0-1% (Figure 25a,b). Compared to wild lemurs, lemurs from the three captivity settings had significantly greater ARG relative abundances (Figure 25a, Appendix A.4.1.); nevertheless, whether considering all of the environments surveyed (LMM; $t = 7.081$, $p < 0.0001$; Figure 25a) or only the wilderness sites (LMM; $t = 2.248$, $p = 0.027$; Figure 25b), anthropogenic disturbance rank significantly correlated with the relative abundance of ARGs in host guts. Qualitatively, among wild lemurs, those from the wilderness sites with the highest disturbance ranks (i.e., BEZ and BER) had the greatest ARG abundances. This trend was significant when comparing BER to four of the other wilderness sites (i.e., IVO, AMO, RAN, and TSI) but not when comparing to ISA (Appendix A.4.1; Table 12). At most of the 10 sites, there

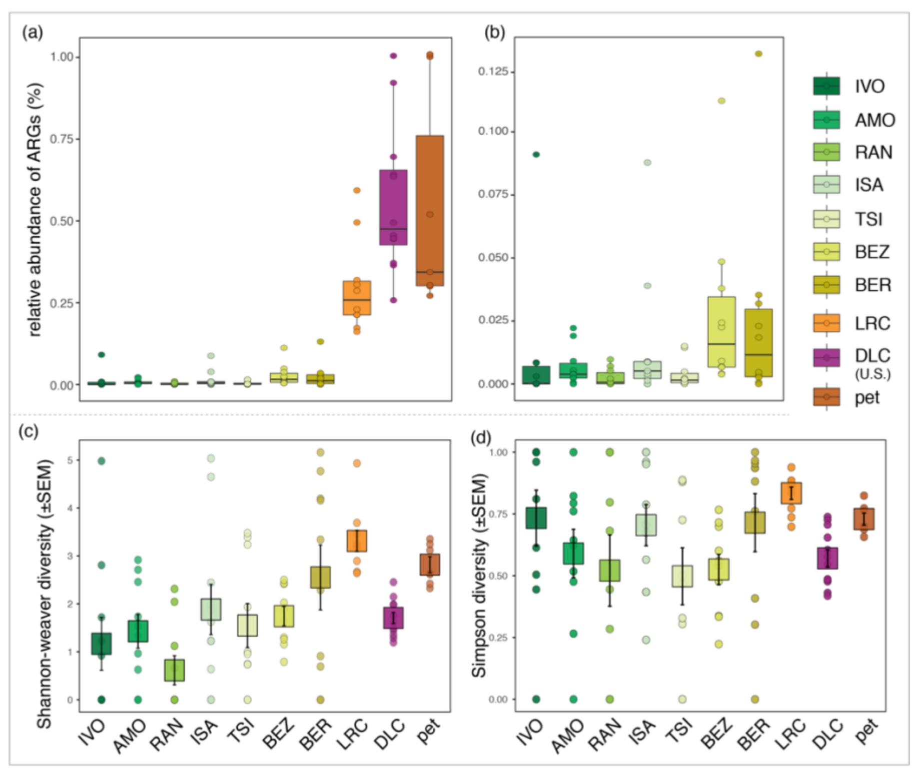


Figure 25. Relative abundances and diversity of ARGs in the gut microbiota of ring-tailed lemurs (*Lemur catta*) living at 10 sites: seven natural habitats, two captive settings in Madagascar, and one captive setting in the U.S. Shown are the relative abundances of ARGs in (a) all lemurs and (b) wild lemurs only. Also shown are (c) Shannon-Weaver diversity and (d) Simpson diversity of ARGs across all lemurs. Sites are presented in ascending order according to their ranked level of anthropogenic disturbance (Table 3).

were outlier individuals that appeared to have substantially greater ARG abundances than their site-mates (Figure 25a,b). For example, at IVO, ISA, BEZ, and BER, the relative abundances of ARGs in outlier individuals were 2-3 standard deviations greater than the average ARG abundance across the site-mates.

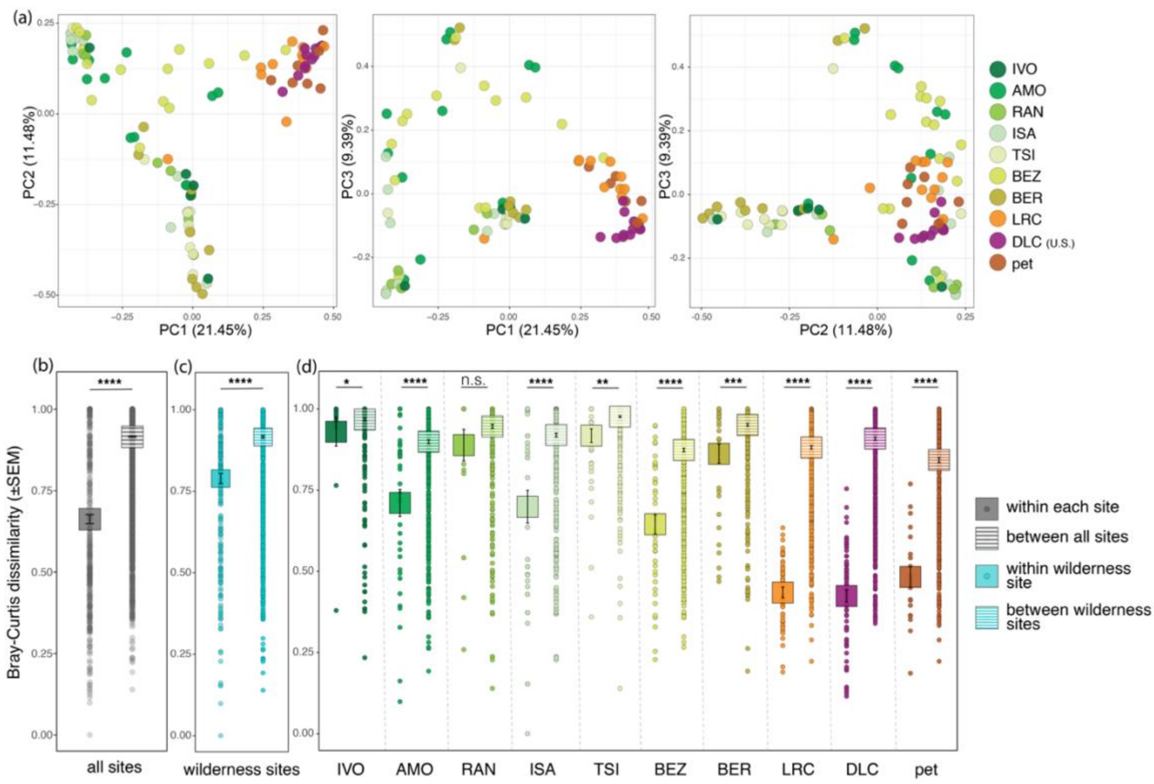


Figure 26. Beta diversity of ARGs in the gut microbiota of ring-tailed lemurs (*Lemur catta*) living at 10 sites (as identified in Table 3). (a) Principal coordinate analyses of Bray-Curtis dissimilarity including combinations of axes 1, 2, and 3. Analyses of dissimilarity within or between sites for (b) all lemurs, (c) only wild lemurs, and (d) by site. Sites are presented in ascending order according to the ranked level of anthropogenic disturbance (Table 3). Kruskal-Wallis test with pairwise comparisons and Benjamini-Hochberg correction; p < 0.05 *, p < 0.01 **, p < 0.001 ***, p < 0.0001 ****, n.s. = nonsignificant.

Compared to ARG relative abundance, the diversity metrics of ARGs in lemur fecal samples were more similar across sites, showing both high inter-individual variation within sites and substantial overlap between sites (Figure 25c,d). Disturbance rank was nonetheless a significant predictor of ARG Shannon diversity across all lemurs

(LMM; all lemurs: $t = 2.505$, $p = 0.034$; Figure 25c), but not across wild lemurs (LMM; wild lemurs: $t = 2.107$, $p = 0.081$; Figure 25c). Disturbance rank did not significantly predict Simpson diversity at any scale (LMM; all lemurs: $t = 0.679$; $p = 0.515$, wild lemurs: $t = -0.428$, $p = 0.685$; Figure 1d).

Overall, lemur resistomes largely clustered according to whether the lemur was wild or captive (Figure 26a). Within a site, wild lemurs had significantly greater interindividual variation than did captive lemurs (i.e., they clustered less tightly; Appendix A.4.2; Table 13). Additionally, variation between lemur resistomes was significantly lower when comparing samples within sites versus between sites, a pattern that held true at all but one site (i.e., RAN; Appendix A.4.2; Table 13). In other words, lemur resistomes were largely site-specific (Figure 26b-d; Appendix A.4.2; Table 13). This pattern was most pronounced in the lemurs from the three captive settings, further indicating that their resistomes differed dramatically from those of wild lemurs.

Although the relative abundances of ARGs varied across lemur populations, lemur resistomes were largely dominated by ARGs in the tetracycline resistance gene family ($\mu = 53.47\% \pm \sigma = 36.12\%$), with additional notable contributions of ARGs from the vancomycin (8.45% ± 21.22%), multi-drug resistant (MDR; 7.96% ± 17.82%), beta-lactam (6.98% ± 16.51%), aminoglycoside (3.45% ± 11.29%), and macrolide (3.40% ± 8.64%) gene families (Figure 27). At the gene level, the five most prevalent ARGs included three tetracycline ARGs (*tetW*, *tetQ*, and *tetO*) and two vancomycin ARGs (*vanH* and *vanS*),

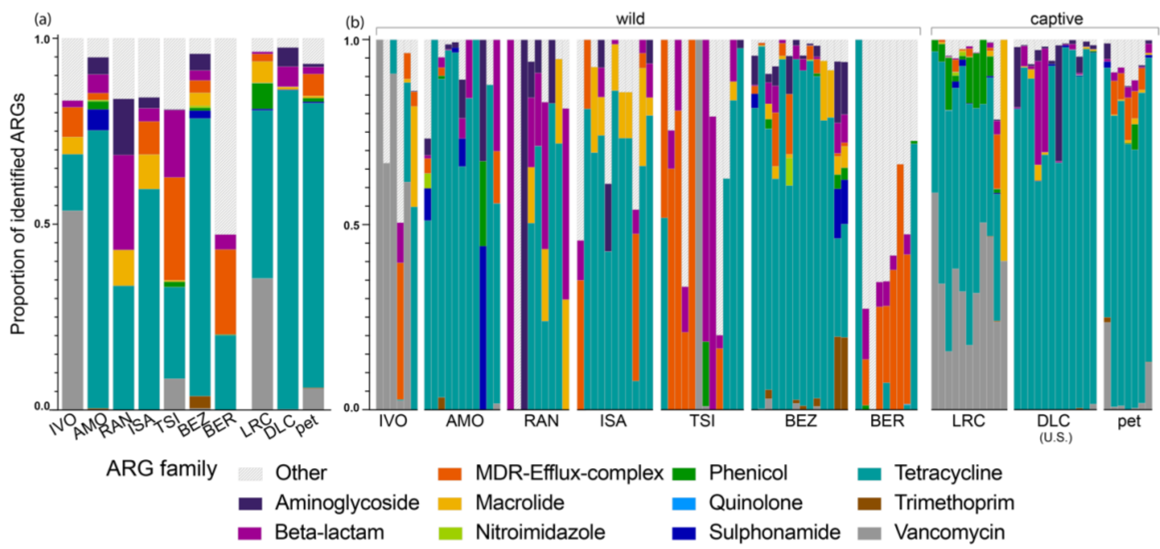


Figure 27. The proportions of identified ARGs, categorized by gene family, in the gut microbiota of ring-tailed lemurs (*Lemur catta*) at seven natural habitats, two captive settings in Madagascar, and one captive setting in the U.S. Data are shown (a) averaged by 'site' and (b) by individual lemur within sites. Gene families were identified by color and those representing < 1% of the ARGs were combined into the category "Other". Sites are presented in ascending order according to the ranked level of anthropogenic disturbance (Table 3).

