MICROBIOMES AND ZOONOSES: DYNAMICS OF THE BLACK FLYING FOX (*PTEROPUS ALECTO*) GASTROINTESTINAL MICROBIOME

by

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DEDICATION

This dissertation is dedicated to my parents, who have loved and supported me unconditionally… Even after being in graduate school for nearly a decade.

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"It takes a village to raise a child." It also takes a team of highly qualified researchers and many friends and family members to help this Ph.D. student get through. I am incredibly fortunate to have had all three throughout my Ph.D.

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ABSTRACT

Land-use change is increasingly recognized as a driver of spillover of zoonotic pathogens. Australian black flying foxes (*Pteropus alecto*) are experiencing extensive loss of habitat which reduces available food, particularly in winter. Hendra virus (HeV, family: Paramyxoviridae) was isolated from horses and humans in 1994 and *P. alecto* was later identified as the reservoir host. As habitat loss threatens these bat populations, and Hendra virus continues to spill over to horses annually, it is important to understand factors that influence bat health and viral shedding. Because gastrointestinal tract (GIT) microbiomes are important for host health and are understudied in flying foxes, the goal of this research was to understand the natural dynamics of the *P. alecto* GIT microbiome and its associations with diet, body composition, markers of inflammation, and viral shedding. We sampled *Pteropus alecto* near Brisbane from 2018-2020. We captured bats returning from foraging and collected rectal swabs to determine the GIT microbiome using 16S rRNA amplicon sequencing. In addition to feces for dietary analysis, we also collected samples to measure health and infection, including blood to measure neutrophil-to-lymphocyte ratios, urine to detect Hendra virus, and bioelectrical impedance analysis to measure body fat. These data enabled us to determine how the *P. alecto* GIT microbiome varied within individuals over time and in the context of physiological, ecological, and dietary shifts. Lastly, we asked if we could predict health outcomes using the GIT microbiome. We found that *P. alecto* GIT microbiomes are highly dynamic over time, through different life stages, between foraging strategies, and that the type of diet is associated with GIT microbiome diversity. Bats consuming native foods had lower GIT microbiome diversity compared to those consuming introduced and cultivated foods. Despite associations between body fat and HeV infection, the GIT microbiome was not able to predict these health outcomes. These results suggest that *P. alecto* GIT microbiomes are highly dynamic and may not contribute significantly to host health. Future research should incorporate more health metrics or other approaches to microbiome profiling to determine if the GIT microbiome could be used as a biomarker of health.

CHAPTER ONE

INTRODUCTION

Microbes inhabiting the gastrointestinal tract (GIT) can have substantial impacts on their hosts' overall health. GIT microbiomes contribute to host physiology, behavior, immune function, and nutrient and energy acquisition (as reviewed in Flint et al., 2012; Martin et al., 2019; Morais et al., 2021; Sommer and Bäckhed, 2013; Zheng et al., 2020 and others). Rapid declines and extinctions of wildlife in the Anthropocene have prompted research to understand the factors that influence wildlife health, including the importance of their microbiomes, especially as land-use change and decreases in biodiversity are associated with increased risk of zoonotic spillover (McFarlane et al., 2013). One group of wildlife in particular, bats, host a number of virulent zoonotic viruses such as SARS, MERS, Marburg virus, and Ebola virus (Letko et al., 2020). However, bats are also essential to their biomes and provide substantial ecosystem services such as pollination, seed dispersal, and insect population control (Kunz et al., 2011). Increased understanding of the importance of GIT microbiomes in combination with the ecological significance of bats and their unique roles as viral reservoirs, has meant that bat GIT microbiome research has gained momentum over the past decade (Jones et al., 2022).

In 1994, a novel paramyxovirus, Hendra virus (HeV; named for the suburb of Brisbane where it was first discovered), was detected as a viral pathogen that resulted in the fatalities of one human and ~20 horses (Plowright et al., 2015). Since 1994, spillovers of HeV into horses have occurred relatively consistently in austral winter months with occasional subsequent transmission to humans. Since the initial spillover, black flying foxes (*Pteropus alecto*) have been identified as reservoirs of Hendra virus (Halpin et al., 2000).

Australian *Pteropus* species feed on native blossom and native and introduced fruits, but they prefer Myrtaceae and Banksia pollen, and then over-ripe commercial crops like stone fruits (Parry-Jones and Augee, 1991). Many native flowering trees from Myrtaceae and Banksia have irregular and unpredictable flowering cycles, and there is a consistent pattern of low resource availability in the Austral winter (Eby and Law, 2008; Páez et al., 2018; Parry-Jones and Augee, 1991). Moreover, Australian flying foxes are undergoing extensive habitat loss due to urbanization and agricultural intensification, leading to shifts in flying fox foraging ecology (Eby et al., in review). Where previously these bats exhibited nomadic behavior during pulses of native flower blossoming, more flying foxes are establishing resident camps in more urban and agricultural areas (Eby et al., in review; Connell et al., 2006; Parry-Jones and Augee, 2001; Puddicombe 1981). These urban and agricultural areas contain cultivated and introduced plants that provide food year-round, including when primary resources are not available (Banack, 1998). However, these cultivated and introduced fruits do not meet the nutritional needs (i.e., deficient in essential mineral concentrations) of flying foxes (Nelson et al., 2000; Pulscher et al., 2021). Pulses of Hendra virus occur most frequently in winter associated with winter bottlenecks in resource availability and when animals are feeding on suboptimal foods in anthropogenic landscapes (Eby et al., in review; Field et al., 2001; Páez et al., 2018; Plowright et al., 2015).

This highly dynamic and disturbed habitat makes an interesting system in which to study bat GIT microbiomes and their associations with physiological, ecological and health markers. To date there are some microbiome studies on *Pteropus* spp. (Henry et al., 2018; Mohd-Yusof et al., 2022), but none that assess the microbiome of *P. alecto* (black flying foxes), which are reservoirs of Hendra virus (Halpin et al., 2000). There are no studies that address changes in bat

foraging behavior with regard to nomadic versus resident behavior, and there is only one study that addresses the effect of introduced diets on bat GIT microbiomes (Ingala et al., 2019).

As of September 15, 2021, there were 65 bat microbiome studies, most of which occurred after 2010 (Figure 1). Bat microbiome studies are primarily cross-sectional; few studies had an experimental or longitudinal design (Jones et al., 2022). For my dissertation, I used longitudinal studies of flying fox populations and recaptured individuals to understand the temporal dynamics of the flying fox GIT microbiome. To our knowledge, this research includes the first analyses of the GIT microbiome from wild, recaptured bats. Additionally, to our knowledge, these analyses are the first to include diet and three potential health metrics in association with the GIT microbiome of bats.

Chapter One discusses the importance of host-associated microbiomes and reviews the current status of bat microbiome research with a focus on the bat GIT microbiome and identified knowledge gaps in the field. We found that while bat microbiome studies span the globe (in 31 countries), most of this research is focused on two families in the western hemisphere (Vespertilionidae and Phyllostomidae). As of publication, 209 species (from a total of 14 families) out of more than 1,400 bat species (batnames.org) have been sampled for microbiome studies. In particular, there are few studies that include samples from Yinpterochiroptera (which includes the family Pteropodidae). Furthermore, the majority of bat microbiome studies to date focus on describing the microbiome diversity composition with cross-sectional methodology; few experimental and longitudinal studies have been completed. Current research on bat GIT microbiomes provides mixed evidence about the stability of bat GIT microbiomes over time. Other than the potential of GIT microbiome to impact nutrient acquisition, few bat microbiome

studies incorporated pathogen dynamics or direct measurements of diet and health. We used this review as a scaffold to determine our questions of interest for this dissertation.

Chapter Two of this dissertation assessed the extent to which physiological and ecological factors affect bat GIT microbiome stability. We used rectal samples collected from bats across five roosts (both resident and nomadic) from the years 2018-2020 to assess the roles of sex, age, reproductive status, season, year, and foraging status (e.g., nomadic vs resident) in shaping the *P. alecto* GIT microbiome. These analyses offer insight into longitudinal dynamics of the GIT microbiome in both individual and populations of flying foxes.

Chapter Three assesses the association between *P. alecto* GIT microbiome alpha- and beta diversity with the proportion of the individual bats' diet that is native to Australia. Here, we have collected both rectal swabs (to determine the GIT microbiome) and fecal samples (used to determine the diet at the time of capture) from the same individual flying foxes. This chapter offers insight into how changing habitat affects the *P. alecto* GIT microbiome and what may be a "normal" state for these bats.

Chapter Four incorporates several metrics to measure flying fox health in association with GIT microbiome diversity and composition. Here, we collaborated with several other students to gather information on leukocyte profiles (as determined with microscopy of blood smears), percent body fat (as determined by bioelectrical impedance), and presence of Hendra virus shedding (as determined by quantitative polymerase reaction; qPCR) for each bat in concordance with microbiome data. We use imputation and Bayesian regression to determine if the *P. alecto* GIT microbiome can predict the health of individuals.

To date, very little work has been done to understand the dynamics of the *P. alecto* GIT microbiome. We describe natural fluctuations in the P. alecto GIT microbiome in the context of host health, physiology, ecology, and viral shedding status. These results integrate rigorous, longitudinal, and quantitative approaches to determine if the GIT microbiome has the potential to serve as a biomarker of viral shedding, inflammation, or body composition for bats.

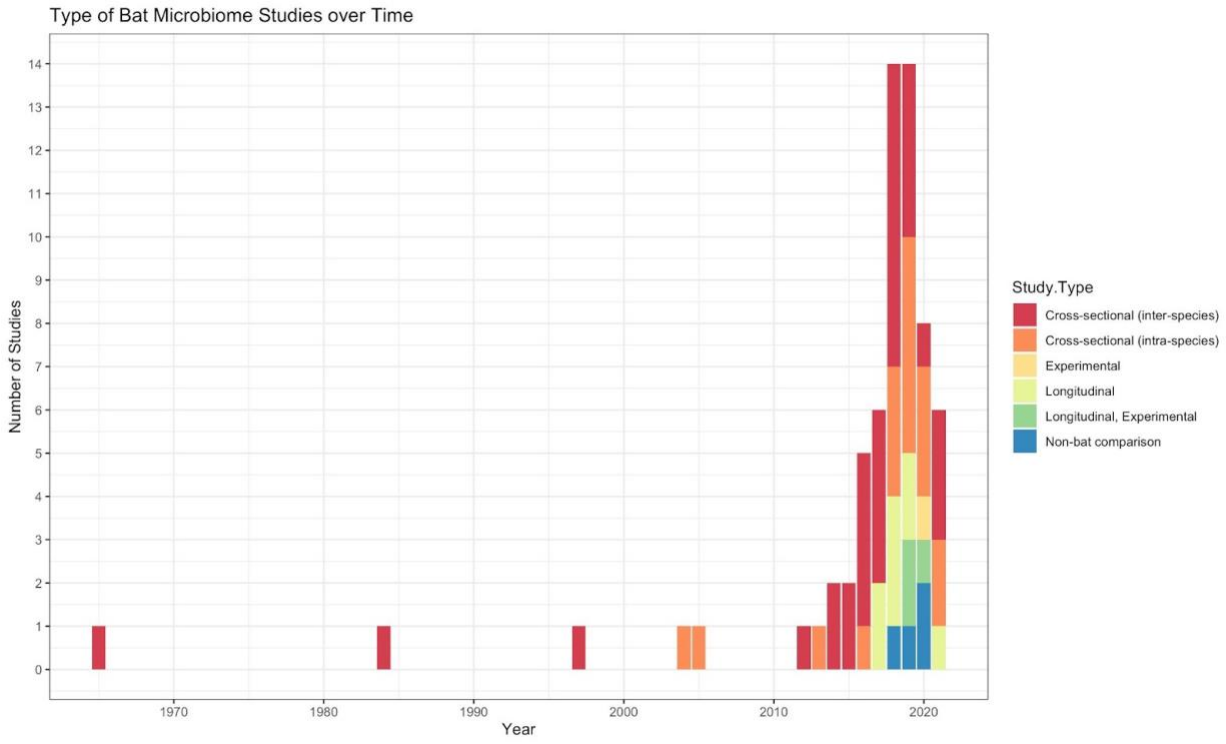

Figure 1: Types (as indicated by color of bar) of published bat microbiome (i.e., microbial flora) studies over time.