which, when summed, accounted for 63.16% of ARGs across all of the lemurs. Certain ARGs were more prevalent in different lemur populations. Notably, lemurs from BER had marked proportions of ARGs (e.g., *evgS*, *mdtF*, and *AcrF* genes) that were not classified under a single gene family, but confer resistance to multiple antibiotic classes, including fluroquinolones, penams, and macrolides ((Alcock et al., 2020); Figure 27). In addition, lemurs from the LRC, predominantly, had notable proportions of phenicol resistance genes (6.88% ± 6.49%; Figure 27). Vancomycin ARGs were found almost exclusively in lemurs from Madagascar, notably in animals at the LRC, as well as in

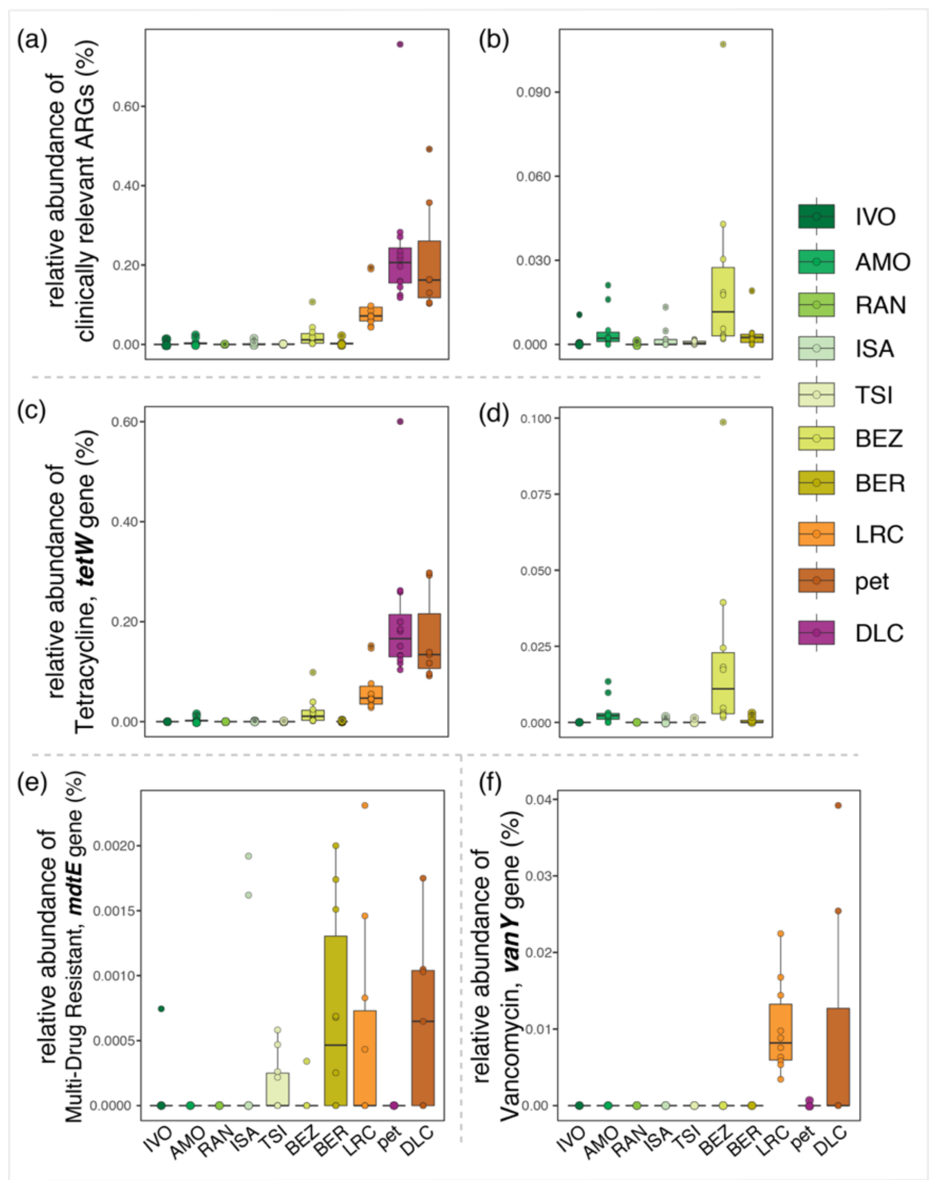


Figure 28. Relative abundances clinically relevant ARGs, including (a,b) all identified clinically relevant ARGs and (c,d) Tetracycline resistance gene, *tetW* in the gut microbiota of (a,c) all ring-tailed lemurs (*Lemur catta*) versus (b,d) only wild lemurs, across sites ranked by increasing level of anthropogenic disturbance (see Table 3). Also shown are the relative abundances of (e) multi-drug resistant gene *mdtE* and (f) Vancomycin-resistant gene, *vanY*, for lemurs at all sites.

some pets (Figure 27). Although vancomycin ARGs dominated the resistomes of lemurs at IVO, it should be noted that their relative abundances were quite low.

With respect to clinically relevant ARGs – those identified as posing significant risk to human health – relative abundance was significantly correlated with disturbance rank when comparing across all lemurs (LMM; $t = 6.201$, $p = 0.0002$; Figure 28a), but not when comparing across wild lemurs only (LMM; $t = 1.459$, $p = 0.204$; Figure 28b). This pattern was mirrored in the relative abundance of the *tetW* gene, the most prevalent of the clinically relevant ARGs (LMM; all lemurs: $t = 5.724$, $p = 0.0003$; wild lemurs: $t = 1.281$, $p = 0.256$; Figures 28c,d). Other clinically relevant ARGs, such *mdtE* and *vanY*, also varied in relative abundance in lemurs across sites (Figures 28e,f).

Soil resistomes and their covariation with lemur resistomes

The relative abundance and diversity of ARGs in soil microbiota appeared to vary between the four sites analyzed, but the differences did not reach statistical significance (Figure 29a,b), likely owing to small sample sizes. One notable pattern consistent with predictions, however, was that the greatest relative abundance and diversity of ARGs occurred at BEZ, the most disturbed of these four sites (Figure 29a,b). Here, the resistomes of two soil samples clustered distinctly from all other samples (arrows in Figure 29c,d), and were the only ones to include notable proportions of phenicol, sulphonamide and trimethoprim ARGs. Otherwise, similar to lemur

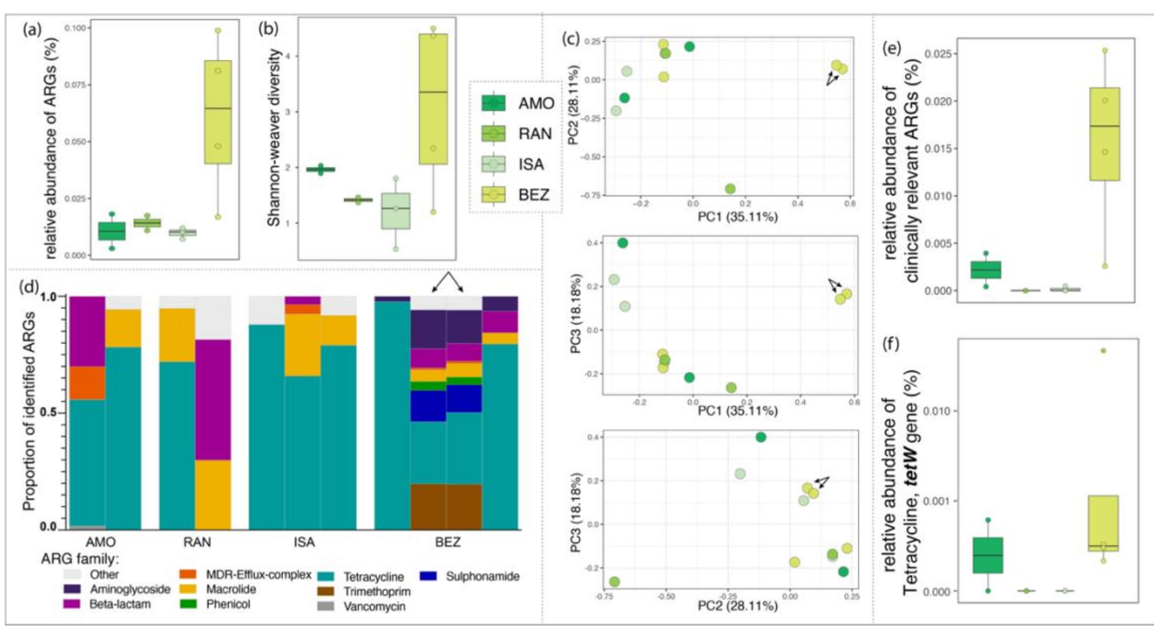


Figure 29. ARGs in soil samples from four sites in Madagascar, representing different levels of anthropogenic disturbance. Shown are (a) relative abundance, (b) alpha diversity, (c) Principal coordinate analyses of Bray-Curtis dissimilarity, including combinations of axes 1, 2, and 3, (d) proportions of identified ARGs by gene family, (e) relative abundances of all clinically relevant ARGs, and (f) relative abundances of the *tetW* gene. Gene families were identified by color and those representing < 1% of the ARGs were combined into the category "Other". Sites are presented in ascending order according to the ranked level of disturbance (Table 3). Arrows in (c and d) point to two soil samples from BEZ that cluster distinctly, reflecting their unique composition.

resistomes, ARGs in soil microbiota were dominated by tetracycline resistance genes (μ= 61.044% ± σ = 30.091%), with additional substantial contributions of ARGs from macrolide (11.22% ± 11.06%) and beta-lactam (10.03% ± 16.39%) ARGs. Notably, soil from BEZ also included substantial proportions of aminoglycoside (3.59% ± 6.17%), trimethoprim (3.58% ± 7.96%), and sulphonamide (2.30% ± 5.13%) families (Figure 29d). Unlike lemur resistomes, however, the soil microbiota had low abundances of ARGs in

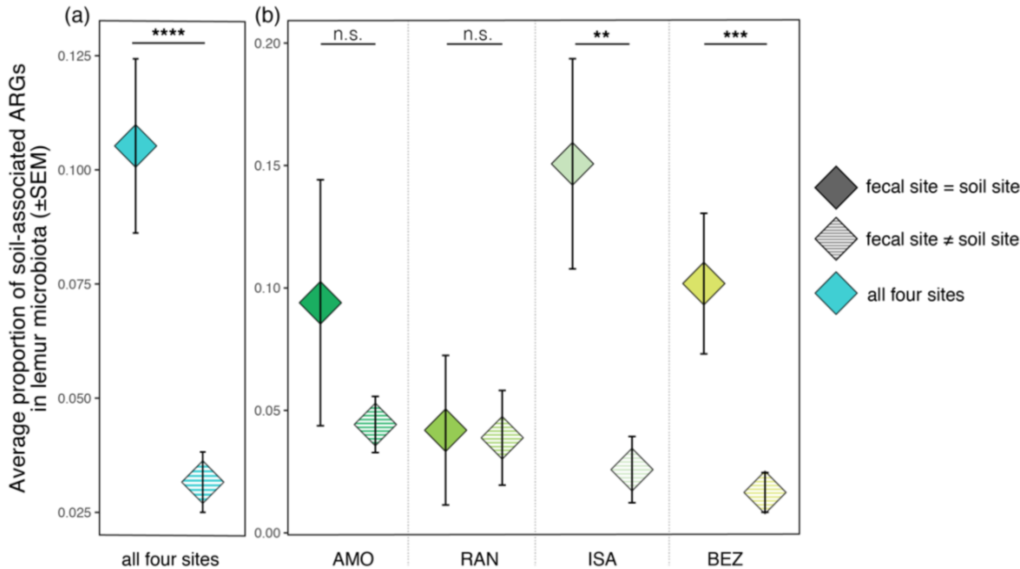


Figure 30. Shown are the proportions of soil-associated ARGs in lemurs depending on whether the soil was from the same site as the lemur (fecal site = soil site; solid color) or from a different site (fecal site ≠ soil site; striped) (a) averaged across all four sites and (b) for each specific site. Kruskal-Wallis test with pairwise comparisons and Benjamini-Hochberg correction; $p < 0.01$**, $p < 0.001$***, $p < 0.0001$****, n.s. = nonsignificant.

the vancomycin and MDR gene families. The relative abundance of clinically relevant ARGs (Figure 29e), including the *tetW* gene (Figure 29e,f) reprised patterns observed in lemur fecal samples (Figure 29a-d).