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CHAPTER TWO

DO GASTROINTESTINAL MICROBIOMES PLAY A ROLE IN BATS' UNIQUE VIRAL HOSTING CAPACITY?

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Opinion

Do gastrointestinal microbiomes play a role in bats' unique viral hosting capacity?

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Bats are reservoirs for zoonotic viruses, which they tolerate without experiencing disease. Research focused on deciphering mechanisms of virus tolerance in bats has rarely considered the influence of their gastrointestinal tract (GIT) microbiome. In mammals, GIT microbiomes influence infections through their effect on host physiology, immunity, nutrition, and behavior. Bat GIT microbiomes more closely resemble the Proteobacteria-dominated GIT microbiomes of birds than those of other mammals. As an adaptation to flight, bats have rapid GIT transit times which may reduce the stability of their microbiome, constrain nutrient uptake, and affect pathogen exposure and evolution of tolerance mechanisms. Experimental and longitudinal studies are needed to understand the function of bats' GIT microbiomes and their role in modulating viral infection dynamics.

Bats are diverse reservoirs of zoonotic viruses

With over 1400 documented species, bats (order Chiroptera) collectively constitute about 20% of mammalian species diversity (https://batnames.org/) and are unique as the only mammals capable of powered flight. Bats are long-lived for their body size and exploit diverse dietary strategies, including frugivory, nectarivory, insectivory, carnivory, and sanguivory [1]. Bats provide crucial ecosystem services such as pollination, seed dispersal, and consumption of agricultural pests and disease vectors [1]. However, bats also act as reservoirs for emerging zoonoses, including Hendra and Nipah henipaviruses, and severe acute respiratory syndrome (SARS), Middle East respiratory syndrome (MERS), and SARS-coronavirus-2 (CoV-2) coronaviruses [2]. Much current research is focused on deciphering the mechanisms by which bats host viruses that are highly virulent to other mammals without themselves experiencing clinical disease. The extent to which GIT microbiomes may modulate bat health and viral hosting capacity is a burgeoning field with many unanswered questions. Here, we postulate how bat GIT physiology may influence their microbiome composition, function, and stability to modulate infection dynamics. Future experimental, longitudinal, and functional research will help to elucidate microbial contributions to bat health and viral infection susceptibility.

Gastrointestinal microbiomes play a role in mammalian health and disease

Microbial communities (**microbiomes**) (see Glossary) that colonize the GIT play a vital role in mammalian health and immune function. Mammalian GIT microbiota have been shown to affect the nutritive, physiological, behavioral, and immunological status of their host in ways that may influence the probability of infection with, and subsequent transmission of, infectious pathogens (Figure 1). Studies performed *in vitro* and in model animals have found that GIT microbes can competitively exclude potential pathogens, directly inhibit pathogen replicative ability and infectious potential [3], and support the development of host mucosal and systemic immunity [4,5]. Intestinal bacteria can also facilitate coinfection of enteric viruses by promoting recombination [6], or providing structures such as lipopolysaccharide (found on the surface of Gram-negative

Highlights

To date, most bat microbiome research has been limited to cross-sectional and descriptive studies but recent work is shifting towards longitudinal and experimental investigations aimed at deciphering bat microbiome function.

Bats and flighted birds have much shorter gastrointestinal tract (GIT) transit times than non-flying vertebrates, likely influencing the composition of their microbial communities.

Like birds (and distinct from other mammals), bat GIT microbiomes are largely dominated by Proteobacteria, early gut colonizers associated with inflammation in other species.

Bat microbiome studies based on intestinal samples show evidence of host–microbiome phylosymbiosis, while those based on fecal or rectal samples do not.

Consensus is emerging that bat GIT microbiomes likely contribute to nutrient acquisition for their hosts, but the role of the GIT microbiome in bat immune function remains largely unexplored.

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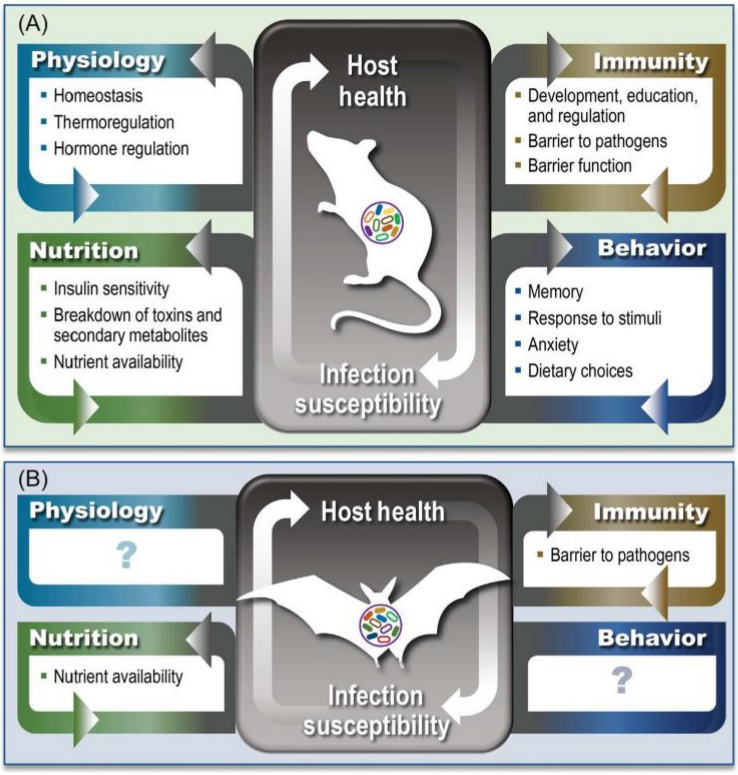

Figure 1. Microbiomes contribute to host health through several mechanisms. Panel (A) shows known bidirectional communication between the model and well-studied system hosts (e.g., mice and humans) and their **host-associated microbiomes.** Host-associated microbiomes are known to contribute to host health by influencing or modulating host physiological processes, immune function, nutritional status, and behavior. Panel (B) shows that, in bats, gastrointestinal tract (GIT) microbes have been shown to contribute to host nutrition, and skin microbes can serve as a barrier to external pathogens (see Box 1 in the main text). However, relative to mice and humans, much less is known about the specific contributions of microbiomes to their chiropteran hosts, highlighting a need for future research in these areas.

Glossary

Dysbiosis: imbalance in the composition and function of the microbiome that may enhance susceptibility to infection by pathogens.
Gut–brain axis: the bidirectional communication between the enteric and nervous systems whereby emotional and cognitive function and intestinal function are linked.
Host-associated microbiome: microbial communities that have been demonstrated to have meaningful, mutualistic relationships with the host that harbors the microbes. The host provides a niche for these microbes to colonize. In turn, the microbes provide essential functions such as metabolite anabolism and catabolism.
Microbiome: communities of microbial organisms residing on and within humans, plants, animals, or other systems. These microscopic communities include bacteria, viruses, archaea, fungi, and protists.
Paracellular absorption: the passage of solutes or molecules between epithelial cells. In the GIT, tight junctions (multiprotein complex) regulate the passage rate of molecules during paracellular transport. Regarding human health, tight junction function is essential in preventing a 'leaky gut' or increased intestinal permeability which is associated with inflammation.
Phylosymbiosis: a pattern of simultaneous host and microbiome phylogenetic branching alignment so that more closely related hosts contain more closely related microbes.
Transcellular transport: the passage of solutes and molecules through a cell either through active transport or passive diffusion.
White adipose tissue: subcutaneous tissue that stores and releases fatty acids to supply fuel.

bacteria), which some viruses co-opt to evade host immune responses [7]. Thus, under differing conditions, intestinal bacteria may either exclude potential pathogens from the host, or, facilitate pathogen persistence [7].

Nutrition plays a critical role in host immunity [8], offering an indirect mechanism by which the GIT microbiome can further modulate infection. Host nutrition is affected by GIT microbiota [9], as GIT-dwelling microbes biosynthesize B and K vitamins [10], which contribute to mineral homeostasis [11] and help to break down dietary fiber to produce short-chain fatty acids (SCFAs) [9]. In particular, SCFAs can directly impede the development of respiratory viral infections [12]. In humans, uptake of B and K vitamins improves immune efficiency [13,14], while micronutrient deficiency impairs cellular immune responses [15].

In addition to nutritional and immunological function, GIT microbes also influence host fitness through behavior and physiology. The **gut–brain axis** describes constant crosstalk between GIT microbes and the host brain [16], which occurs through several mechanisms. For example, humans with inflammatory bowel syndrome, characterized by perpetual GIT microbiome **dysbiosis**, are more likely to experience anxiety [17], and the transplantation of 'depression microbiota' into healthy mice can produce depression-like behaviors [18]. The GIT microbiome is also important for maintaining homeostasis. Alterations in the GIT microbiome during cold temperatures can expand GIT size and absorptive capacity, improve insulin sensitivity, and increase the metabolic activity of **white adipose tissue** through a process known as 'browning' [19]. Thus, through both direct and indirect mechanisms, microbiomes play a large role in maintaining health and preventing infection in mammalian hosts. While most mammalian microbiome research has been focused on human and mouse model systems, exciting new research is beginning to unravel these processes in non-model species, including bats.

Bat microbiome research is a growing field

We systematically reviewed the literature on bats and microbiomes. Most studies on bat microbiomes were published in 2016 or after, likely as a result of increasing global access to sequencing technologies and increasing awareness of the importance of microbiomes more generally. Most bat microbiome literature focuses on the GIT microbiome (78%) with a smaller number of studies investigating external microbiomes (e.g., skin; Box 1) or microbiomes of other organs or fluids (e.g., heart, eyes, urine). Because the first line of mammalian pathogen defenses is often local (e.g., respiratory mucosal surface [21]), the microbes that colonize these other sites are important for understanding host health.

To date, the microbiomes of 205 bat species from 14 bat families have been sampled from 32 countries (Figure 2). Phyllostomidae and Vespertilionidae are highly represented relative to other bat families, respectively accounting for 30% and 32% of all bat species sampled for microbiome analyses. This research skew may be due in part to geographic convenience for North and South American research groups, and could reflect the unique diversity of feeding strategies (nectarivory, frugivory, sanguivory, carnivory, and insectivory) that exist within the Phyllostomidae – thus enabling researchers to control for phylogeny while testing associations between diet type and microbial community [22]. Much microbiome research has focused on insectivorous bats, probably because two-thirds of bats globally are insectivorous [1].

Box 1. Bat microbiomes beyond the gastrointestinal tract

In humans, the skin microbiota varies by site (e.g., forearm versus armpit) and tends to remain stable over time in healthy adults [77]. These resident bacteria protect the host by preventing the colonization of pathogenic bacteria [77]. In humans, dysbiosis of skin microbiota may result in skin disorders such as acne, eczema, malodor, and chronic wounds [77]. In reptiles and amphibians, changes in the skin microbiota have been associated with sometimes-fatal fungal infections [78].

Bacterial communities on bat skin and fur are dominated by microbes derived from the classes Actinobacteria, Gammaproteobacteria, and Firmicutes [55,79–84]. Microbial richness associated with bat skin is greater than that of the GIT and oral communities [38]. The external microbiota of bats is host-specific [82,84] and reflects the host environment [79], but can change at the roost-level over time [55]. Bats roosting together tend to have external microbiomes more similar to each other over time rather than themselves from earlier time points [55].

Bats' skin microbes also protect from pathogens (see Figure 1 in the main text). Bacteria isolated and cultured from bat skin inhibit the growth of *Pseudogymnoascus destructans* (*Pd*), the causative agent of white-nose syndrome (WNS) [85]. The skin microbiome of WNS-positive bats is typically enriched with bacteria with the ability to inhibit *Pd* growth [81] but *Pd* growth can also destabilize skin microbiomes [82]; Grisnik *et al.* [83] found reduced populations of antifungal bacteria on the skin of bats infected with *Pd*, perhaps evidence of *Pd*'s successful overpowering of the skin microbiome. In addition to acting as a barrier to pathogens, microbes on bat skin likely aid in reproduction. Sex-related differences in bats' skin microbiota are associated with specific odors and corresponding sex-selected scent organs [86,87].

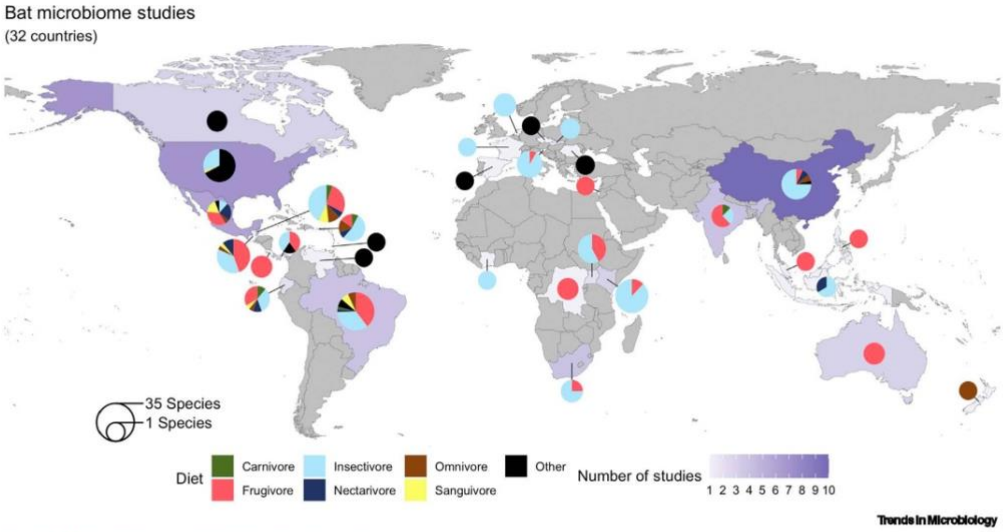

Figure 2. Map of the current bat microbiome studies. Shading of the countries shows the global distribution of bat microbiome studies, where darker shading indicates a greater number of studies from that country. The pie charts show the number of bat species sampled from each country (as indicated by the circumference of the pie) where smaller pies indicate a smaller number of species sampled. The corresponding proportions of the pie show the diet types of the bats for which there are gastrointestinal tract (GIT) microbiome studies; studies focused on non-GIT microbiomes (e.g., skin, urine, or other organs or fluids) are shown in black as 'other'.

More longitudinal and experimental studies will elucidate bat GIT microbiome dynamics

Most bat microbiome studies (74%) have been cross-sectional, with few longitudinal or experimental studies. Future longitudinal studies will be important in providing insight into the stability of bat GIT microbiomes across seasons and the extent to which bats experience dysbiosis following pathogen infection or dietary shifts. Vouchering specimens in collaboration with natural history museums will further support the establishment of bat microbiome records over longer time horizons [23]. Additionally, experimental studies will be crucial for deciphering the directionality in pathogen–microbiome interactions. For example, adenovirus infection was associated with altered GIT microbial communities in juvenile *Artibeus jamaicensis*, but not adults, and subsequently predicted downstream impacts on nutritional and immunological functions for infected versus uninfected juveniles [24]. Without experimentation, it is impossible to distinguish whether viral infection drives a change in the GIT microbiome or whether the presence of certain microbial communities render the host more susceptible to infection. Admittedly, experimental approaches in bat microbiome research are challenging as captive bat colonies are rare, and difficult to maintain, and experimental infections with virulent human pathogens require high biosafety containment [25]. Nonetheless, simple experiments can be undertaken on wild bats held temporarily in field settings [26], and experimental infections can be accomplished using non-zoonotic pathogens [27].

Most bat microbiome studies describe the microbial community (42%), but few study the functional role of that community. Nonetheless, consensus concerning the state and function of bat GIT microbiomes is beginning to emerge.

Bats break the mammalian mold with bird-like GIT microbiomes

At a broad taxonomic level, bat GIT microbiomes are unique from those of other mammals. Bat GIT microbiomes are dominated by microbes in the phylum Proteobacteria, including families Enterobacteriaceae and Streptococcaceae, [22,28–34], whereas most mammalian GITs are primarily colonized by microbes from the phyla Bacteroidetes and Firmicutes [32]. Bat GIT microbiomes more closely resemble those of flying birds than those of other mammals, though the species-level composition of specific GIT Proteobacteria – and their concomitant functions – may diverge in these two hosts [34]. In humans and mice, increased proportions of Proteobacteria signify dysbiosis and associated diseases [inflammatory bowel disease (IBD), diabetes, obesity, and some neurological disorders [35]], but in bats, Proteobacteria stably dominate apparently healthy GITs, suggesting that model systems cannot be universally extrapolated. Proteobacteria likely do not equate to dysbiosis in bats.

As in other mammal hosts, a combination of intrinsic (e.g., genetics, sex, reproductive status) and extrinsic (e.g., geographic location) factors shape bat GIT microbiomes [28,30,36–41]. Across mammals, GIT microbial diversity decreases as dietary strategies shift from herbivory to carnivory [32,42]. By contrast, bats primarily exhibiting herbivorous (nectarivorous or frugivorous) feeding strategies have lower microbial diversity than bats exhibiting carnivorous (insectivorous, sanguivorous, or omnivorous) feeding strategies [22,28,29,43] regardless of phylogenetic and geographic separation. Early bat microbiome research (primarily within the Phyllostomidae) revealed that closely related bats share closely related microbiota (**phylosymbiosis**) [22,28,36,44]. However, more recent work, including a broader range of bat families, suggests that bat GIT microbiomes – like those of birds – host communities that vary geographically but do not stably track host phylogeny [34,38], but see Box 2 for discussion of the role that sampling methods may play in these results.

Bat GIT microbiome stability – and its effect on bat health – remains unclear

In humans, lack of stability of the GIT microbiome is linked to disease [45]. Human GIT microbiomes exhibit successional changes over the first 3–4 years of age before reaching climactic adult-like microbiomes largely resilient to perturbation [45]. Currently, the literature concerning the stability of bat GIT microbiomes is indeterminant: bat GIT microbiome diversity appears to shift seasonally during/after hibernation [46,47], and during reproduction in females [39,40]. Some studies have shown age-related changes in bat GIT microbiome diversity [39], though patterns are not consistent. For example, young *Rhinolophus hipposideros* bats host an overrepresentation of *Klebsiella* bacteria relative to adults [33], while *Vespertilio sinensis* bats show increasing GIT microbial diversity from birth until 4 weeks of age [48]. Habitat fragmentation and dietary shifts may also impact the variability of bat GIT microbiomes [37] and even within bat species, GIT microbiomes can differ between roosts [49].

Box 2. Sampling technique matters when defining bat GIT microbiomes

Sampling the intestinal microbiota of bats requires euthanizing the animal, whereas fecal and rectal samples may be collected from live bats. Fecal samples can be collected noninvasively from underneath roosting bats [49,88]. However, microbial composition differs between bat intestinal and fecal samples (i.e., guano) [22,89]. Shannon richness has shown to be higher in bat fecal samples, but intestinal samples harbor greater microbial phylogenetic diversity (via Faith's Phylogenetic Diversity index), a measure of diversity within a target (in this case, microbial) class [22]. Importantly, intestinal microbiota is more phylogenetically constrained than fecal microbiota and better reflects the evolutionary history of the bat host (phylosymbiosis) [22]. Feces may not necessarily be a good proxy for understanding the microbes that colonize the bat GIT [22,89] – in contrast to taxa, such as primates, with longer GIT transit times, for which phylosymbiosis can be deduced from feces [90]. Intestinal samples likely offer a better medium for investigating questions in the context of host evolution, whereas fecal samples may be better suited to addressing questions related to host diet and ecology [22]. The differences in microbiome diversity among feces and intestines, relative to rectal swabs, has not been tested. Rectal swabs may offer better insight into the bacteria colonizing bats' intestines and can be collected sans euthanasia.

By contrast, one study of *Myotis myotis* demonstrated identical rectal microbiomes in juveniles and adults, indicating a lack of age-related shift in this species [50]. However, all bats used in the study [50] were volant and therefore at least partially weaned. Because early successional dynamics of the GIT microbiome often correspond to dietary transitions [51], it would be interesting to assess whether differences manifest in the GIT microbiomes of nonvolant pups, which consume milk instead of adult foods. These transitions may be particularly important for the development of bat immune systems as bats inherit maternal immunity via milk [52]. As mentioned previously, adenovirus infection in bats is associated with altered GIT microbiomes in juvenile bats, but not adults [24]. This work highlights the importance of successional changes in the GIT microbiome that could modulate viral shedding events that are associated with the juvenile–adult transition in other bat systems [53,54]. Adult *Rousettus aegyptiacus* GIT microbiomes more closely reflect microbial communities from previous samples of the same individual than temporally coincident samples from other bats [55], highlighting the compositional stability of the microbial community in mature bat GITs. Finally, it should be noted that, like the microbiomes of other mammals, bat GIT microbiomes converge in captivity [56,57].

Bat microbiomes likely benefit their hosts

In many mammals, GIT microbes provide a myriad of essential functions to the host (Figure 1), while receiving habitat and resources in return. Though support for a hypothesis of bat–microbe mutualism is mounting, several studies document evidence of commensal and parasitic interactions between bats and their microbiota, as well. Understanding these relationships is essential for understanding how GIT-dwelling microbes could modulate bat virus infection dynamics.

Bat GIT microbiomes likely contribute to nutrient acquisition

In support of a more mutualistic bat–microbiome relationship, recent work suggests that bat GIT bacteria play a role in host nutrient acquisition. Unique communities of bacteria that inhabit different body sites suggest that site-specific microbiomes have specialized needs, functions, or competitional differences [30]. Numerous studies have shown that bat GIT microbiome composition is associated with host dietary strategy [22,28,36,38,43,58], and Phillips *et al.* [59] demonstrated that, while three sympatric insectivorous bat species had varying GIT bacterial composition, the predicted functions of these communities were consistent across species. Sanguivorous vampire bats (*Desmodus rotundus*) have GIT microbiomes that are distinct from those of insectivorous bats in both composition and predicted function [44]. Indeed, the GIT microbiomes of vampire bats are more similar to those of sanguivorous birds than they are to those of other bats [60]. Similarly, whole-genome sequencing of the common vampire bat and its GIT microbes show that microbial genes are enriched for functions contributing to nutrient acquisition, metabolism, and homeostasis [44]. Specifically, these functions are complementary extensions of endogenously encoded host gene functions, suggesting that vampire bats depend on their microbes to function optimally.

Bats with frugivorous or nectarivorous dietary strategies also host bacteria that have predicted functions that may contribute to host nutrient acquisition. Chiefly, *Enterobacter* identified in the GIT of frugivorous *Cynopterus brachyotis* are known to metabolize sugars (including xylose, which comprises a large portion of most plant carbohydrates) [61,62], and GIT bacteria isolated from nectarivorous/frugivorous *Pteropus giganteus* have cellulolytic capabilities which help to break down plant cell walls [63]. Nonetheless, studies have yet to categorically demonstrate the digestive functions of these microbes in bat hosts. Additional functional studies are needed to determine whether microbes identified in frugivorous bat GITs truly support nutrient acquisition in their respective hosts.

Bat GIT microbiomes may play a role in host longevity

Further evidence suggests that the functions of bat GIT bacteria may extend beyond nutrient acquisition. Hughes *et al.* [50] postulated that bats' GIT microbes may contribute to longevity after identifying stable GIT microbes in long-lived bat GITs with functions including DNA replication and repair, metabolism, and oxidative phosphorylation. However, these processes are ubiquitous across all living organisms, making it difficult to pinpoint unique differences for bats. It should be noted that this study, and many other bat microbiome studies summarized here, use the program PICRUSt [24,46,48,50,57–60] to predict functions from 16S rRNA sequence data. PICRUSt is a less accurate predictor for non-human samples [64] and does not account for active microbial metabolism further complicated by transcriptional and post-translational regulatory processes. To our knowledge, no studies have assessed the fecal or intestinal transcriptome or metabolome of bats to determine the specific metabolic pathways encoded in bat GIT microbial genomes. Though more work is needed to accurately assess the direct contributions of the microbiome, evidence to date suggests that GIT microbes support nutrient acquisition in bats which may impact their abilities to host or resist pathogens.

Simple digestive tracts in bats and birds may facilitate pathogen exposure

Both bats and birds have rapid gut transit times, a hypothesized adaptation to facilitate low body mass for flight [65]. Indeed, both bats and birds have simplified digestive tracts which are short in length and have reduced intestinal nominal surface areas (excluding contributions of villi and microvilli) and less intestinal tissue relative to nonvolant mammals [66,67]. Furthermore, most bats lack a cecum or appendix, an organ that houses bacteria beneficial for digestion in several bird species [68]. In general, consumption of difficult-to-digest, highly fibrous foliage is rare in both taxa [65]. Frugivorous bats are known to disgorge most of the fibrous material (pulp) in their diets and concentrate consumption on nectar and juice [1].