Regarding covariation between lemur and soil resistomes, there were no significant differences between the total proportion of soil-associated ARGs in lemur guts across the four sites from which soil samples were analyzed (Kruskal-Wallis test; $\chi^2 = 2.781$, $p = 0.426$; Figure 30a). Nevertheless, when comparing all lemur and soil samples from the four sites, soil resistomes from within a population's site accounted for, on

average, significantly greater proportions of lemur ARGs than did soil resistomes from other sites (Kruskal-Wallis test; all sites: $\chi^2 = 20.91$, $p < 0.0001$; Figure 30b). However, when comparing lemur and soil ARGs within and between specific sites, this pattern was not significant for all sites (Figure 30c); whereas soil resistomes from ISA and BEZ had significantly greater representation in ISA and BEZ lemurs than did soil resistomes from other sites, the same patter was nonsignificant from soil and lemurs from AMO and RAN (Pairwise Wilcoxon tests with Benjamini-Hochberg adjustment; ISA: $p = 0.005$; BEZ: $p = 0.0001$; AMO: $p = 0.896$; RAN: $p = 0.896$; Figure 30c).

Discussion

By broadly assessing ARGs in multiple ring-tailed lemur populations living under variably disturbed conditions, we (a) shed light on the breadth of ARG presence and diversity outside of traditionally studied settings, (b) highlight the potentially different routes by which ARGs may be acquired by wild versus captive animals, and (c) demonstrate covariance between lemur and soil resistomes in certain wild populations. Consistent with previous findings on natural resistomes in animal (Kipkorir, Ang'ienda, Onyango, & Onyango, 2019; Marcelino et al., 2019; Vittecoq et al., 2016) and environmental microbiota (Esiobu et al., 2002), ARGs were present in nearly all lemur and soil samples collected, including those from animals that had minimal potential exposure to introduced antibiotics (C. M. McCann et al., 2019; Pallecchi, Bartoloni,

Paradisi, & Rossolini, 2008; Van Goethem et al., 2018). As one of the largest examinations of ARGs in an endangered species and its habitat, our study adds to the growing recognition that wildlife-associated and environmental consortia may act as inextricable reservoirs of ARGs, exemplifying both the ecological and conservation concerns associated with the resistance crisis.

At the lowest end of the anthropogenic disturbance gradient, relatively undisturbed, wild lemur populations (e.g., at IVO and AMO) nonetheless harbored diverse, low-abundance ARGs that likely reflect natural resistomes. Even in these low-risk populations, the composition of lemur resistomes were site-specific, indicating that naturally occurring ARGs are neither randomly present across individuals nor are they homogenized across populations. Thus, under conditions of minimal anthropogenic impacts, selective pressures other than human influence may shape animal resistomes.

One such pressure may involve host sociality. Notably, the site-specificity in resistomes mirrors the site- or troop-specificity in the composition of primate gut microbiota (Bornbusch, Greene, Rahobilalaina, Calkins, et al., n.d.; Grieneisen et al., 2017; Moeller et al., 2016; Tung et al., 2015). A widely accepted mechanism underlying these patterns is the sharing of microbes between hosts, particularly between conspecific social partners (Archie & Tung, 2015; Moeller et al., 2016; Tung et al., 2015). Ring-tailed lemurs are highly social, spending sizeable portions of their time in physical contact with groupmates (e.g., grooming, resting, playing; (Jolly, 1998; C. P. van Schaik &

Kappeler, 1993)). If social vectors for microbial transmission have a homogenizing effect on the overall gut microbiomes of group members, the same pattern would be expected for specific microbial genes, such as ARGs. That we see site-specific patterns in lemur resistomes supports the potentially homogenizing impact of ARG social transmission.

Another pressure may involve components of host diet. As an omnivorous and sometimes geophagic host, the ring-tailed lemur ingests naturally occurring biomolecules and chemical elements, such as plant secondary compounds (e.g., tannins) and heavy metals, that can modify or co-occur with bacterial resistance mechanisms (Berendonk et al., 2015; Compean & Ynalvez, 2014; Farha et al., 2020; Hatano et al., 2005; Pal, Bengtsson-Palme, Kristiansson, & Larsson, 2015; Seiler & Berendonk, 2012). The presence and abundance of these compounds could vary with food availability and dietary preferences across the wide range of habitats occupied by wild ring-tailed lemurs, including across the populations within this study (Canington, n.d.). The social transmission of microbes and habitat-specific diets are plausible mechanisms for shaping natural resistomes in wild primate populations and warrant further research to better characterize the potential for 'unperturbed' wildlife populations to serve as reservoirs of ARGs.

Unexpectedly, most of our wild lemur populations, including those in the most undisturbed habitats according to our criteria, included specific individuals that appeared more enriched for ARGs than their site-mates. To the extent that these

individuals may represent 'sentinel' animals – a concept widely used in epidemiology to describe animals or species that manifest a specific risk and thus provide advance warning (Aguirre, 2009; Schwacke, Gulland, & White, 2013; Van der Schalie et al., 1999) – whose resistomes may reflect increased potential of ARG exposure at given sites (Blanco & Bautista, 2020; Plaza-Rodríguez et al., 2021; Sacristán et al., 2020). Sentinel individuals or species may be differentially exposed to ARGs via different behavioral patterns or biogeographical ranges (Rabinowitz et al., 2005; Vittecoq et al., 2016). Given the probability of host-host transmission of ARGs, sentinels may act as propagators of ARG within their population (Vittecoq et al., 2016). Alternatively, whereas healthy hosts may be able to combat colonization by resistant bacteria, sentinel individuals may have been those most susceptible to infection (Schwacke et al., 2013; Taur & Pamer, 2013). In this case, ARG enrichment might be confined to vulnerable individuals. Although we were unable to pinpoint the cause of this variation in our study populations, both of these scenarios are plausible and longitudinal data on ARG prevalence in wildlife population would help disentangle these two possibilities.

Although degree of anthropogenic disturbance has been suggested as a driver of ARG enrichment in other wildlife populations (Sacristán et al., 2020; Tripathi & Cytryn, 2017), most ARG studies have been limited to single or few host populations. Here, we show that, across seven populations of wild lemurs, the abundance of ARGs in host gut microbiota was correlated with our assessments of four anthropogenic components.

Compared to lemurs from other wilderness sites, lemurs from BER and BEZ (i.e., the sites with the greatest disturbance rankings) had significantly greater abundances of overall and clinically relevant ARGs. Notably, because both of these sites have well-established, long-term research stations (Jolly, 2012; Jolly, Koyama, et al., 2006; Sauther et al., 1999; Robert W Sussman et al., 2012), certain lemur groups have been habituated, captured, handled for research purposes, and, at BEZ, on rare occasions in the recent past, treated with antibiotics (personal communication with M. Sauther). Because routine captures ceased almost a decade ago, it is improbable that the lemurs sampled for this study received direct antibiotic treatment. Nonetheless, given the persistence and propagation of ARGs, earlier research practices may have left a signal in the lemur resistomes that could be perpetuated, albeit dampened, over time. Lemurs at TSI also were handled for research purposes, yet they showed no signal of ARG enrichment. Whereas researcher presence and animal handling began relatively recently at TSI (i.e., in the 10 years before sampling), researcher activity has been occurring for ~47 and ~60 years, respectively, at BEZ and BER. Perhaps our findings suggest that, when human contact is infrequent and sporadic, long-term exposure may be required for ARGs to accumulate and disseminate within wildlife populations.

Compared to the ARGs found in wild lemurs, those in captive lemurs were greatly enriched, in some cases by multiple orders of magnitude. Unsurprisingly, this trend reflected antibiotic treatment received by captive animals at the LRC and DLC.

Moreover, the types of ARGs present in these two populations reflected the differences in antibiotic availability and use between the two countries. The resistomes of lemurs from the LRC, for example, included phenicol ARGs, which most commonly confer resistance to chloramphenicol, an antibiotic that is rarely used in developed countries (Fernández et al., 2012; Wareham & Wilson, 2002), but that is available in Madagascar, specifically for treating the plague (Chanteau et al., 2000; Godfred-Cato et al., 2020; McCrumb Jr et al., 1953). In addition, LRC lemurs harbored notable proportions of ARGs conferring resistance to vancomycin, which, until recently, was considered a last-resort antibiotic for severe bacterial infections, including *Staphylococcus aureus* (i.e., MRSA; (Gardete & Tomasz, 2014; Koch et al., 2014)). A recent increase in vancomycin use has led to increased vancomycin resistance and decreased efficacy (Dhanda, Sarkar, Samaddar, & Haldar, 2018), including in isolates from humans and animals in Madagascar (Gay et al., 2017).

By contrast, for lemurs at the DLC, tetracycline, aminoglycoside, and beta-lactam ARGs were the first, second, and third most prevalent, respectively, potentially reflecting the common use of all three antibiotic families in veterinary care (Sarmah, Meyer, & Boxall, 2006; Schwarz & Chaslus-Dancla, 2001); personal communication with veterinarians at the DLC). Nonetheless, not all lemurs in these captive settings had received equivalent antibiotic treatment. Moreover, lemurs at the DLC without any history of antibiotic treatment still harbored substantial ARGs that rivaled those seen in

conspecifics with numerous previous antibiotics treatments (Bornbusch, Harris, et al., 2020). In combination, these results indicate that antibiotic treatment produced predictable patterns in ARG enrichment, but those patterns are also present in non-treated lemurs. The presence of specific types of ARG enrichment in antibiotic treatment-naïve individuals again suggests an independent mechanism of social or environmental transmission.

Another line of evidence in favor of social or environmental transmission is the finding that, despite a lack of antibiotic treatment, pet lemurs harbored some of the greatest abundances of ARGs. Similar to the resistomes of wild lemurs and lemurs at the LRC, those of pet lemurs included ARGs that reflected the use of antibiotics in Madagascar, but at significantly higher abundances than that shown by their wild conspecifics. In a recent report, researchers indicated that ARGs associated with virtually every class of antibiotics have increased in Madagascar, both in humans and in domestic animals (Gay et al., 2017). For pet lemurs, constant contact with humans and domestic animals likely serves as a vector for ARG transmission, akin to social transmission. Pet lemurs live under unsuitable conditions (LaFleur et al., 2021; Reuter, Gilles, Wills, & Sewall, 2015; Reuter & Schaefer, 2016); their poor health may present a particular vulnerability to ARG enrichment. Moreover, because the transmission of ARGs is not host-specific, ARG enrichment in pet lemurs poses a significant risk of transmission to those humans who come into contact with the lemur.