Regardless of diet, shorter intestine lengths and reduced gut transit times should pose constraints on both bird and bat nutrient uptake within the gut. Most nonvolant vertebrates acquire nutrients via a process known as **transcellular transport**, through which nutrients (e.g., glucose) are transported by cells across the gut epithelium. By contrast, **paracellular absorption** is the process by which nutrients are acquired via passive transport across the gut epithelium's intracellular spaces [69]. Both birds and bats show evidence of expanded intestinal villi lining the gut epithelium, which could functionally increase the inner surface area of the small intestine and amplify opportunities for both transcellular and paracellular nutrient absorption. Indeed, previous work demonstrates that paracellular absorption is enhanced in both flying bird and bat taxa [66,69], which could reduce the reliance of these taxa on microbiota to enhance these processes. Additionally, heightened use of paracellular absorption may place species at risk of higher toxin or pathogen exposure [70]. Theory predicts that higher pathogen exposure should favor the evolution of tolerance mechanisms in a host [71]; therefore, paracellular absorption could have important implications for bats' evolution as tolerant viral reservoirs [2]. Given bats' association with several emerging pathogens of importance to human health [2], the particular rapidity of GIT transit exhibited by Chiroptera, with implications for the composition of the GIT microbiome, is of special interest.

Rapid GIT transit times in bats and birds may select for commensal GIT microbiota

The extent to which different hosts rely on GIT microbiota for various functions varies widely across the animal kingdom, with some animals not requiring a microbiome at all [72]. In animals with low microbial biomass and rapid GIT transit times, it can be difficult to tease apart which microbes are transient or resident, and to identify the relative strength of microbial functional

contributions to the host [72]. Recent research reporting lack of phylosymbiosis in bat and bat GIT microbiome phylogenies [34,38] may suggest a low reliance of bats on their GIT microbes, but the contributions of the bat GIT microbiome to essential host functions are still being elucidated.

Rapid GIT transit times [67] may limit the extent to which some bat GIT bacteria are able to provide beneficial metabolites to their hosts as many of these metabolites (e.g., SCFAs) are produced through food fermentation in the GIT, which is generally a time-consuming process (depending on GIT transit time and amount of fiber ingested [73]). Consequently, it is possible that bat–GIT microbiome relationships are largely commensal, with bats providing habitat for GIT microbes but receiving few essential functions in return. Low bacterial biomass in oral and fecal samples [30] and high interindividual variation in bat GIT microbiomes [22,36,55] offer evidence of a lack of dependence of bat hosts on their microbiota, but samples with low biomass are likely to be overwhelmed by contaminants during processing and may be more reflective of environmental (i.e., transient) microbes from diet and grooming [72]. Furthermore, some work indicates that microbiome composition and abundance appear relatively consistent throughout various regions of the bat GIT (excepting some frugivores which demonstrate moderate shifts in relative abundance of different microbial taxa across the length of the GIT [28]). In many other taxa, distinctive GIT microbial communities colonize different regions of the digestive tract and perform specific digestive functions [20]. The homogeneity of bat GIT microbiomes could indicate a more commensal host–microbe relationship or could simply highlight that the low fecal biomass of bat GITs [30] has, to date, hindered our ability to identify important differences in microbial community composition and function.

In humans, microbiota inhabiting the more rapidly transiting small intestine demonstrate faster growth rates and more rapid utilization of simple substrates to avoid expulsion with transit [74]. Rapid transit rates in bird and bat GITs could alter the abiotic conditions in the GIT to select for 'weedy' microbial taxa able to quickly utilize available substrates [72]. Support for this hypothesis in bats is seen in the dominance of bat GITs with Proteobacteria, which are highly represented in the human small intestine. Some species within Proteobacteria have some of the fastest growth rates, and commonly employ phosphoenolpyruvate transport systems that enable rapid utilization of simple carbohydrate sources [75]. Proteobacteria may offer substantial benefits to chiropteran hosts, but these benefits are not fully characterized in the scientific literature. Without understanding which microbes are transient versus resident, it is difficult to tease apart the contributions of the GIT microbiomes to bat hosts.

More work is needed to characterize the contributions of Proteobacteria to bat health

As mentioned previously, high proportions of Proteobacteria that dominate bat GITs are associated with inflammation and poor health outcomes (e.g., IBD) in humans and mice [76]. Nonetheless, no study identifying the presence of these GIT microbial communities in bats has reported that any of the bat hosts investigated appeared unhealthy. Bats' unique anti-inflammatory mechanisms, evolved to cope with flight, may also help them to tolerate GIT bacteria associated with inflammation in other mammals. Even so, fitness effects are notoriously difficult to assess in wild animals, and few studies on this topic have been conducted, making it challenging to assess whether high proportions of Proteobacteria are indicative of dysbiosis in bats. Studies interrogating the functional role that these GIT microbes play in bat health and disease thus represent a critical research priority.

Concluding remarks

The field of bat microbiome research is growing rapidly, and exciting new breakthroughs in technique, approach, and understanding are emerging. As evidence of bat–microbe mutualisms

Outstanding questions

How stable are bat GIT microbiomes over time? Are these dynamics sex- or species-specific?

Are rapid gut transit times the causal factor in the dominance of Proteobacteria in the bat GIT microbiome?

Do high proportions of Proteobacteria in the bat GIT microbiome signify a 'dysbiotic state'? To what extent do dietary shifts (e.g., from nectar to fruit) perturb the GIT microbiome?

Do Proteobacteria play a role in GIT 'leakiness' in bats and enable increased rates of paracellular transport through the epithelium for quicker access to nutrients from food?

Does increased paracellular transport in bat GITs increase their exposure to pathogens and potentially influence tolerance?

Do bat GIT microbes contribute to tolerance or resistance of pathogens?

How heritable are GIT microbiomes from mothers to pups, and are there successional changes associated with weaning?

How do bat GIT microbes affect intestinal permeability and barrier function? How much does this matter for bats?

Does the GIT microbiome play a role in educating or modulating bats' immune systems?

continues to grow, future work in this area should progress towards a deeper understanding of the functional contributions offered by bat microbial communities in the GIT and elsewhere. Adoption of more 'omics-based approaches (i.e., transcriptomics, metabolomics, proteomics, lipidomics) applied to both bat hosts and their microbiomes will help to generate insights concerning microbial contributions to bat health and the dynamics of bat infections. Moreover, inclusion of techniques to assess host viromes and mycobiomes will provide insight into microbial dynamics within hosts. A future emphasis on longitudinal and experimental work will help to reveal whether the concept of a 'dysbiotic' state applies to bats and, if so, what health implications result from this disturbance (see Outstanding questions). Experimental studies may also enable better assessment of the causal underpinnings of observed associations between bat pathogen infection and changes in the microbial community. Most likely, bat microbiomes play a critical role in bat health, physiology, and immunity. It will be up to future research to demonstrate how.

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Declaration of interests

No interests are declared.

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CHAPTER THREE

BLACK FLYING FOX (*PTEROPUS ALECTO*) GASTROINTESTINAL MICROBIOMES DEMONSTRATE SHORT-TERM, PHYSIOLOGICAL, AND ECOLGOGICAL SHIFTS IN ALPHA DIVERSITY AND COMPOSITION

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Abstract

In humans, stability is one of the characteristics associated with a healthy gastrointestinal (GIT) microbiome. Current evidence for bat GIT microbiome stability is mixed, and it is unclear if and which microbes and their functions are essential to bat hosts. Here, we use rectal microbiomes of flying foxes (*Pteropus* spp.) to assess changes in the GIT microbiome composition associated with sex, age, reproduction, and within individuals over time. Additionally, we assessed the stability of the GIT microbiomes of black flying fox (*P. alecto*) populations over three years, summer and winter seasons, and in relation to resident status in urban areas. We found that *P. alecto* GIT microbiomes vary with respect to age and reproduction, and that GIT microbiomes within individuals change over short periods of time. Variation in GIT microbiome composition may enable *P. alecto* to respond to dynamic nutritional resources to cope with their high energetic needs. Elucidating the GIT microbiome dynamics of flying foxes may ultimately enable us to improve bat health. To our knowledge, this study is the first to sample the GIT microbiome of wild recaptured bats.

Introduction

In many mammalian systems, the microbial communities colonizing the gastrointestinal tract (the GIT microbiome) have been shown to make important contributions to host health. This includes both direct and indirect contributions to nutrition through the liberation and biosynthesis of essential nutrients and effects on host behavior, immune function, and physiology (as reviewed in Flint et al., 2012; Martin et al., 2019; Morais et al., 2021; Sommer and Bäckhed, 2013; Zheng et al., 2020 and others). These contributions result from the collective actions of a diversity of microbes that interact symbiotically with the host and each other.

However, the role of the GIT microbiome in modulating bat health is speculated but has not been demonstrated. Bats provide numerous ecosystem services ((Kunz et al., 2011)), but have also been identified as the reservoirs of many zoonotic pathogens such as Middle East Respiratory Syndrome (MERS), Severe Acute Respiratory Syndrome (SARS), henipaviruses, and filoviruses (Letko et al., 2020) and it is hypothesized that disruptions to the GIT microbiome may influence spillover events (Jones et al., 2022). Pulses of viral shedding associated with season and parturition are associated with spillover of these zoonotic viral pathogens into new hosts, including humans (Plowright et al., 2015; Ruiz-Aravena et al., 2022). Thus, it's important to understand factors affecting bat health, including GIT microbiomes, which may in turn affect pathogen shedding.

Globally, bat populations face numerous threats, including habitat loss (Frick et al., 2020). As their access to historic resources are changing, it's crucial to parse out mechanisms underlying bat health. Habitat degradation is associated with decreased diversity in wildlife GIT microbiomes (e.g., in howler monkeys; Amato et al., 2013). Bat microbiomes contribute to host

physiology and nutrient acquisition and may contribute to their role as reservoir hosts (Jones et al., 2022). To elucidate the role of the GIT microbiome in bat health through energy and nutrient acquisition, and identify whether their changing ecology is affecting their role as reservoirs of zoonotic pathogens (perhaps even through changing microbiomes), we must understand the temporal dynamics of bat GIT microbiomes.

Currently there is mixed evidence on the longitudinal dynamics of bat GIT microbiomes. Some studies have suggested compositional stability of bat GIT microbiomes over time. For example, Hughes et al. (2018) found no difference in the composition or predicted function of juvenile and adult *Myotis myotis* rectal microbiomes, indicating a lack of age-related changes in this species. Similarly, Kolodny et al. (2019) showed that over time, captive *Rousettus aegyptiacus* GIT microbiomes more closely resemble microbial communities from previous samples of the same individual than temporally-coincident samples from other bats, thus indicating short-term stability of these bat GIT microbiomes within individuals.

By contrast, several studies show significant seasonal, reproduction- and age-related changes in bat GIT microbiome diversity and composition. Some bat GIT microbiomes (e.g., *Rhinolophus euryale* and *Rhinolophus ferrumequinum*) can shift seasonally around hibernation (Maliničová et al., 2017; G. Xiao et al., 2019) or during reproduction, though reproductive changes may be limited to females (Gaona et al., 2019). *Vespertilio sinensis* also demonstrate increasing GIT microbial diversity during the first four weeks after birth (Yin et al., 2020) and young bats (*Rhinolophus hipposideros*) have a greater abundance of some bacterial taxa (i.e., *Klebsiella*) relative to adults (Vengust et al., 2018).

Furthermore, viral infection in bats has been associated with different GIT microbiome compositions in juvenile bats, but not adults (Wasimuddin et al., 2018). Though the cause and effect in pathogen-microbiome relationships cannot be demonstrated in these observational studies (Dietrich et al., 2017a; Wasimuddin et al., 2018), these results highlight the potential importance of age-related changes in the GIT microbiome and consequences for viral infection. It is also important to understand how the degree of turnover in the GIT microbiome may enable hosts to better adapt to new resources.