With the growing recognition that animal hosts represent only one component of microbial landscapes, researchers are increasingly probing the relationships between host-associated and environmental microbiota. In a previous study on these ring-tailed lemur populations, we showed that exposure to and acquisition of soil microbes likely contributed to interpopulation variation in gut microbiota (Bornbusch, Greene, Rahobilalaina, Calkins, et al., n.d.). Here, we expand on this finding, showing that the same pattern holds for ARGs: significant covariation between lemur gut and soil resistomes was found in two disturbed sites, ISA and BEZ. Although based on a directional, source-sink analysis, this covariation is likely to be bidirectional, such that lemurs can acquire ARGs from their environment and also shed them into the environment. Although ISA lemurs had relatively low-abundance resistomes and neither human contact nor antibiotic exposure, the significant covariation between lemur and soil ARGs indicates that the presence of non-wildlife animals and humans might contribute to ARG reservoirs in both lemurs and their environments.

Although antibiotic stewardship has rightly become a focus of western medicine, our study highlights that the resistance crisis extends outside of traditional clinical settings; antibiotic treatment should be considered as only one of many possible mechanisms that facilitate ARG enrichment. Indeed, unlike the curbing of antibiotic use, the exploitation and anthropogenic disturbance of natural ecosystems is accelerating. We show that an endangered primate harbors diverse and, in some cases,

anthropogenically enriched resistomes. As a premier One Health issue and impending threat to global health, antibiotic resistance demands greater study in a wider range of systems, specifically in the context of wildlife and environmental reservoirs of ARGs.

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Chapter 6. Conclusions

Summary, synthesis, and broader implications

By integrating results across all four data chapters, I see three main conclusions stemming from this research: (1) lemur microbiota are sensitive to environmental variation across multiple scales (e.g., across individual hosts, populations, and groups of populations), (2) this sensitivity reflects interactions between lemur-associated and external or environmental microbiota, and (3) analyses of different facets of microbiota (e.g., bacterial diversity, composition, membership, and resistomes) are necessary to generate a holistic perspective of host-microbe dynamics. I will extrapolate on these three conclusions and discuss additional, specific takeaways from certain chapters.

First, microbiota varied widely (a) within an individual ring-tailed lemur over time, (b) across individuals within a population, (c) across populations, and (d) between conglomerate 'populations' encompassed under wild or captive categorizations. A major implication of these findings is that there is not a single microbial community that can be ascribed as representative of ring-tailed lemur microbiota at any scale. This finding applies to both wild and captive lemurs; there was neither an 'optimal' microbial community that defined wild lemurs nor was there a universal 'signal of captivity' among captive lemurs. These patterns of variation are relevant both for within-species and cross-species comparative studies, particularly those that include only a single or a few populations of each host. For comparative studies across host taxa, it is often

suggested that interspecific differences will overshadow intraspecific variation, and so accounting for within-species variation is not a priority; however, the results of my dissertation demonstrate that intraspecific variation, regardless of the magnitude, is relevant to microbe-host-environment relationships.

Second, interactions between lemur-associated microbiota and external consortia (e.g., environmental microbes) contribute to the variation mentioned above. Much of the evidence for the interplay between consortia comes from my investigations of matched lemur gut and soil microbiota (in Chapters 2 and 5); lemurs harbor microbes and ARGs that are correlated with the microbiota and resistomes of relevant soil sources. This concept of external acquisition is reinforced by the finding in Chapter 4 that lemurs with no antibiotic treatment harbored substantial abundances of ARGs that were likely acquired from their environment or from their conspecifics. Interestingly, these patterns also seem to hold true for non-gut microbiota: The proportion of known environmental microbes in lemur armpit microbiota was correlated with the lemurs' access to natural forest enclosures. In combination, these results suggest that varying exposure to external microbes drives the rates of microbial transmission between sources; environmental acquisition may play a greater role in structuring or augmenting the microbiota of animals in natural, microbe-rich environments compared to those in environments with altered or minimized microbial presence. I initially approached this aspect of my research with the perspective that lemurs are recipients of microbes from external

sources. However, these relationships are likely multi-directional; hosts can share microbes among themselves and with their environments, creating networks of microbial transmission that transcend trophic scales (Christian et al., 2015; León-Zayas, McCargar, Drew, & Biddle, 2020; Trinh, Zaneveld, Safranek, & Rabinowitz, 2018). As with many aspects of my research, these results emphasize the need to consider microbial phenomena outside of "the lab." Typically, explanatory frameworks for microbiota dynamics have emerged from clinical studies with that aim to control for as many variables as possible and reduce external, microbial contamination. Although, clinical studies have immense value, this simplification of microbial environments likely limits detection of biologically relevant interactions between host-associated and external microbiota; controlled studies can be informed by or expanded through the generation of data that reflect naturally complex and 'messy' systems.

Third, analyses of different aspect of microbial communities, such as bacterial diversity, composition, membership, associations, and resistomes, may each provide different 'snapshots' of the microbial dynamics. For example, in Chapter 2, I showed that, whereas a lemur's environmental condition (e.g., wild or captive) was not correlated with microbiota alpha diversity, it was tightly correlated with microbiota composition. This pattern was further emphasized by the results of Chapter 4: following antibiotic treatment, the recovery of alpha diversity vs. microbial composition was driven by different ecological processes. Whereas alpha diversity was seen to rebound

rapidly, suggesting an important role for sheer numbers of taxa in initial recovery, the composition of microbiota showed long-term instability, indicating that microbial membership was more important in maintaining community stability over time. Moreover, biologically meaningful, yet different, patterns in microbial dynamics can emerge from different analyses of the same samples. For example, when qualitatively comparing the results of Chapters 2 and 5 (which stem from the same samples), patterns of microbial diversity and composition do not mirror patterns of ARG enrichment; metrics of microbial identity produced by 16S sequencing do not necessarily follow the same patterns as metrics of microbial function produced by shotgun sequencing. Ultimately, to determine the biological relevance of these different aspects of microbiomes, metrics of microbial identity and function should be considered holistically.

A few other takeaways have emerged from my dissertation research. Namely, a greater understanding of the concept of a "core microbiome." As traditionally defined, a core microbiome comprises microbial members that are fairly ubiquitous across the hosts of interest and have relevance to host function (Risely, 2020). Whereas I found evidence of a core microbiome in the same body site between two lemur species (e.g., in the labial microbiota of ring-tailed lemurs and Coquerel's sifakas; Chapter 3), I found little support for a core microbiome across wild and captive populations of ring-tailed lemurs (Chapter 2). These two sets of results indicate that the presence of a core

microbiota may be driven less by similarities between the hosts and more by functional constraints of the specific microbial community. For example, we hypothesized that labial microbiota of ring-tailed lemurs and sifakas play an important role in the production of "honest" olfactory signals – a necessary, conserved function that may limit the flexibility of that microbial community and select for specific microbial members (e.g., certain aerobic, fermentative bacteria). By contrast, the lack of a core gut microbiome across ring-tailed lemur populations likely reflects functional flexibility that is necessary for an omnivorous host that has a wide dietary range. So, the likelihood and significance of core microbiomes vary between hosts of different groups and also between the microbial communities in which they are defined.

Another facet of my dissertation that warrants further discussion is the presence of ARGs in wildlife and their environments and the associated implications this observation has for animal care and health. By characterizing the breadth of ARGs across different scales and using descriptive and experimental approaches, I have highlighted two key points that are important for understanding ARG dynamics. First, significant ARG enrichment does not result solely from antibiotic treatment and, thus, other routes of enrichment (e.g., social or environmental) should be equally considered across environments. Lemurs that never received antibiotics (at the DLC), and those that had almost no potential to receive antibiotics (pets in Madagascar), had marked enrichment of diverse and clinically relevant ARGs. Second, and building off the

previous point, ARGs can be acquired from and shared with external ARG reservoirs; ARGs co-occurred between lemurs and their soil environments (in Chapter 5) and were likely shared between conspecific hosts (in Chapter 4). Together, these two points demonstrate that, similar to the concept of microbial landscapes, the resistomes of wildlife and their environments likely form dynamic networks of ARG transmission between sources. They also highlight the need for increased awareness and study of ARGs in wild and captive animals; akin to efforts to promote human health, in situ conservation efforts and ex situ animal care practices would benefit from global antibiotic stewardship and greater investigation of antibiotic alternatives.

Future directions and applied value

There are multiple avenues of research that could be pursued to follow-up on this dissertation. Below, I highlight theoretical new directions and those that I hope to pursue myself. Namely, I aim to continue doing animal microbiome research with equal focus on the basic science and the applied value of microbial ecology; there have been recent and urgent calls from scientists and animal care experts alike to better integrate microbiome science into animal care and conservation. Moreover, the questions that underlie my research are not host-driven, but rather informed by broader ecological processes and potential implication, and so, could be tested in a wide range of hosts.

One theoretical future direction would be the use of traditional model systems to more directly test mechanisms of microbial dynamics. Similar to the point I make earlier about combining field and laboratory studies, my dissertation research has provided broad-scale perspectives of microbial dynamics in 'real life,' but more controlled settings are often necessary to probe mechanistic relationships. For example, my results on environmental acquisition are correlative and could be augmented by experimental studies that provide specific, yet varying, exposures to or inoculations of external microbes. Under this scenario, a combination of cross-sectional and longitudinal data could be used to test for the selective incorporation and persistence of microbes into host-associated communities.

Another area of my research that warrants further investigation is the potential interplay between different bodily microbiota. For example, in Chapter 3, the relationships between axillary, labial, and vaginal microbiota demonstrated that varied host traits and ecological processes differentially structured the communities of specific body sites. Although we did not analyze gut microbiota in this study, there is intriguing evidence of cross-talk between gut and reproductive microbiota, e.g., vaginal microbiota (Amabebe & Anumba, 2020; K. L. Chen & Madak-Erdogan, 2016; Quaranta, Sanguinetti, & Masucci, 2019). Moreover, similar to the acquisition of external microbes, it is likely that certain bodily microbes are sourced from or shared with other body sites.

Ultimately, the burgeoning study of microbial source tracking would benefit from considering both internal and external sources of microbes.

Regarding the directions I wish to pursue in my own career, I first aim to expand my research to include non-lemur hosts in a variety of environments. I have ongoing collaborations with the Smithsonian's National Zoological Park, Nutrition Laboratory, Center for Conservation Biology, and Center for Conservation Genomics to perform microbiome research with numerous endangered species, including black-footed ferrets (Mustela nigripes), cheetahs, (Acinonyx jubatus), and various primates species, including lemurs. Furthermore, I will continue to work with the colony of lemurs at the Duke Lemur Center.

One research area I hope to further explore is the relationship between host-associated and environmental microbiota in wild and captive animals. For example, in captive animals kept indoors or in artificial habitats, does a lack of exposure to environmental microbes influence microbiota structure and function (or potentially contribute to captivity-associated disorders, e.g., obesity and diabetes)? Furthermore, what non-soil sources of external microbes might contribute to host-associated communities? In carnivores, for example, are prey-associated microbes incorporated into predator gut microbiota? How might this relationship differ between captive carnivores fed processed or sanitized diets vs. wild carnivores that feed on whole carcasses? Black-footed ferrets are an ideal system in which to test these questions. Ferrets are born in and

mature in captivity settings, e.g., at a zoo, where they are kept solitarily in cleaned, indoor environments and are fed processed meat or dead whole prey. Once they are sexually mature, ferrets are transferred to semi-natural settings where they live solitarily, outdoors in conditioning pens and are fed live prey. Ultimately, ferrets are released into the wild where they live in social groups and prey almost exclusively on other small mammals. Throughout this process, I aim to collect microbial samples from ferrets and from sources of environmental and prey-associated microbes to test for relationships between microbial communities. Beyond the applied value to ferret biology and conservation, the results of this project have the potential to highlight the transmission of microbes across trophic scales, with implications for understanding carnivore biology.