Historically, most Australian flying foxes exhibited nomadic foraging ecologies and would follow pulses of flowering in native forests (Eby et al. in review). These pulses of flowering are ephemeral, irregular, and patchily distributed across the landscape, and many critical flowering species have been extensively cleared (Eby et al. in review). To cope with ongoing loss of natural food resources, flying foxes are increasingly form smaller, 'resident' camps in anthropogenic landscapes (Eby et al. in review). Over the past several decades, concordant with significant habitat loss, there has been a significant shift in favor of residency in urban and agricultural areas to feed on cultivated and introduced plants (Eby et al. in review). Unlike native flowering trees and fruits, these marginal food sources are available all year round (Mcdonald-Madden et al., 2005). It is currently unknown how these dietary shifts impact the *Pteropus alecto* GIT microbiome.

The aim of this study was to assess the temporal dynamics of the *P. alecto* GIT microbiome, and specifically the stability of the GIT microbiome associated with host physiology from three different perspectives: reproductive status, age, and short-term changes within and between recaptured individuals. Additionally, we assessed the GIT microbiome

stability of *P. alecto* populations over three years, summer and winter seasons, and in relation to resident status in urban areas.

We hypothesize that: 1) *P. alecto* will exhibit annual, seasonal, reproductive, and age-related shifts in alpha and beta diversity of the GIT microbiome; 2) GIT microbiomes from recaptured *P. alecto* will be more similar to samples from the same bat than to other bats (i.e., there will be minimal shifts in alpha diversity, and minimal shifts in composition); and 3) *P. alecto* sampled in continuously-occupied urban resident camps ('resident individuals') will have greater GIT microbiome alpha diversity and different composition relative to *P. alecto* sampled in seasonally-occupied nomadic camps ('nomadic individuals'). These analyses will provide insight into the dynamics of the *P. alecto* GIT microbiome and determine whether or not these bats' GIT microbiomes tend to remain stable or experience significant turnover across short periods of time.

Methods

Capture and sampling of bats

We captured resident and nomadic black flying foxes (*P. alecto*) at five sites in the greater Brisbane area (MSU IACUC #201750 and Griffith University Animal Ethic Committee ENV/10/16/AEC). Nomadic camps were identified by rapid large influxes of flying foxes to a roost (>10,000 individuals). Resident camps were identified as having at least 100 *P. alecto* present year-round. We captured bats via mist nets at dawn upon return from foraging. During processing (within 6 hours of capture), we anesthetized bats with isoflurane (starting at 5% then reducing to 1.5%) and used sterile synthetic cotton swabs to collect rectal swabs into 70% ethanol. Samples were stored on ice or in liquid nitrogen until they could be stored at -80C. For

each individual, we collected forearm length, mass, and identified the sex, age class, and reproductive status. We assigned an age group classification (i.e., juvenile, subadult, or adult) by using a combination of forearm length, mass, and other visible characteristics such as toothwear and color, and evidence of past reproduction. Juveniles were considered to be the young of the year, subadults were considered to be young bats 1-2 cohorts older than the juveniles and generally lacked evidence of previous reproduction. Upon capture, all bats were checked for a Passive Integrated Transponder (PIT) tag. If no PIT tag was present, we inserted a PIT tag (RFID, ZD Tech Group China) under the skin between the scapulae while the bat was anesthetized. All bats were given fluids (one part 2.5% glucose and 0.45% NaCl solution and one part Hartmann's solution) subcutaneously and monitored for at least half an hour before release back into their roost.

Lab methods

To prepare rectal swabs for processing, we evaporated the ethanol from the tubes using an Eppendorf Concentrator 5301 at 60°C for approximately 30-45 minutes until dry. We extracted DNA from the rectal swabs using the ZymoBIOMICs DNA Miniprep Kit (D4300). ZR BashingBeads and ZymoBIOMICS Lysis Solution were added directly to the dried field tube containing the rectal swab. We then used a Mini-Beadbeater-16 (Biospec Products) at 3500 oscillations per minute to bead-beat samples for 1 minute and a wait period of 5 minutes, repeating this beat-wait cycle for a total of five cycles. We followed the rest of the protocol for the extraction kit and used 100uL of DNase-free water to elute the DNA in the final step.

We confirmed the presence of bacterial DNA in each sample with a PCR using the primer set 27F/1492R (Frank et al., 2008) to amplify an ~1,500bp section of the microbial 16S

rRNA gene and visualize the bands with gel electrophoresis. We then sent samples to the University of Michigan Microbiome Core to amplify and bidirectionally sequence variable region 4 of 16s rRNA gene with Illumina MiSeq according to (Kozich et al., 2013).

Preliminary processing of 16S sequence data

We used R (v4.1.2, R Core Team 2021) for all bioinformatics and analyses. We used DADA2 (Callahan et al. 2016) to filter, trim (to 220bp size), assess sequencing error rates, remove chimeras, and form amplicon sequence variants (ASVs). ASVs were taxonomically assigned using SILVA (nr99_v1381.1, Quast et al. 2013). We used *phyloseq* (McMurdie and Holmes 2013) and the frequency approach in *decontam* (Davis et al 2017) to identify and remove contaminants. After removing mitochondrial and chloroplast sequences, we calculated relative abundance of each ASV and we then used a 0.25% cutoff (Reitmeier et al., 2021) to remove any ASVs that were not present in at least 0.25% abundance in any sample.

Statistical methods

We used *vegan* (Oksanen et al. 2020) and *picante* (Kembel et al. 2010) to calculate alpha diversity metrics (Shannon's diversity index, species richness, and Faith's PD) for each rectal sample. Each alpha diversity metric gives us insight into differences in the microbial communities. Species richness represents the number of microbial ASVs per sample. Shannon's diversity index represents both the microbial species richness and evenness (abundances within taxa). Lastly, Faith's PD takes into account the phylogenetic relatedness of the microbial taxa. We used three linear models (one for each alpha diversity metric as a response variable) to assess changes in alpha diversity with respect to sex, age, and reproductive status. We used three more

linear models to assess the effect of our three ecological factors (season, year, and nomadism) on each alpha diversity of the GIT microbiome.

To determine physiologically- and ecologically-based changes in microbiome composition (i.e., beta diversity), we used the *adonis* function (in the *vegan* package, Oksanen et al. 2020) to run a permutational multivariate analysis of variance (PERMANOVA) with 999 permutations (set.seed=406 for reproducibility) with Bray-Curtis distance calculated from the relative abundance of ASVs as a response variable and tested the difference of the physiological factors (sex, age, and reproductive status) and ecological factors (season, year, nomadism) while accounting for site variation and the date of DNA extraction. Bats with unknown reproductive status were removed from the dataset only for analyses of associations of reproductive status and microbiome. For the aforementioned linear models, we did not include individuals recaptured across multiple capture events.

To assess the differences in GIT microbiome composition between bats and within bats (at different time points), we used a PERMANOVA with 999 permutations with Bray-Curtis distance calculated from the relative abundance of ASVs from only the recaptured bats as a response variable and the PIT tag number as an ID (for between bat comparisons) and capture time point (for within bat comparisons).

We identified the bacterial genera that were differentially abundant between *P. alecto* in different sex, reproductive, and age groups as well as between *P. alecto* captured in summer (December-February) and winter (May-July) seasons. We fit a Bayesian regression model with a Bernoulli response distribution using Hamiltonian Monte Carlo as implemented in Stan (Stan Development Team 2022). Three chains were run for 4,000 iterations after 2,000 burn-in

iterations. Convergence was checked using trace plots and R-hat values (RStan, Stan Development Team 2022). For all effect estimates, a normal prior distribution with a mean of zero and standard deviation of 0.5 was used. All relative values were scaled to a mean of zero and a standard deviation of 1 before model fitting.

Results

Flying fox demographics

We captured 195 individuals at 6 six sites in the greater Brisbane area from 2018-2020 (Table 1). Of these 195 bats, seven *P. alecto* were recaptured twice and one individual was recaptured three times.

Table 1: Summary of the captured *P. alecto* demographics by site, season, year, sex, age, and reproductive status. For reproductive status, NR= not reproductive, R=reproductive (males only), L=lactating, P= pregnant, PL=post-lactating (not reproductive at time of capture, but showed evidence of past reproductions), and NA=not determined.

Site	**Accession**	**Year**	**Season**	**Males**				**Females**			
				Juvenile	**Sub-Adult**	**Adult**	**Total**	**Juvenile**	**Sub-Adult**	**Adult**	**Total**
Bundaberg (Nomadic)	ACBBG001	2020	Summer	NR: 1	0	R: 6	7	NR: 1	NR: 3	L: 4	8
Gympie (Nomadic)	ACGMP001	2019	Summer	NA: 2 NR: 3	0	NA: 5	10	NR: 2	NR: 1	L: 8	11
Hervey Bay (Nomadic)	ACHVB001	2018	Winter	NA: 2	0	NA: 1	3	0	P: 1	NA: 1	2
	ACHVB002	2020	Winter	NR: 1	NR: 2	R:5	8	NR: 1	NR: 1	P: 5	7
Mt. Ommaney (Nomadic)	ACMOM001	2019	Summer	NR: 3	0	NA: 4	7	0	NA: 1	P: 3 NR: 2	6
Redcliffe (Resident)	ACRED002	2018	Winter	NR: 2	0	NA: 1	3	NR: 5	0	P: 2 NA: 1	8
	ACRED004	2018	Summer	0	0	NR: 10	10	0	0	L: 4	4

Table 1 Continued

	ACRED006	2019	Winter	0	0	0	0	0	0	P: 1	1
	ACRED007	2019	Winter	0	0	R: 2	2	0	0	0	0
	ACRED008	2019	Spring	0	0	R: 1	1	0	0	P: 2	2
	ACRED009	2019	Summer	0	NR: 2	R: 6	8	0	0	L: 8	8
	ACRED011	2020	Spring	0	0	R: 8	8	0	0	L: 1	1
	ACRED012	2020	Winter	0	NR: 2	R: 6	8	0	NR: 2	P: 5	7
Toowoombah (Resident)	ACTOW002	2018	Winter	NR: 1	NR: 2	0	3	NR: 1	NR: 2	P: 2 NR: 1	6
	ACTOW004	2018	Summer	0	0	NA: 8	8	0	L: 1 NR: 1	L: 2	4
	ACTOW008	2019	Winter	0	0	R: 1	1	0	0	0	0
	ACTOW011	2019	Summer	0	0	R: 6 NR: 1	7	0	NR: 1	L: 4 PL: 3	8
	ACTOW013	2020	Winter	0	0	R: 1	1	0	0	0	0
	ACTOW014	2020	Winter	NR: 2	0	R: 4	6	NR: 1	0	P: 10	11

Pre-processing

From a total of 175 individual *P. alecto*, we had a total of 6,377,434 reads and the mean number of reads per sample was 27,608 (SE=686) before filtering. We were left with a total of 5,366,110 reads across all samples (mean = 23,229 reads, SE = 574) after filtering. This resulted in the identification of 4,834 ASVs. Of these, 34 were identified as contaminants and removed. An additional 867 ASVs were identified as mitochondria and chloroplasts and also removed, resulting in 3,942 ASVs. Applying the 0.25% cut-off resulted in a final dataset of 797 ASVs.

Nineteen bacterial genera were detected in at least 50% of captured bats (Fig. 1). Of these 19, only 10 bacterial genera were detected in more than 75% of sampled individuals. Streptococcus was the only genus present in all flying foxes. There were 57 bacterial genera that were not shared and detected in only a single flying fox. The bacterial genera that are shared among the proportion of captured bats declines rapidly in the number of bacterial genera shared across individuals (Fig. 2).