Another focus of my dissertation that I am eager to further pursue is the use of microbial therapies, such as fecal microbiota transfaunations (FMT), as tools for animal care. Whether via prophylactic or therapeutic use, microbial therapies have the potential to minimize the overuse of prescription medications (e.g., antibiotics) and ameliorate the impacts of microbial dysbiosis (Guo et al., 2020; Niederwerder, 2018). I am currently setting up projects to examine the use of FMT to treat microbial declines seen in lemurs undergoing environmental shifts, cheetahs undergoing antibiotic treatment, and black-footed ferrets undergoing reintroduction to the wild. The last of these projects, which is focused on FMT in the context of reintroduction, is of particular interest. Reintroduced

animals often suffer high rates of morbidity and mortality stemming, in part, from pathogenic infections and nutritional deficiencies (Teixeira, De Azevedo, Mendl, Cipreste, & Young, 2007; Venesky, Mendelson, Sears, Stiling, & Rohr, 2012; Viggers, Lindenmayer, & Spratt, 1993); these detrimental health outcomes may be underpinned by dysbiotic microbiota. If we can use FMT to emulate the microbiota of wild animals in the captive animals slated to be released, we could prime their microbiota to withstand the environmental changes and reduce negative health consequences. Similar to the carnivore project described above, the transfer and reintroduction of black-footed ferrets provides an intriguing system to test the value for ex situ and in situ conservation efforts.

Throughout all of these future projects, I will be further expanding my skillset to include new methodological approaches in microbial science and other areas. For example, I will be learning the entire pipeline for 16S and shotgun sequencing, allowing me to perform microbial sequencing "in-house". Moreover, I hope to expand my use of shotgun sequencing to include greater investigation of microbiota function. In addition, I aim to expand my expertise in bioinformatics, namely, by working with 'source tracking' tools to determine dynamics between microbial communities, which have relevance to many of my future research interests. I also hope to gain novel skills and knowledge in animal nutrition. Although my dissertation research was largely focused on non-dietary variables, nutrition is an undeniably powerful force that structures

microbiota across host taxa. By collaborating with the Smithsonian Nutrition Lab, I will gain laboratory skills to analyze nutritional content of, e.g., food items, feces, and milk, and will also greatly benefit from increasing consideration of nutrition in my study of microbiota. Ultimately, I hope to combine analyses of host-associated and external microbiota, with data on microbial function and nutritional content to form a more holistic perspective on host-microbe symbiosis across varying environments.

Appendix

Appendix 1. Supplemental material for Chapter 2

A.1.1. Statistical results on alpha diversity in lemur gut microbiota

The full statistical results for two metrics of alpha diversity (Shannon and Faith's Phylogenetic diversity) in lemur gut microbiota are shown in Tables 5-8. For each metric, we (a) used a generalized linear mixed model (GLMM) to determine the predictive value of environment condition (wilderness; wild lemurs, captivity in Madagascar; captive$_{M}$ lemurs, and captivity in the U.S.; captive$_{U.S.}$ lemurs; Table 5, 7) and setting (See Table 1 for names and descriptions of the 13 different settings; Table 6, 8), and (b) Kruskal-Wallis rank sum tests and pairwise comparisons using Wilcoxon rank sum exact tests, with Benjamini-Hochberg adjustment for post-hoc and pairwise statistical comparisons.

Table 5. Supplementary table for Chapter 2. Shannon diversity, by condition. GLMM: Shannon diversity ~ condition + sex.

	Df	Deviance	AIC	F	p-value
<none>		29.243	192.27		
condition	2	38.099	230.86	23.773	9.57E-10
sex	1	29.304	190.61	0.33	0.566

<u>Kruskal-Wallis rank sum test</u>

Kruskal-Wallis chi-squared = 30.694, df = 2, p-value = 2.162e-07

<u>Pairwise comparisons using Wilcoxon rank sum exact test, with Benjamini-Hochberg adjustment:</u>

	captivity: Madagascar	captivity: US
captivity: US	0.00023	-
wilderness	1.30E-07	0.635

Table 6. Supplementary table for Chapter 2. Shannon diversity, by setting.
GLMM: Shannon diversity ~ condition + sex

	Df	Deviance	AIC	F	p-value
<none>		21.291	155.18		
setting	9	38.099	230.86	13.157	2.35E-15
sex	1	21.337	153.53	0.325	0.569

Kruskal-Wallis rank sum test

Kruskal-Wallis chi-squared = 89.371, df = 12, p-value = 6.535e-14

Pairwise comparisons using Wilcoxon rank sum exact test, with Benjamini-Hochberg adjustment:

	AMO	BER	BEZ	DLC	FIH	ISO	IVO	LRC	NZP	NCZ	pet	RAN
BER	0.777	-	-	-	-	-	-	-	-	-	-	-
BEZ	0.017	0.000	-	-	-	-	-	-	-	-	-	-
DLC	0.688	0.840	0.000	-	-	-	-	-	-	-	-	-
FIH	0.688	0.707	0.975	0.737	-	-	-	-	-	-	-	-
ISO	0.000	0.001	0.000	0.002	0.120	-	-	-	-	-	-	-
IVO	0.100	0.061	0.000	0.110	0.299	0.021	-	-	-	-	-	-
LRC	0.000	0.000	0.000	0.000	0.108	0.647	0.022	-	-	-	-	-
NZP	0.171	0.100	0.001	0.114	0.408	0.707	0.552	0.694	-	-	-	-
NCZ	0.863	0.778	0.463	0.647	0.843	0.071	0.197	0.061	0.361	-	-	-
pet	0.157	0.173	0.000	0.295	0.547	0.171	0.975	0.108	0.519	0.242	-	-
RAN	0.688	0.934	0.000	0.777	0.688	0.002	0.098	0.001	0.110	0.519	0.286	-
TSI	0.197	0.207	0.000	0.361	0.519	0.003	0.302	0.000	0.202	0.427	0.688	0.302

Table 7. Supplementary table for Chapter 2. Faith's phylogenetic diversity, by condition. GLMM: Faith's phylogenetic diversity ~ condition + sex

	Df	Deviance	AIC	F	p-value
<none>		1361.5	810.62		
condition	2	1438	815.43	4.415	0.013
sex	1	1361.6	808.64	0.018	0.891

Kruskal-Wallis rank sum test

Kruskal-Wallis chi-squared = 10.383, df = 2, p-value = 0.005563

Pairwise comparisons using Wilcoxon rank sum exact test, with Benjamini-Hochberg adjustment:

	captive$_{M}$ lemurs	captive$_{U.S.}$ lemurs
captive$_{U.S.}$ lemurs	0.021	-
wild lemurs	0.022	0.056

Table 8. Supplementary table for Chapter 2. Faith's phylogenetic diversity, by setting. GLMM: Shannon diversity ~ condition + sex

	Df	Deviance	AIC	F	p-value
<none>	1075	786.59			
setting	9	1438	815.43	5.628	1.08E-06
sex	1	1075.3	784.63	0.041	0.839

Kruskal-Wallis rank sum test

Kruskal-Wallis chi-squared = 64.518, df = 12, p-value = 3.347e-09

Pairwise comparisons using Wilcoxon rank sum exact test, with Benjamini-Hochberg adjustment:

	AMO	BER	BEZ	DLC	FIH	ISO	IVO	LRC	NZP	NCZ	pet	RAN
BER	0.019	-	-	-	-	-	-	-	-	-	-	-
BEZ	0.003	0.886	-	-	-	-	-	-	-	-	-	-
DLC	0.019	1.000	0.886	-	-	-	-	-	-	-	-	-
FIH	0.913	0.767	0.705	0.751	-	-	-	-	-	-	-	-
ISO	0.210	0.000	0.000	0.000	0.714	-	-	-	-	-	-	-
IVO	0.705	0.015	0.000	0.018	1.000	0.015	-	-	-	-	-	-
LRC	0.851	0.015	0.005	0.018	0.886	0.680	0.412	-	-	-	-	-
NZP	0.633	0.016	0.009	0.041	0.705	0.886	0.190	0.705	-	-	-	-
NCZ	0.061	0.310	0.251	0.251	0.600	0.012	0.018	0.106	0.120	-	-	-
pet	0.633	0.169	0.031	0.080	1.000	0.029	0.861	0.395	0.203	0.106	-	-
RAN	0.014	0.886	0.847	0.886	0.886	0.000	0.001	0.018	0.015	0.251	0.060	-
TSI	0.251	0.018	0.001	0.022	1.000	0.000	0.406	0.179	0.148	0.015	0.847	0.002

A.1.2. Statistical results on covariation between lemur gut and soil microbiota

Table 9. Supplementary table for Chapter 2. Statistical results on covariation between lemur gut and soil microbiota. Full statistical results (Wilcoxon rank sum exact tests, with Benjamini-Hochberg adjustment) for comparing mean proportion of soil-associated microbes in the gut microbiota of lemurs within and between the three conditions (wilderness, captivity in Madagascar, and captivity in the U.S.; Chapter 2; Figure 9)

	captive$_{M}$ vs. captive$_{M}$	captive$_{M}$ vs. captive$_{U.S.}$	captive$_{M}$ vs. wild	captive$_{U.S.}$ vs. captive$_{M}$	captive$_{U.S.}$ vs. captive$_{U.S.}$	captive$_{U.S.}$ vs. wild	wild vs. captive$_{M}$	wild vs. captive$_{U.S.}$
captive$_{M}$ vs. captive$_{U.S.}$	8.90E-08	-	-	-	-	-	-	-
captive$_{M}$ vs. wild	2.00E-16	6.60E-13	-	-	-	-	-	-
captive$_{U.S.}$ vs. captive$_{M}$	0.0367	0.00018	4.30E-11	-	-	-	-	-
captive$_{U.S.}$ vs. captive$_{U.S.}$	2.20E-16	9.30E-05	0.136	1.60E-12	-	-	-	-
captive$_{U.S.}$ vs. wild	2.00E-16	2.00E-16	1.30E-05	4.80E-12	0.001	-	-	-
wild vs. captive$_{M}$	0.8311	4.60E-15	2.00E-16	0.004	2.00E-16	2.00E-16	-	-
wild vs. captive$_{U.S.}$.	2.00E-16	3.30E-10	0.084	3.30E-11	0.064	0.403	2.00E-16	-
wild vs. wild	3.50E-15	3.50E-15	2.00E-16	4.60E-10	0.00051	0.00011	2.00E-16	0.007

A.1.3. Supplementary Figure: Differential abundance of soil microbes

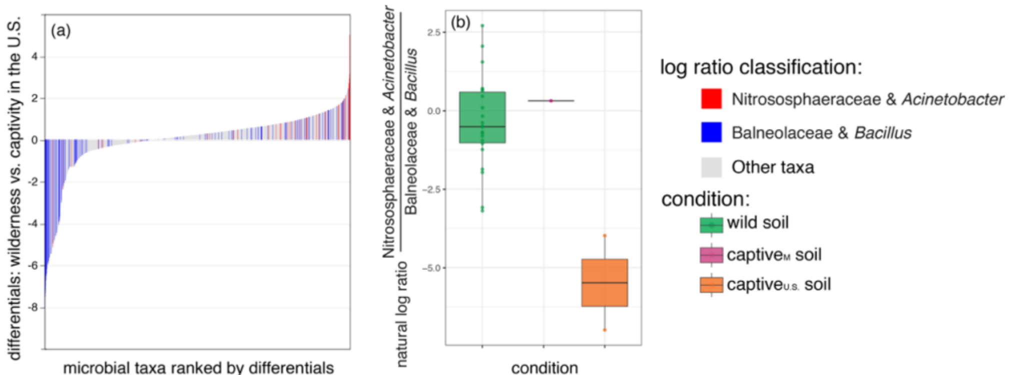

Figure 31. Supplementary figure for Chapter 2. Differential abundance analysis of soil microbiota. Differential abundance analysis of the family Nitrososphaeraceae and genus *Acinetorbacter* vs. the family Balneolaceae and genus *Bacillus* in soil microbiota (a) Differential rank plot showing all microbial taxa (x-axis) ranked by their differentials (y-axis; the estimated log-fold changes for taxa abundances across sample groups) from wilderness vs. captivity in the U.S. Those taxa that are more abundant in the wild lemurs compared to captive lemurs in the U.S. appear on the right side of the plot whereas those that are less abundant in wild lemurs appear on the left side. Taxa belonging to family Nitrososphaeraceae and genus Acinetorbacter are highlighted in red whereas those in the family Balneolaceae and genus Bacillus are in blue (other soil taxa are in gray). (b) Natural log ratios of relative abundances of the two groups of taxa across soil from the three conditions.