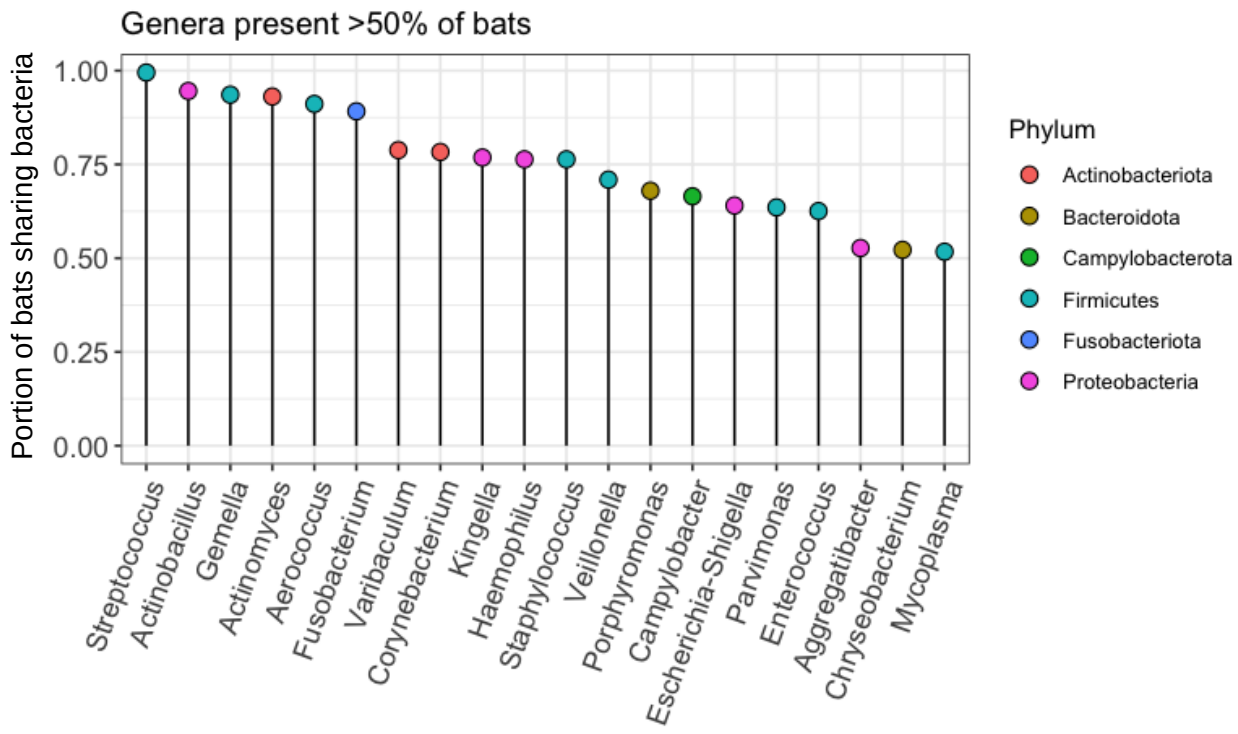

Figure 1: The microbial genera present in the percent of *P. alecto* sampled. Lollipop colors show the Phylum of each microbial genus.

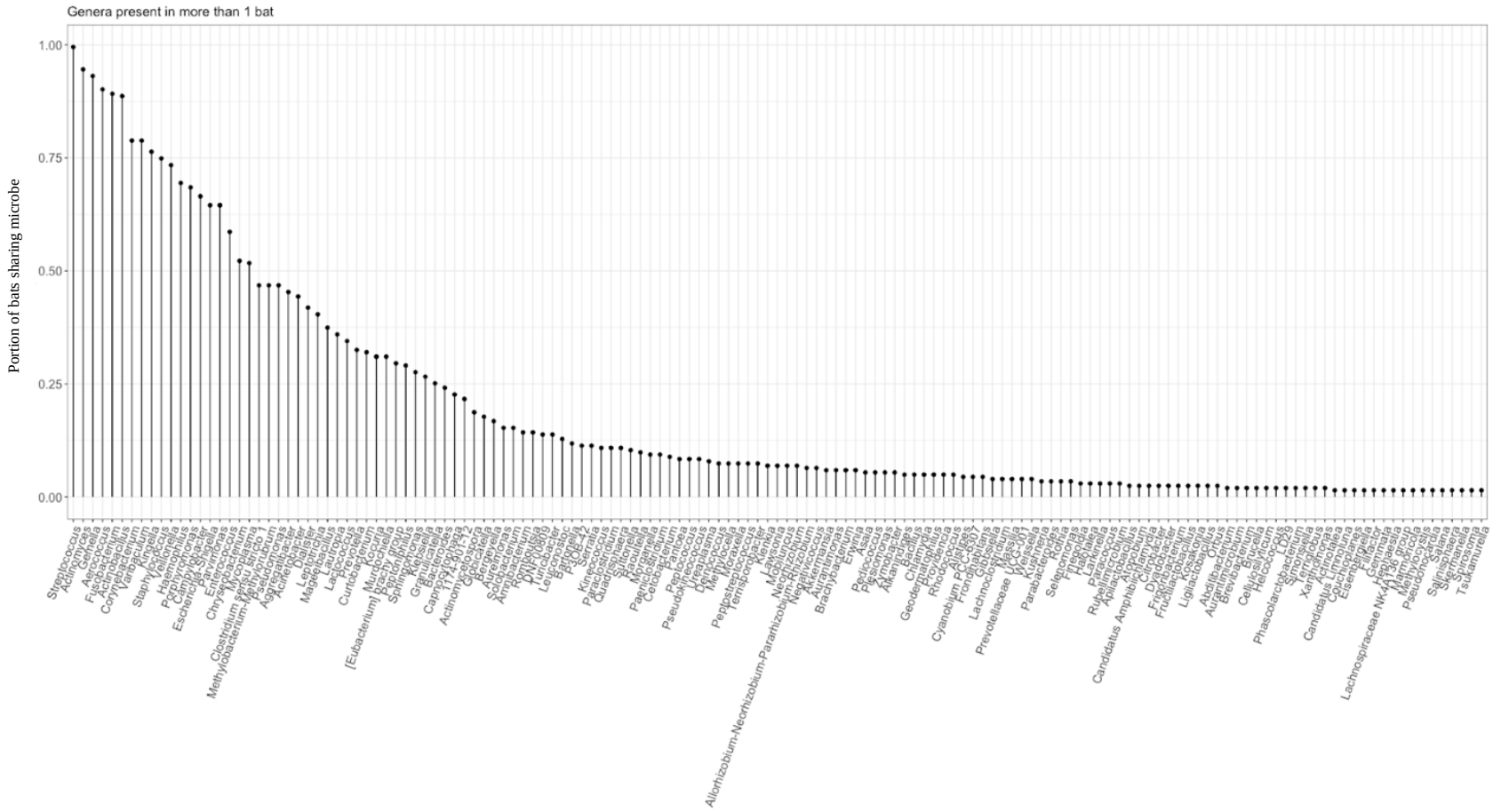

Figure 2: The portion of all sampled *P. alecto* (y-axis) that share each microbial genus. These microbial genera represent ASVs (on the x-axis) that are present in more than one bat.

Physiological shifts in GIT microbiome alpha diversity and composition

We found no evidence for a difference in species richness of the *P. alecto* GIT microbiome between sexes, among age classes, or by reproductive status (Table 2). Similarly, there was no evidence of a difference in Faith's PD of the microbiota among age classes or reproductive status; and no evidence that Faith's PD may be lower in males compared to females (Table 2). By contrast, there was strong evidence that Shannon's diversity of the *P. alecto* GIT microbiome was lower in subadults compared to juveniles and adults, and higher in lactating females compared to non-reproductive, post-lactating, and pregnant females and males of any reproductive status (though only three pregnant females included in this analysis). There was strong evidence for a difference in the microbiome composition between sexes ($p=0.001$, Figure 3), among age classes ($p=0.007$, Figure 4), and among animals with a different reproductive status ($p=0.006$, Figure 5). Sex, age class, and reproductive status accounted for 5.6%, 2.5%, and 4.6% of the total GIT microbiome compositional variation.

Table 2: Results from multiple linear models with sex, age, and reproductive status as factors and their effects on three alpha diversity metrics. P-values indicative of strong evidence are bolded and noted with an asterisk. P-values indicative of moderate evidence are bolded without an asterisk.

Alpha Diversity Metric	**Physiological Factor**	**Pairwise Comparison**	**P-Value**
'Species' (ASV) Richness (SR) df=141 F=2.452	Sex	Female:Male	0.127
	Age	Adult:Juvenile	0.592
		Adult:Sub-Adult	0.499
	Reproductive Status	Lactating:Non-Reproductive (Males)	0.483
		Lactating:Post-lactating females	0.351
		Lactating:Pregnant	0.778
		Lactating:Reproductive (males)	0.476
Shannon Diversity df=141	Sex	Female:Male	0.1833
	Age	Adult:Juvenile	0.9389
		Adult:Sub-Adult	**0.0439***

Table 2 Continued

F=3.584	Reproductive Status	Lactating:Non-Reproductive (Males)	0.2248
		Lactating:Post-lactating females	0.4090
		Lactating:Pregnant	**0.0436***
		Lactating:Reproductive (males)	0.1048
Faith's PD df=141 F=2.741	Sex	**Female:Male**	**0.0787**
	Age	Adult:Juvenile	0.5499
		Adult:Sub-Adult	0.3868
	Reproductive Status	Lactating:Non-Reproductive (Males)	0.3942
		Lactating:Post-lactating females	0.4210
		Lactating:Pregnant	0.9618
		Lactating:Reproductive (males)	0.5960

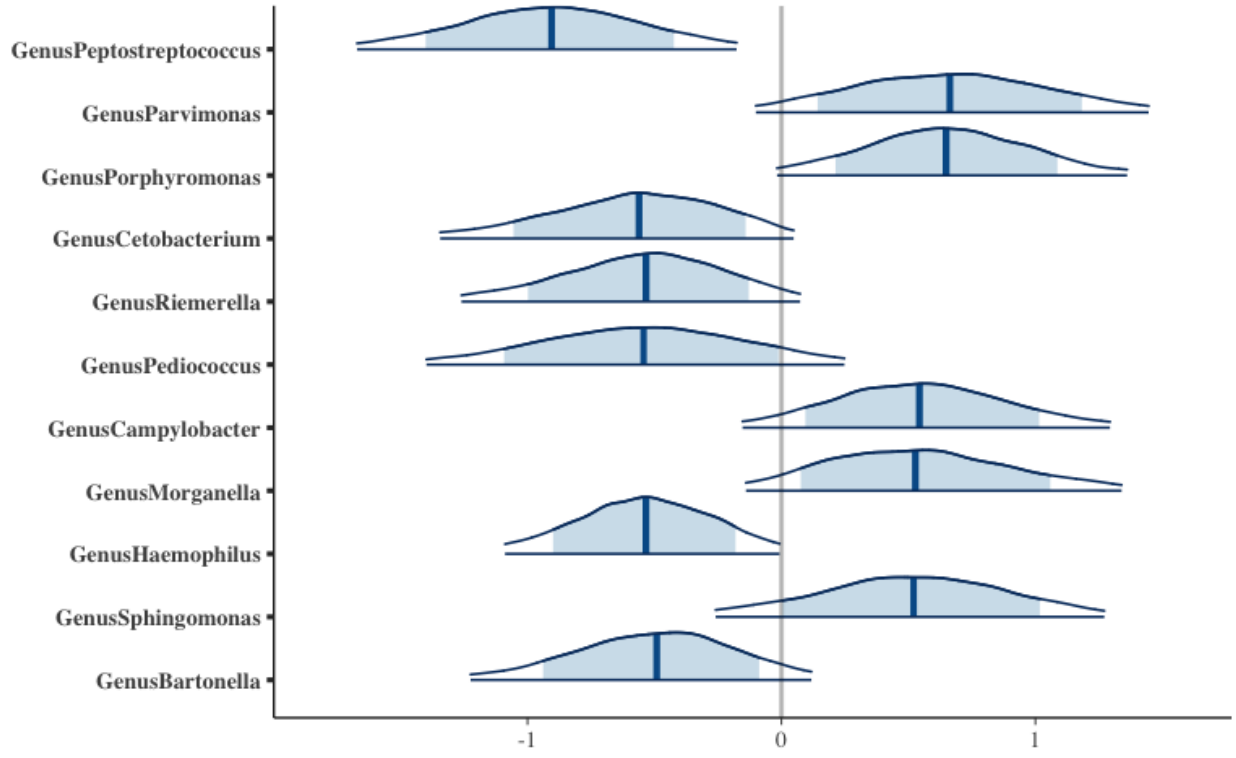

Figure 3: The posterior probability distributions for bacterial genera that are differentially abundant between males (left) and females (right). There was strong evidence for a greater abundance of *Peptostreptococcus* in males relative to females, as the 95% interval around the mean of the distribution does not contain zero. The other bacterial genera shown have distributions cross show moderate evidence of a difference in abundance between males and females.