Appendix 2. Supplemental material for Chapter 3

A.2.1. Model results: LMM3

Table 10. Supplementary table for Chapter 3. Results for LMM3: relative abundance ~ species*body site + free-ranging + body site*P4 + body site*E2 + (1|Animal/BodySite)

o_Lactobacillales:	*T*	unadjusted *P*	
(Intercept)	3.984	0.000164	***
SpeciesP.coquereli	1.026	0.308331	
BodySiteLabial	-4.387	3.97E-05	***
BodySiteVaginal	-3.994	0.000158	***
FreeRangingYes	-1.037	0.303365	
E2	-2.34	0.022166	*
P4	0.776	0.440626	
SpeciesP.coquereli:BodySiteLabial	2.957	0.004234	**
SpeciesP.coquereli:BodySiteVaginal	0.475	0.636575	
BodySiteLabial	4.924	5.45E-06	***
BodySiteVaginal:E2	4.4	3.79E-05	***
BodySiteLabial	-1.203	0.232876	
BodySiteVaginal:P4	-1.128	0.263072	
f_Streptococcaceae.g_*Streptococcus*	*T*	unadjusted *P*	
(Intercept)	2.546	1.31E-02	*
SpeciesP.coquereli	1.026	0.30833	
BodySiteLabial	-2.612	0.01102	*
BodySiteVaginal	-2.372	0.02045	*
FreeRangingYes	-1.037	0.30336	
E2	-2.34	0.02217	*
P4	0.776	0.44063	
SpeciesP.coquereli:BodySiteLabial	2.957	0.00423	**
SpeciesP.coquereli:BodySiteVaginal	0.475	0.63657	
BodySiteLabial	4.924	5.45E-06	***
BodySiteVaginal:E2	4.4	3.79E-05	***
BodySiteLabial	-1.203	0.23288	
BodySiteVaginal:P4	-1.128	0.26307	
f_Bacteroidaceae.g_*Bacteroides*	*T*	unadjusted *P*	
(Intercept)	0.2300	0.8192	
SpeciesP.coquereli	-1.1340	0.2734	
BodySiteLabial	0.1200	0.9048	

BodySiteVaginal	0.8660	0.3898	
FreeRangingYes	1.7160	0.1033	
E2	0.5720	0.5706	
P4	-0.1870	0.8523	
SpeciesP.coquereli:BodySiteLabial	2.6000	0.0196	*
SpeciesP.coquereli:BodySiteVaginal	3.3510	0.0043	**
BodySiteLabial	-1.0820	0.2865	
BodySiteVaginal:E2	-1.6360	0.1107	
BodySiteLabial	-0.3250	0.7462	
BodySiteVaginal:P4	-0.3640	0.7172	

f_Prevotellaceae.g_*Prevotella*	T	unadjusted *P*	
(Intercept)	3.752	0.000361	***
SpeciesP.coquereli	-6.006	3.26E-05	***
BodySiteLabial	-2.058	0.043397	*
BodySiteVaginal	-2.209	0.030535	*
FreeRangingYes	-2.321	0.035767	*
E2	-0.978	0.339631	
P4	-1.245	0.217756	
SpeciesP.coquereli:BodySiteLabial	4.133	0.001039	**
SpeciesP.coquereli:BodySiteVaginal	4.76E+00	0.000353	***
BodySiteLabial	1.81E-01	0.857951	
BodySiteVaginal:E2	0.556	0.585428	
BodySiteLabial	0.71	0.48049	
BodySiteVaginal:P4	0.308	0.758938	

f_Family.XI	T	unadjusted *P*	
(Intercept)	-0.158	8.75E-01	
SpeciesP.coquereli	0.004	0.9965	
BodySiteLabial	1.00E+00	0.32	
BodySiteVaginal	1.163	0.2489	
FreeRangingYes	1.167	0.2609	
E2	0.107	0.9153	
P4	0.322	0.7484	
SpeciesP.coquereli:BodySiteLabial	0.635	0.5361	
SpeciesP.coquereli:BodySiteVaginal	-0.267	0.7934	
BodySiteLabial	0.005	0.9959	
BodySiteVaginal:E2	-4.65E-01	0.6449	
BodySiteLabial	2.025	0.0475	*
BodySiteVaginal:P4	0.708	0.4816	

A.2.2. Model results: LMM4

Table 11. Supplementary table for Chapter 3. Results for LMM4: relative abundance ~ free-ranging + P4 + E2 + (1|Animal)

sifaka axillary taxa	*T*	unadjusted *P*
f_Pasteurellaceae.g_*Actinobacillus*		
(Intercept)	1.667	0.121
Free-ranging_yes	-0.424	0.679
E2	-0.647	0.53
P4	1.041	0.318
f_Pasteurellaceae; g_unidentified		
(Intercept)	0.252	0.806
Free-ranging_yes	1.242	0.267
E2	0.471	0.646
P4	-0.424	0.68
Unassigned		
(Intercept)	1.212	0.249
Free-ranging_yes	-0.168	0.8691
E2	2.464	0.0298*
P4	-2.561	0.025*
f_Beijerinckiaceae.g_*1174.901.12*		
(Intercept)	1.407	0.185
Free-ranging_yes	-0.271	0.791
E2	1.216	0.247
P4	-1.251	0.235
f_Streptococcaceae.g_*Streptococcus*		
(Intercept)	0.381	0.711
Free-ranging_yes	1.181	0.289
E2	0.01	0.992
P4	0.171	0.868
Other		
(Intercept)	4.388	0.00614**
Free-ranging_yes	-1.142	0.32703
E2	-0.812	0.44383
P4	-1.151	0.27744
sifaka labial taxa	*T*	unadjusted *P*
f_Fusobacteriaceae.g_*Fusobacterium*		
(Intercept)	2.84	0.0206*
Free-ranging_yes	-1.614	0.1704
E2	-1.743	0.1101
P4	0.522	0.6144

f_Bacteroidaceae.g_*Bacteroides*		
(Intercept)	1.277	0.245
Free-ranging_yes	0.878	0.424
E2	-0.406	0.695
P4	-0.547	0.597
f_Porphyromonadaceae.g_*Porphyromonas*		
(Intercept)	1.623	0.156
Free-ranging_yes	-0.359	0.744
E2	-1.481	0.17
P4	1.366	0.205
f_Aerococcaceae.g_*Facklamia*		
(Intercept)	-0.488	0.638
Free-ranging_yes	0.662	0.543
E2	1.418	0.184
P4	-0.357	0.73
f_Family.XI.g_*Anaerococcus*		
(Intercept)	-0.283	0.784
Free-ranging_yes	1.195	0.292
E2	1.005	0.337
P4	0.502	0.627
Other		
(Intercept)	1.587	0.141
Free-ranging_yes	-0.549	0.594
E2	0.549	0.594
P4	-1.41	0.186

sifaka vaginal taxa	*T*	unadjusted *P*
f_Bacteroidaceae.g_*Bacteroides*		
(Intercept)	0.95	0.369
Free-ranging_yes	1.09	0.341
E2	0.106	0.917
P4	-0.477	0.643
f_Fusobacteriaceae.g_*Fusobacterium*		
(Intercept)	2.512	0.0322*
Free-ranging_yes	-1.359	0.2313
E2	-1.062	0.3095
P4	0.064	0.9504
f_Porphyromonadaceae.g_*Porphyromonas*		
(Intercept)	1.253	0.2482
Free-ranging_yes	-0.296	0.7844

E2	-1.208	0.2521
P4	2.437	0.0348*
f_Streptococcaceae.g_*Streptococcus*		
(Intercept)	1.197	0.254
Free-ranging_yes	-1.277	0.226
E2	0.628	0.542
P4	-1.402	0.186
f_Campylobacteraceae.g_*Campylobacter*		
(Intercept)	0.966	0.36
Free-ranging_yes	-0.048	0.964
E2	0.637	0.537
P4	0.687	0.508
Other		
(Intercept)	1.414	0.202
Free-ranging_yes	-0.384	0.724
E2	0.545	0.598
P4	-1.288	0.229

ring-tailed lemur axillary taxa	*T*	unadjusted *P*
f_Pasteurellaceae.g_*Actinobacillus*		
(Intercept)	1.5310	0.1642
Free-ranging_yes	-1.4240	0.1924
E2	-0.5050	0.6273
P4	3.1040	0.0146*
f_Neisseriaceae.g_*Alysiella*		
(Intercept)	1.4290	0.1910
Free-ranging_yes	-1.0920	0.3070
E2	-0.2780	0.7880
P4	1.0370	0.3300
f_Bacteroidaceae.g_*Bacteroides*		
(Intercept)	1.0210	0.3370
Free-ranging_yes	0.6700	0.5210
E2	0.6720	0.5210
P4	-1.5580	0.1580
f_Lachnospiraceae.g_unidentified		
(Intercept)	1.1120	0.2980
Free-ranging_yes	-0.5200	0.6170
E2	0.4960	0.6330
P4	-1.0880	0.3080
f_Streptococcaceae.g_*Streptococcus*		
(Intercept)	3.1080	0.0146*
Free-ranging_yes	-0.7720	0.5921
E2	-2.2290	0.0588.
P4	1.9320	0.0905.