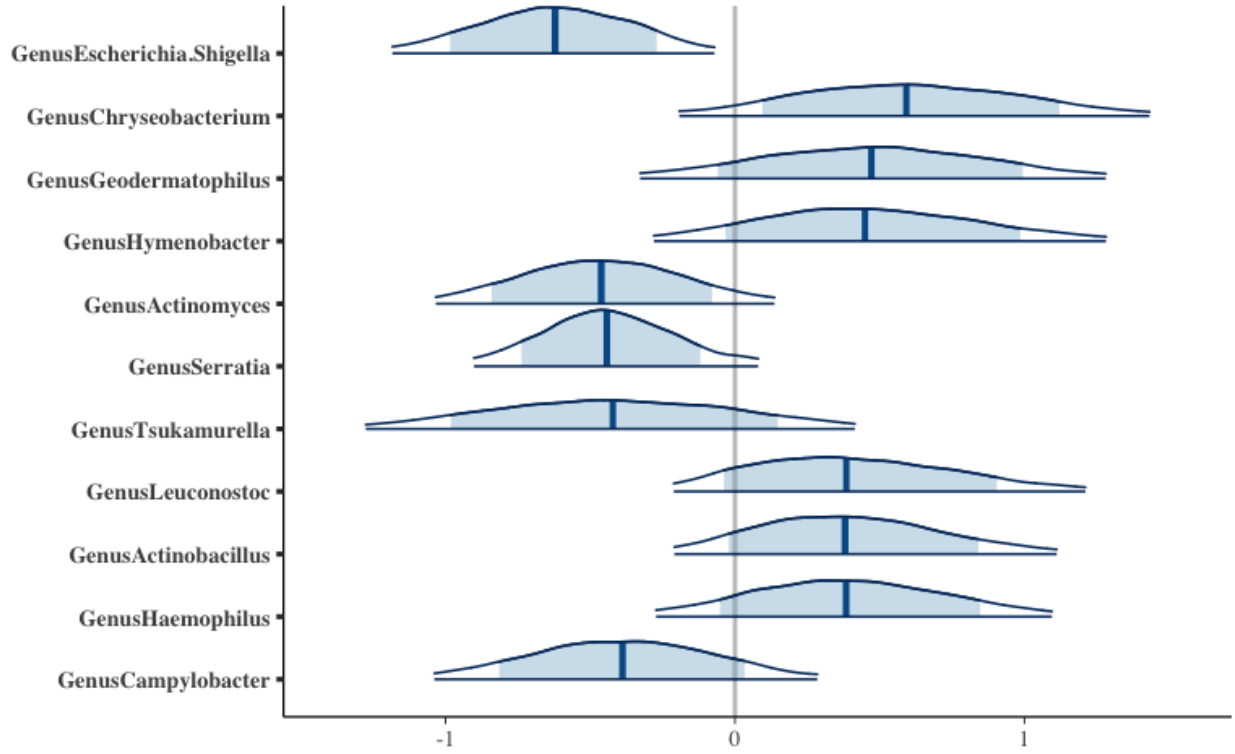

Figure 4: The posterior probability distributions for bacterial genera that are differentially abundant between subadults (left) and juveniles/adults (right). We have strong evidence for a greater abundance of *Escherichia/Shigella* in subadults relative to adults and juveniles, as 95% interval around the mean of the distribution does not contain zero. The other bacterial genera shown have distributions show moderate evidence of a difference in abundance.

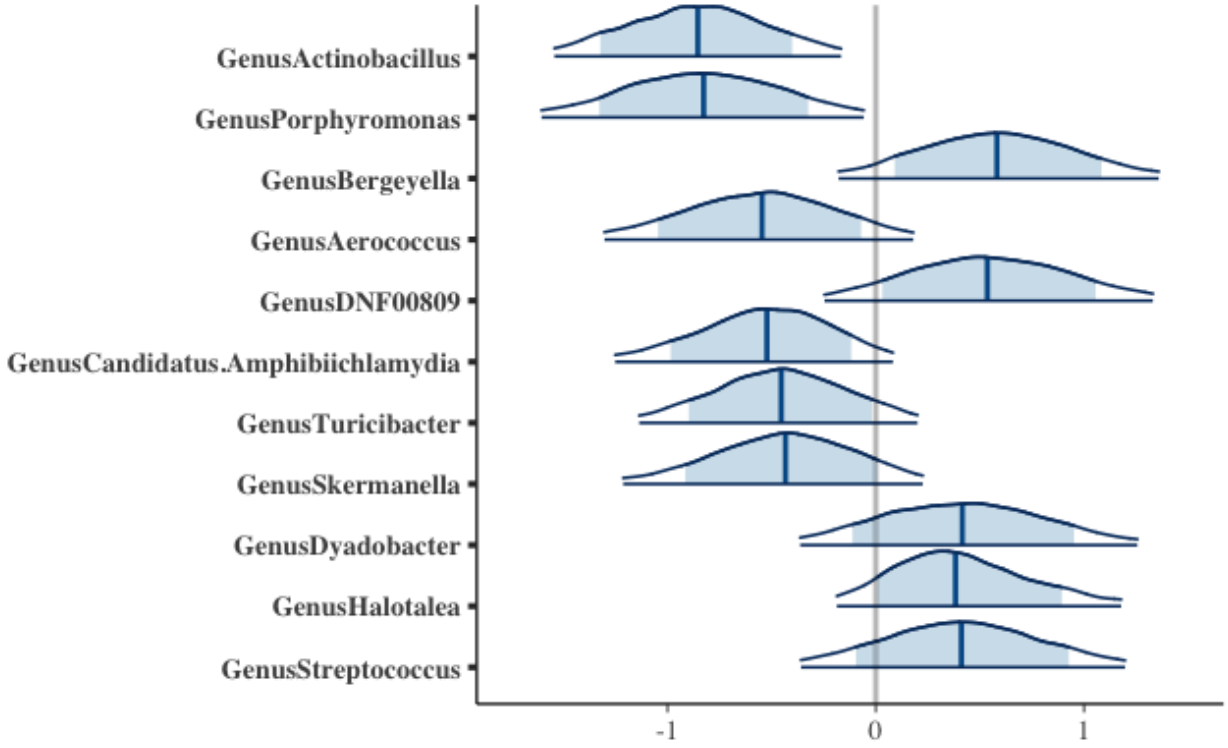

Figure 5: The posterior probability distributions for bacterial genera that are differentially abundant between lactating females (left) and all other females (right). There was strong evidence for a greater abundance of *Actinobacillus* and *Porphyromonas* in lactating females relative to all other females, as the 95% interval around the mean of the distribution does not contain zero. The other bacterial genera displayed have distributions cross zero and show moderate evidence of a difference in abundance between lactating females and all other females.

Ecological shifts in GIT microbiome alpha diversity and composition

There was strong evidence for a difference in the species richness and Faith's PD of the *P. alecto* GIT microbiome between summer and winter seasons (Table 3) but not Shannon diversity (Table 3, Figure 6). There is weak evidence for a difference in Shannon diversity, but not richness or Faith's PD, of the *P. alecto* GIT microbiome among years (Table 3). There was no difference in any alpha diversity metric between the GIT microbiomes of nomadic and resident *P. alecto* (Table 3). PERMANOVA results showed that there is strong evidence for a difference in the microbiome composition between nomadic and resident flying foxes (p=0.015) and among seasons (p=0.027, Figure 7). There was weak evidence of a difference in the microbiome composition between years (p=0.094). Together, nomadism, season, and year account for 1.4%, 2%, and 0.8% of the variation in the GIT microbiome compositional variation respectively.

Table 3: Results from multiple linear models with season, year, and site classification as factors and their effects on three alpha diversity metrics. P-values indicative of strong evidence are bolded and noted with an asterisk. P-values indicative of at least moderate evidence are bolded.

Alpha Diversity Metric	**Ecological Factor**	**Pairwise Comparison**	**P-Value**
'Species' (ASV) Richness df=171 F=1.873	Season	**Summer:Winter**	**0.019***
	Year	Year (2018-2020)	0.399
	Site classification	Nomadic:Resident	0.484
Shannon Diversity	Season	Summer:Winter	0.962

Table 3 Continued

df=171 F=1.459	Year	**Year**	**0.0629**
	Site classification	Nomadic:Resident	0.96
Faith's PD df=171 F=1.437	Season	**Summer:Winter**	**0.0424***
	Year	Year	0.6009
	Site classification	Nomadic:Resident	0.3871

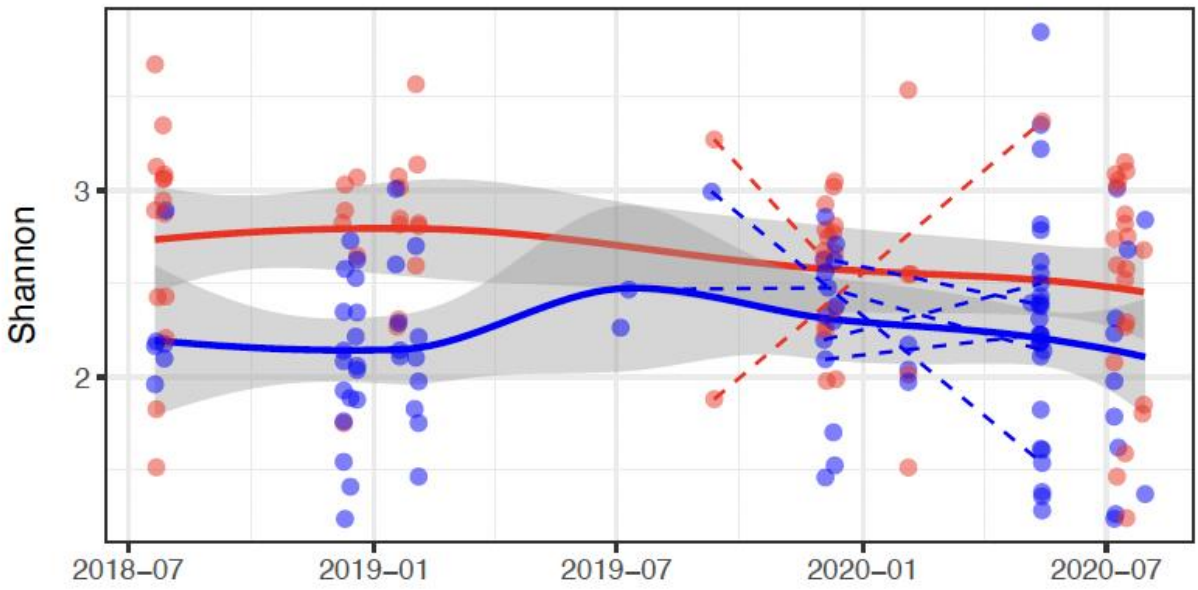

Figure 6: The change in Shannon diversity of sampled male (blue) and female (red) *P. alecto* GIT microbiome over time. Smoothed lines were added to highlight seasonal and annual variation. Dashed lines represent the Shannon diversity of recaptured bats at first and second/third time points.

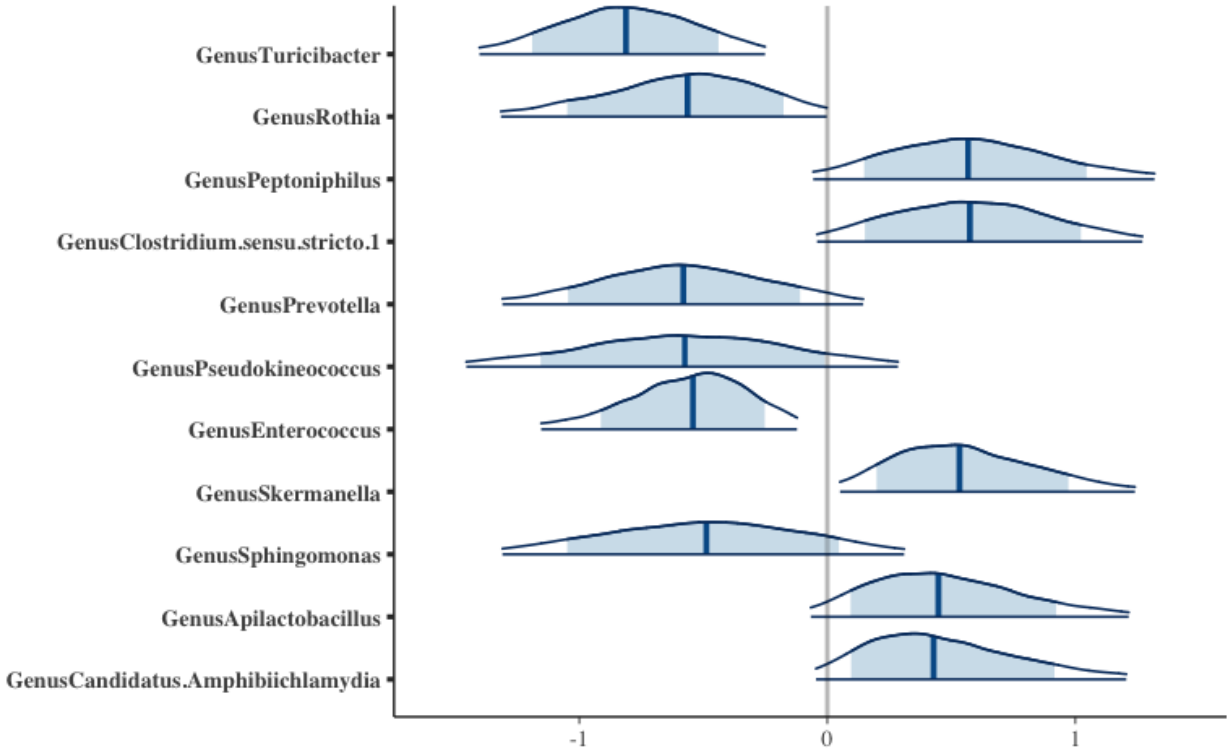

Figure 7: The posterior probability distributions for bacterial genera that are differentially abundant between winter (left) and summer (right) seasons. We have strong evidence for a greater abundance of *Turicibacter*, *Rothia*, and *Enterococcus* in winter and *Skermanella* in summer, as the 95% intervals around the means of these distributions do not contain zero. The other bacterial genera displayed have distributions which include zero but show moderate evidence of a difference in abundance between winter and summer.