Other		
(Intercept)	6.4150	0.0002***
Free-ranging_yes	3.6260	0.0067**
E2	-1.5480	0.1601
P4	-0.2770	0.7891

ring-tailed lemur labial taxa	*T*	unadjusted *P*
f_Bacteroidaceae.g_*Bacteroides*		
(Intercept)	2.747	0.0252*
Free-ranging_yes	1.711	0.3605
E2	-2.266	0.0551.
P4	-0.624	0.55
f_Fusobacteriaceae.g_*Fusobacterium*		
(Intercept)	3.663	0.00637**
Free-ranging_yes	0.179	0.8621
E2	-1.969	0.08447.
P4	-1.651	0.13728
f_Porphyromonadaceae.g_*Porphyromonas*		
(Intercept)	2.513	0.0391*
Free-ranging_yes	-1.254	0.437
E2	0.779	0.4607
P4	-2.215	0.0599.
f_Christensenellaceae.g_*Christensenellaceae*		
(Intercept)	0.574	0.582
Free-ranging_yes	-0.082	0.937
E2	1.05	0.324
P4	-1.226	0.255
f_Spirochaetaceae.g_*Treponema.2*		
(Intercept)	-1.071	0.315212
Free-ranging_yes	0.4	0.699631
E2	1.353	0.213073
P4	6.013	0.000319***
Other		
(Intercept)	0.92	0.384
Free-ranging_yes	-1.5	0.172
E2	3.004	0.017*
P4	-0.958	0.366

Appendix 3. Supplemental material for Chapter 4

A.3.1 Descriptions of statistical models

Hierarchical Generalized Additive Models (HGAMs): We used HGAMs to test for variation in the trajectories of alpha and beta diversity, over time, between the three experimental groups of lemurs: CON, ABX, and ABXFT. All models are structured following Pedersen et al., 2019 and we report model syntax for use in the R (ver. 4.0.2) via the gam() function in package {mgcv}. The models were run on data spanning the entire experiment and on subsets of data spanning the treatment period and/or recovery period. The model for testing variation in alpha and beta diversity was as follows:

Full Model: Diversity_metric ~ Experimental_group * Year + s(Day, by = Experimental_group) + s(Animal, by = Year, bs = "re") + s(Experimental_group, bs = "re"), method = "REML"

Model term explanations:

Experimental_group * Year: Testing for fixed effects of experimental group, year, and the interaction between the two.

s(Day, by = Experimental_group): provides a group-specific smoothing spline for each experimental group that accounts for differences in response trajectory over time (Day).

s(Experimental_group, bs = "re"): because group-specific intercepts are not incorporated into factor-by-variable smoothers (e.g., the term described above), this term represents random effects for the intercepts (bs="re" term) of the different experimental groups.

s(Animal, by = Year, bs = "re"): similar to the above term, this term provides random effects for the intercepts of each animal with a smoothing spline that is specific to the year (to account for different patterns of the same animal across the two different years).

Bayesian multivariate Gaussian process regression: We used this statistical method to test for covariation between the log-ratios of microbial taxa. Log ratios reflect the abundance of specific taxa relative to the mean abundance of all other taxa, thus negating concerns on the compositional nature of microbiome data. Counts in each sample were resampled in a procedure similar to that performed by Fernandes et al. (2014). This procedure was repeated 500 times, yielding 500 resampled instances of the original data set with bacterial sequence variants rendered as log-ratios.

Per-cohort observation matrices were constructed by aggregating samples within a cohort (i.e. CON, ABX, ABXFT) to give one such observation matrix Y per cohort per resampling instance. We independently fit each of these resampled instances using the following Bayesian multivariate Gaussian process regression model:

$$\mathrm{Y} \sim \mathrm{N}(\Lambda[\mathrm{X}], \Sigma, I) \tag{1}$$

$$\Lambda[X] \sim N(\Theta[X], \Sigma, \Gamma[X]) \tag{2}$$

$$\Sigma \sim IW(\Xi, \upsilon) \tag{3}$$

Here X is vector of sample times indices (days).

Line (1) describes the deviation in observed values from their moving averages (latent parameter Λ). Parameter Σ describes the covariation between log-ratio sequence variants.

Line (2) describes the deviation in the moving averages from their baseline values (specified by Θ). Again, Σ mediates covariation between log-ratio sequence variants. Parameter Γ is a kernel matrix that encodes (autoregressive) covariance between samples.

Line (3) specifies a prior belief about the scale and structure of the log-ratio covariance matrix Σ. Parameters for this prior were chosen to be effectively non-informative.

This model fits a smooth trajectory, varying around Θ, to each log-ratio, enforcing the between-sample correlation specified by Γ. Inference on the remaining parameters, Λ and Σ, can be performed by treating this model as an instance of Bayesian multivariate linear regression, which gives closed-form estimates for the posterior (data-informed) distributions of Λ and Σ.

For each resampled instance of the data set, we calculated the *maximum a posteriori* estimate for Σ, yielding a single, most probable estimate for the covariance across log-ratio sequence variants given a single resampled instance of the data set.

A.3.2 Supplementary figures of beta diversity (Figures 32, 33, and 34)

Figures 32, 33, and 34 show beta diversity (i.e., community composition) for all subjects (CON, ABX, and ABFT). In the main text we present the model-predicted values of unweighted UniFrac distances (Figure 3). In Figure 32, we present the raw beta diversity data for

unweighted UniFrac. These data reflect the same patterns seen in Figure 3 and demonstrate the minimal variation seen in CON animals.

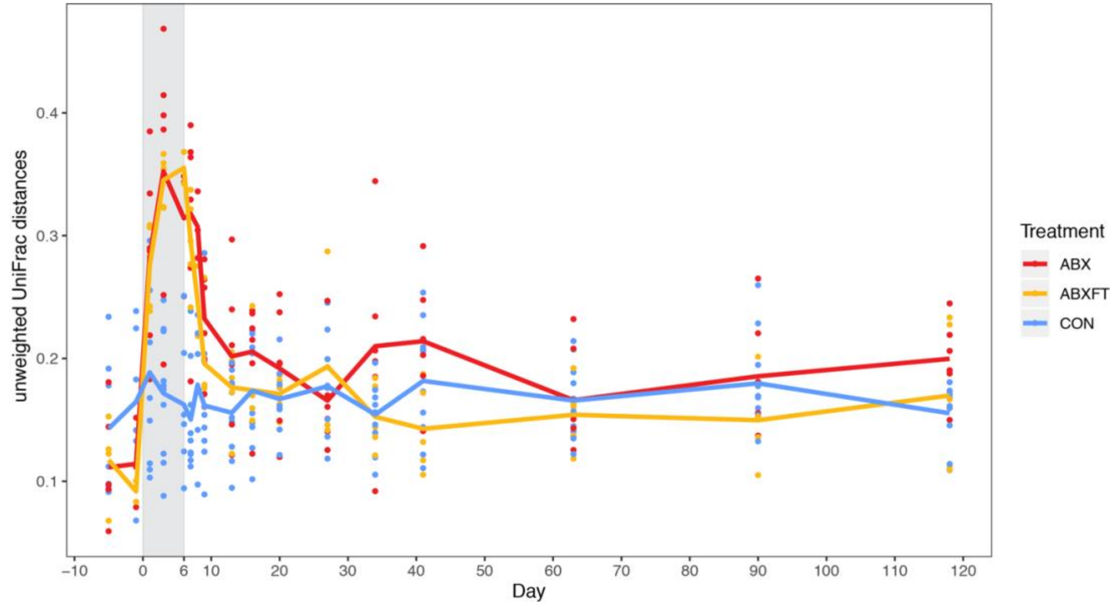

Figure 32. Supplementary figure for Chapter 4. Beta diversity over time in lemurs in three experimental groups. Change in bacterial composition (Unweighted UniFrac beta diversity) over time relative to a pretreatment, baseline sample collected 4 days before the onset of treatment for all animals and used for FT. Shown are the values for healthy, male ring-tailed lemurs (*Lemur catta*) that received no treatment (CON), antibiotics only (ABX), or antibiotics plus fecal transfaunation (ABXFT). Dots represent individual data points and lines connect the mean values of alpha diversity across individuals at each time point. The shaded window represents the period of antibiotic treatment (days 0-6), with fecal transfaunation administered on day 7; all values prior to the onset of treatment represent baseline values and all values post-treatment represent the period of recovery.

In figures 33 and 34, we present principal coordinate plots of unweighted and weighted UniFrac diversity, respectively. In each figure, the top plot (a) shows all data points colored by experimental group. The three plots below (b, c, d) are the data from a representative individual in each of the three experimental groups. These figures show that, for both metrics of beta diversity, the microbiota of CON animals vary little over time, whereas the animals in both

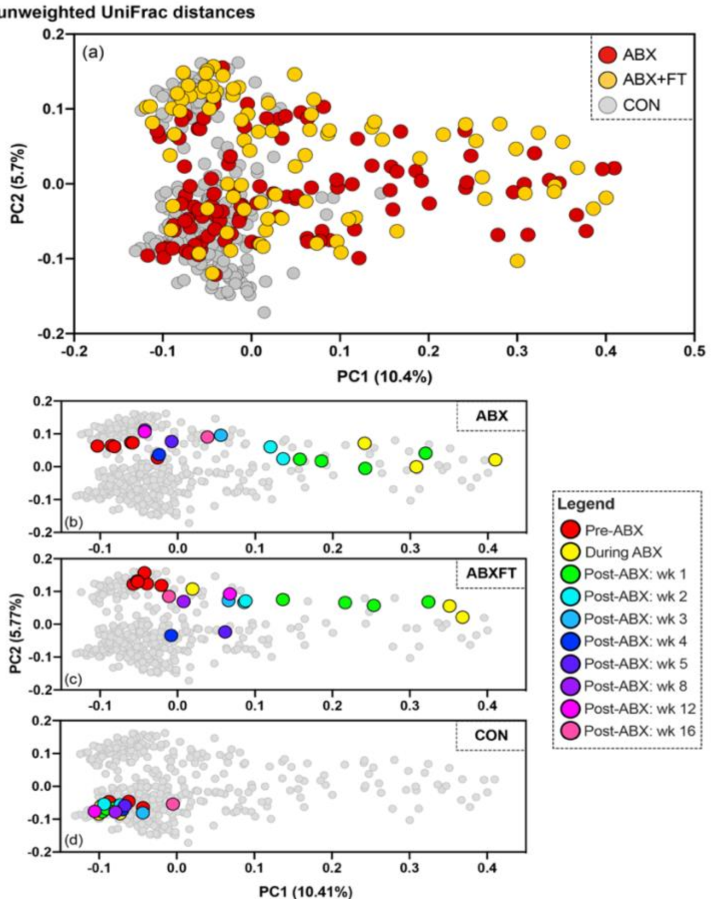

Figure 33. Supplementary figure for Chapter 4. Principal coordinate analyses of unweighted UniFrac beta diversity including (a) all samples colored by the three experimental groups: healthy, male ring-tailed lemurs (*Lemur catta*) that received no treatment (CON), antibiotics only (ABX), or antibiotics plus fecal transfaunation (ABXFT). (b, c, d) beta diversity of a representative animal from each of the three experiment groups with data points colored according time of collection (pre-, during, and post-antibiotic treatment; gray points are data from all other animals).

treatment groups experience a large shift in gut microbiota composition (to the right of the plot) associated with antibiotic treatment, followed by recovery of community composition akin to their pre-treatment microbiota (the left of the plot).

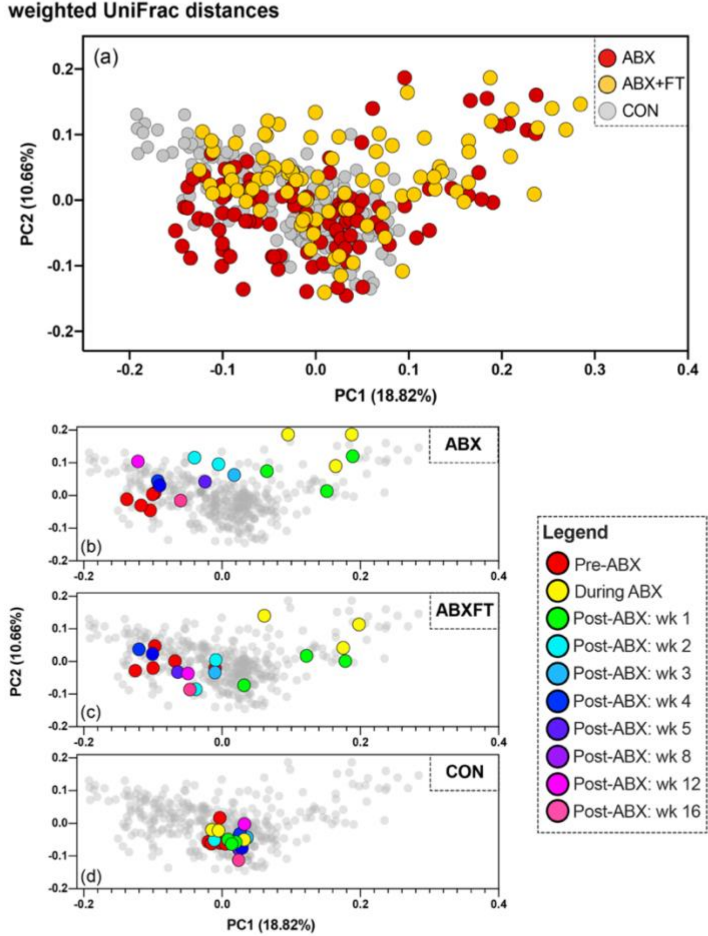

Figure 34. Supplementary figure for Chapter 4. Principal coordinate analyses of weighted UniFrac beta diversity including (a) all samples colored by the three experimental groups: healthy, male ring-tailed lemurs (*Lemur catta*) that received no treatment (CON), antibiotics only (ABX), or antibiotics plus fecal transfaunation (ABXFT). (b, c, d) beta diversity of a representative animal from each of the three experiment groups with data points colored according to time of collection (pre-, during, and post-antibiotic treatment; gray points are data from all other animals)

Appendix 4. Supplemental material for Chapter 5

A.4.1. Statistical results on the relative abundance of lemur ARGs between sites

Table 12. Supplementary table for Chapter 5. Here we report the full statistical results for comparisons of the relative abundance of lemur ARGs between sites. We performed pairwise comparisons using Wilcoxon rank sum exact tests, with Benjamini-Hochberg adjustment for pairwise statistical comparisons. P-values reported at 0.0000 are less than 0.0001.

	IVO	AMO	RAN	ISA	TSI	BEZ	BER	LRC	pet
AMO	0.2347	-	-	-	-	-	-	-	-
RAN	0.9078	0.1761	-	-	-	-	-	-	-
ISA	0.2182	0.8234	0.1761	-	-	-	-	-	-
TSI	0.3527	0.2720	0.3870	0.3344	-	-	-	-	-
BEZ	0.0236	0.0207	0.0067	0.1640	0.0059	-	-	-	-
BER	0.1129	0.3431	0.0560	0.6768	0.1129	0.3871	-	-	-
LRC	0.0005	0.0001	0.0005	0.0004	0.0001	0.0001	0.0001	-	-
pet	0.0016	0.0003	0.0016	0.0014	0.0003	0.0003	0.0003	0.1129	-
DLC	0.0003	0.0000	0.0003	0.0003	0.0000	0.0000	0.0000	0.0067	0.5741

A.4.2. Statistical results on comparisons of ARG composition within and between sites

Table 13. Supplementary table for Chapter 5. Full statistical results for comparisons of ARG Bray-Curtis distance within and between sites. We performed pairwise comparisons using Wilcoxon rank sum exact tests, with Benjamini-Hochberg adjustment for pairwise statistical comparisons. P-values reported at 0.0000 are less that 0.0001

	IVO (w/i)	IVO (btw)	AMO (w/i)	AMO (btw)	RAN (w/i)	RAN (btw)	ISA (w/i)	ISA (btw)	TSI (w/i)	TSI (btw)	BEZ (w/i)	BEZ (btw)	BER (w/i)	BER (btw)	LRC (w/i)	LRC (btw)	pet (w/i)	pet (btw)	DLC (w/i)
IVO (btw)	0.0196	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AMC (w/i)	0.0009	0.0000	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AMC (btw)	0.5284	0.0000	0.0000	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
RAN (w/i)	0.2107	0.9504	0.0009	0.0637	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
RAN (btw)	0.0852	0.0547	0.0000	0.0000	0.7435	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ISA (w/i)	0.0201	0.0000	0.7944	0.0001	0.0036	0.0000	-	-	-	-	-	-	-	-	-	-	-	-	-
ISA (btw)	0.8300	0.0000	0.0000	0.0606	0.0644	0.0000	0.0000	-	-	-	-	-	-	-	-	-	-	-	-
TSI (w/i)	0.7739	0.0308	0.0000	0.1555	0.5847	0.1842	0.0004	0.3138	-	-	-	-	-	-	-	-	-	-	-
TSI (btw)	0.0041	0.1021	0.0000	0.0000	0.7435	0.0001	0.0000	0.0000	0.0067	-	-	-	-	-	-	-	-	-	-
BEZ (w/i)	0.0000	0.0000	0.1217	0.0000	0.0000	0.0000	0.1555	0.0000	0.0000	0.0000	-	-	-	-	-	-	-	-	-
BEZ (btw)	0.0343	0.0000	0.0000	0.0000	0.0056	0.0000	0.0064	0.0000	0.0019	0.0000	0.0000	-	-	-	-	-	-	-	-
BER (w/i)	0.1960	0.0000	0.0038	0.1845	0.0635	0.0000	0.0401	0.0554	0.0620	0.0000	0.0000	0.8313	-	-	-	-	-	-	-
BER (btw)	0.4552	0.0000	0.0000	0.0000	0.2951	0.0000	0.0000	0.0000	0.7338	0.0000	0.0000	0.0000	0.0009	-	-	-	-	-	-
LRC (w/i)	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0001	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	-	-	-	-	-
LRC (btw)	0.0473	0.0000	0.0000	0.0000	0.0025	0.0000	0.0051	0.0000	0.0015	0.0000	0.0000	0.3029	0.9468	0.0000	0.0000	-	-	-	-
pet (w/i)	0.0000	0.0000	0.0004	0.0000	0.0000	0.0000	0.0059	0.0000	0.0000	0.0000	0.0011	0.0000	0.0000	0.0000	0.2951	0.0000	-	-	-
pet (btw)	0.0216	0.0000	0.0008	0.0000	0.0008	0.0000	0.0339	0.0000	0.0001	0.0000	0.0000	0.0708	0.3696	0.0000	0.0000	0.0072	0.0000	-	-
DLC (w/i)	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.8400	0.0000	0.2550	0.0000	-
DLC (btw)	0.6938	0.0000	0.0000	0.7101	0.0201	0.0000	0.0001	0.2223	0.0801	0.0000	0.0000	0.0000	0.1551	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000

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Biography

Sarah (Sally) Lyons Bornbusch attended the College of William and Mary (Williamsburg, VA) from 2009-2013 and received a Bachelor of Science degree with Honors in Biology. For her undergraduate thesis research, she worked with Dr. Emmett Duffy at the Virginia Institute of Marine Science (VIMS) to study eusociality in marine shrimp (genus *Synalpheus*). For this research, she was awarded an Undergraduate Honors Research Fellowship and an International Scholarship. Following graduation, Sally continued to work at VIMS as a Research Technician on several marine ecology projects. In late 2013, she began a Volunteer Research Assistant position at the Kalahari Mole-rat Project in South Africa. Throughout her 10 months at the mole-rat project, she assisted in studies of Damaraland mole-rat physiology, behavior, and eusociality. It was during this work in the Kalahari that Sally met her future PhD advisor, Christine Drea.

In 2015, Sally joined the Drea lab at as a PhD student in the Evolutionary Anthropology Department at Duke University. As a PhD student, Sally received the following fellowships and awards: An NSF Graduate Research Fellowship with a supplemental INTERN award, an NSF-funded Training Fellowship in the Integrative Bioinformatics for Investigating and Engineering Microbiomes program, a Luce Anthropocene Graduate Fellowship, two Duke Summer Research Fellowships and a Susan Crissey Memorial Scholarship for Managed Animal Nutrition Research from the Comparative Nutrition Society. To support her research, Sally was awarded multiple

research grants, namely, an NSF Doctoral Dissertation Research Improvement Grant, a Triangle Center for Evolutionary Medicine Graduate Student Research Award, and multiple, Duke Lemur Center Director's Fund grants. Following graduate school, Sally has been awarded a George E. Burch Postdoctoral Fellowship for 2 years of research with the Smithsonian Institution's Conservation Biology Institute and Center for Conservation Genomics.

Sally had published one of her dissertation chapter with two other chapters under revision and review. A full list of Sally's publications is as follows:

Bornbusch S.L.*, Grebe N.M.*, Lunn S., Southworth C., Dimac-Stohl K., Drea C.M. 2020. Stable and transient structural variation in lemur vaginal, labial, and axillary microbiomes: patterns by species, body site, ovarian hormones, and forest access. *FEMS Microbiology Ecology, 96*(6).

Fomsgaard A., Rosenstierne M.W., Rasmussen M., Wright P., Bueno G.L., Noromalala E., **Bornbusch S.L.**, Stensvold C.R., Poulsen M., Hvilsom C. 2020. Prevalence, infection intensity, and genotyping of *Giardia duodenalis* in ring-tailed lemurs (*Lemur catta*) from European zoos and wild populations. *Journal of Zoo and Aquarium Research*.

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Bornbusch S.L.., Greene L.K., McKenney E., Volkoff S.J., Midani F.S., Joseph G. Gerhard W.A., Iloghalu U., Granek J., Gunsch C.K. 2019. A comparative study of gut microbiomes in captive nocturnal strepsirrhines. *American Journal of Primatology*. e22986.

Bornbusch S.L., Lefcheck J. S., Duffy J.E. 2018. Allometry of individual reproduction and defense in eusocial colonies: A comparative approach to trade-offs in social sponge-dwelling shrimps. *PLoS One*.

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Bornbusch S.L., Greene L.K., Clarke T.A., Rothman R., Calkins S., Drea C.M. *Under review.* Gut microbiota of ring-tailed lemurs (*Lemur catta*) vary across natural and captive populations and correlate with environmental microbiota. *Animal Microbiome*.

Waters, S., K. Valenta, **S.L. Bornbusch**, Z. Randriana, Z. Farris, and T. Clarke. "Dog-primate interactions: Implications for coexistence." In *Primates in Anthropogenic Landscapes: Exploring Primate Behavioral Ecology Across Human Contexts*, T. McKinney, S. Waters, and M. Rodrigues, eds. Springer. *Invited, Submitted*

Valenta K., **Bornbusch S.L.**, Jacques Y.D., Nevo O. *In revision.* In the eye of the beholder: Variance in subjective color classification, and its implications for ecological research. *Scientific Reports*

Bornbusch S.L., Drea C.M. *In preparation.* Antibiotic resistance genes in lemur gut and soil microbiota along a gradient of anthropogenic disturbance. *abstract accepted to special issue of Frontiers in Ecology and Evolution*

Bornbusch S.L., Harris R., Dimac-Stohl K., Drea C.M. *In preparation.* Diversity of fungi in the gut mycobiomes of ring-tailed lemurs (*Lemur catta*).

Comizzoli P., Power M., **Bornbusch S.L.**, Muletz-Wolz C. *In preparation.* Relationships between microbiomes and reproductive health and success in rare and endangered animal species.

Grebe N.*, **Bornbusch S.L.***, Boulet M., Greenwald L., Dimac-Stohl K., Drea C.M. *In preparation.* Covariation between chemical signals and glandular microbiota in two lemur species: effects of natural and synthetic hormones.

Harris R. **Bornbusch S.L.**, Dimac-Stohl K., Drea C.M. *In preparation*. Antibiotic treatment influences scrotal gland chemical signals and microbiota in ring-tailed lemur (*Lemur catta*).

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