<u>Short-term shifts in GIT microbiome diversity and composition of recaptured flying foxes</u>

All recaptured *P. alecto* were resampled within a year of initial tagging. There was high variation in how much alpha diversity (both Shannon and Faith's PD) changed among individuals (Fig. 8). Some *P. alecto* experienced dramatic changes (either increased or decreased alpha diversity) while other *P. alecto* GIT microbiome diversity remained somewhat constant,

though this apparent stability depended on the alpha diversity metric used. Differences in changes in Shannon diversity and Faith's PD indicate that there were shifts in microbial abundance that may or may not be closely related. There was no evidence that differences in microbiome composition within ($p=0.964$) or between ($p=0.733$) bats are any different (Figure 9). Neither bat identity nor capture point explained variation in the GIT microbiome.

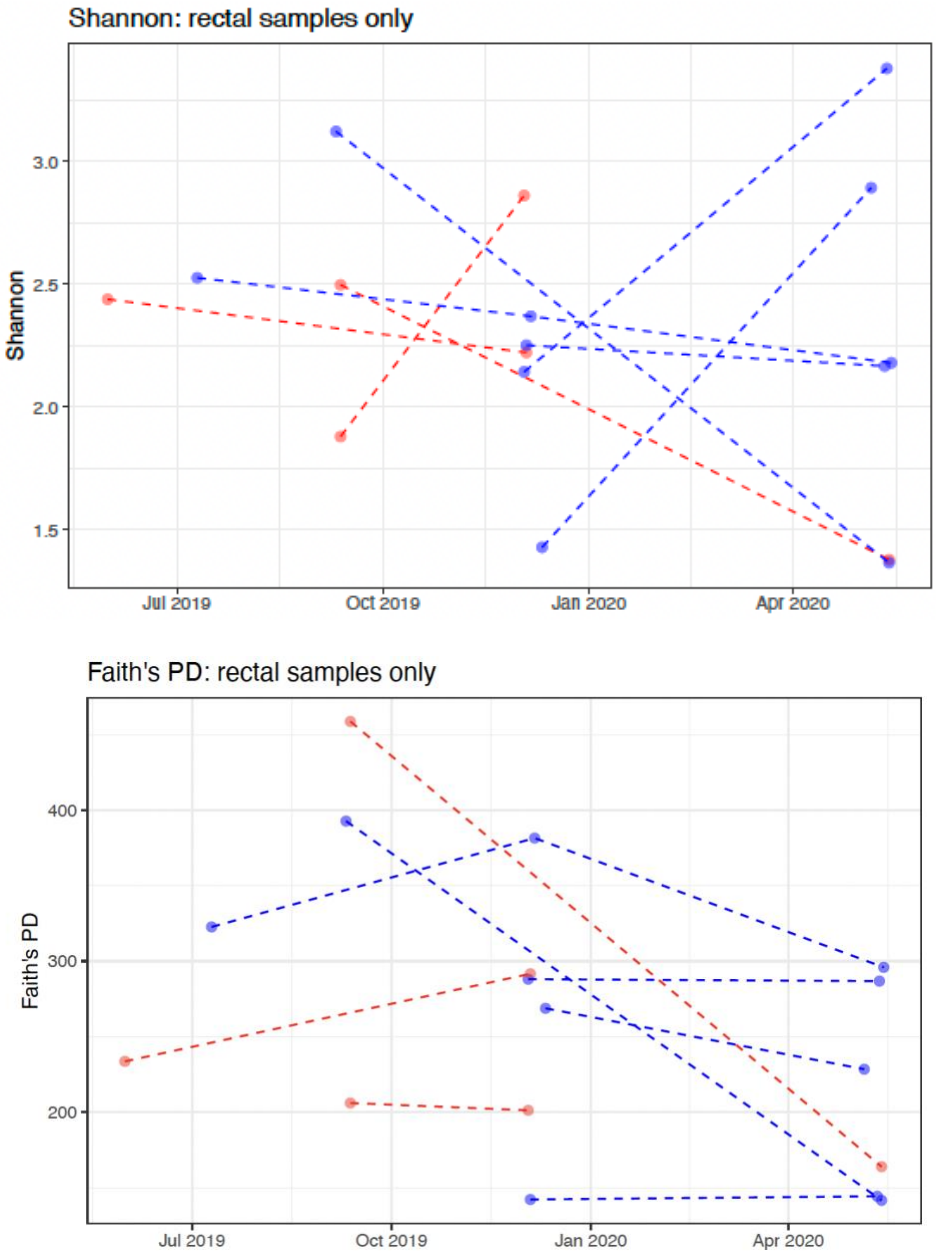

Figure 8: The changes in alpha diversity (Shannon and Faith's PD) of the recaptured bats' GIT microbiomes over time. Blue points are males and red points are females. Dashed lines track an individual's alpha diversity measurements at different time points.

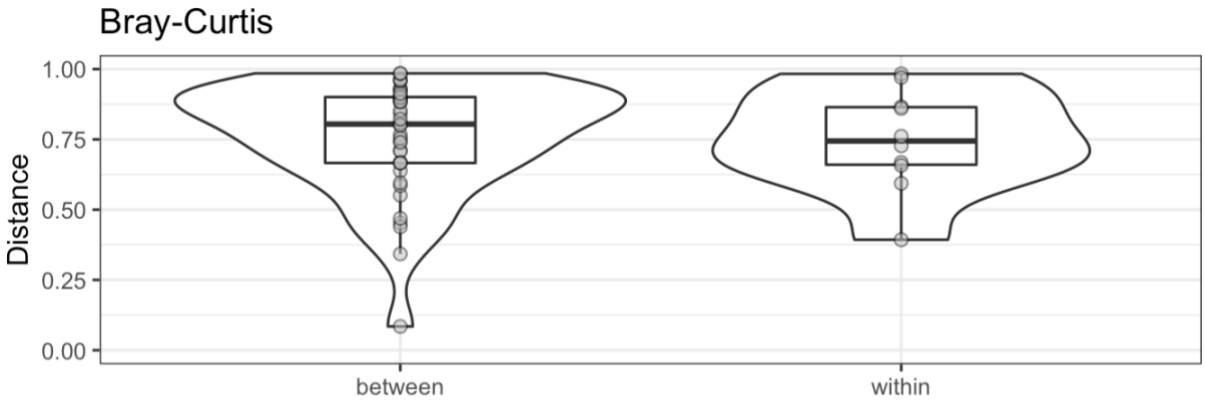

Figure 9**:** Pairwise comparisons of the between-bat beta diversity (where individual's GIT microbiome composition is compared to that of other bats) and within-bat beta diversity (where an individuals' GIT microbiome diversity is compared to its own sample at another time point). We looked at the between- and within-bat distances for each of the three beta diversity distances (Bray-Curtis)

Discussion

These results offer insight into the dynamics of the *P. alecto* GIT microbiome. We show that these flying foxes exhibit annual, seasonal, short-term, sex, age, and reproductive-related shifts in their GIT microbiome alpha diversity and composition. *Pteropus alecto* demonstrate high intra- and interindividual variation.

The differences in GIT microbiome alpha diversity in subadults (which are smaller than adults), but not juveniles or adults, is surprising and may be a reflection of foraging differences. Todd et al. (2022) showed differences in foraging strategies between larger- and smaller-bodied Christmas Island flying foxes (*Pteropus natalis*). Similarly, the lack of difference in alpha diversity between juveniles and adults is surprising. If *P. alecto* experience age-related successional changes in their microbiomes, we'd expect that as their diet becomes more adult-

like, their microbiomes would reflect this change. Consequently, we would expect juveniles' GIT microbiomes to be dissimilar to adults, and for the GIT microbiomes of sub-adults to be somewhere in between juveniles and adults. It should be noted that all bats used in our study (like Hughes et al., 2018) were volant and therefore at least partially weaned and thus, our study may not have captured very early GIT microbiome shifts when pups are dependent on their mothers for milk and do not yet consume adult foods. These age-related differences may play a role in satisfying the differing nutritional demands of these life stages (Amato et al., 2014).

While alpha diversity patterns were inconsistent, the composition of the GIT microbiomes of *P. alecto* is different among age classes even when controlling for sex and reproductive status and stratifying by site. Yin et al. (2020) showed that within the first month of birth, *Vespertilio sinesis* experienced no change in GIT microbiome alpha diversity. However, after the 5th week, alpha diversity increased and then finally stabilized around week 6. Thus, the lack of change in GIT microbiome alpha diversity in our study between juveniles and adults may be due to the fact that all juveniles were greater than six weeks old and volant. Though, we do not know if or when *P. alecto*, a much larger and distantly related species, may experience substantial shifts in their GIT microbiome with regard to weaning and volancy.

With regard to reproduction, there was no evidence to suggest a difference in bacterial species richness among reproductive classes. Taken together with high Shannon diversity and no difference in Faith's PD in lactating females, it is possible that certain closely related bacteria may have differential abundances. When accounting for sex and age, and stratifying by site, there was strong evidence for compositional differences among reproductive status. This is not surprising given that lactating females have high energetic needs relative to non-reproductive and

post-lactating bats (Millar, 1977). Thus, increased alpha diversity of the GIT microbiome may reflect greater foraging effort and perhaps necessary eclectic food choices of lactating bats, and may also contribute to nutrient and energy acquisition. For example, Phillips et al. (2017) showed that pregnant and lactating females have the greatest GIT microbiome diversity relative to other groups. Similarly, Gaona et al. (2020) showed reproductive differences in the GIT microbiome of a population of *Leptonycteris yerbabuenae*.

The strong evidence for a difference in species richness, Faith's PD, and composition between summer and winter seasons and a difference in Shannon's diversity among years indicates that there is substantial temporal variation in the *P. alecto* GIT microbiome. These results are similar to those found for other bat species (Gong et al., 2022; Víquez-R et al., 2021; Xiao et al., 2019). These seasonal shifts in diversity and composition may be in response to seasonal variation in food availability. Or these changes may be tied to reproduction as *P. alecto* reproduce once per year and have synchronous birth pulses in approximately October (Vardon and Tidemann, 1998).

The difference in GIT microbiome composition between nomadic and resident flying foxes may indicate that these bats' foraging strategies may play a role in shaping their GIT microbiomes; or that *P. alecto* may source many of their GIT microbes from their environment or that diet may play a large role in shaping the microbiome. Research in Australian shorebirds has shown that other than an increase in one bacterial genus (i.e., Corynebacterium), migratory birds generally maintained their GIT microbiome composition relative to residents (Risely et al., 2018). Risely et al. (2018) postulates that this maintenance may allow bird GIT microbiomes to recover after migration. Though it should be noted that these birds have consistent seasonal

migrations, whereas *P. alecto* are traditionally nomadic, not migratory, so their movements are less predictable (Welbergen et al., 2020). These compositional differences may be reflective of different foraging strategies as observed with changes in *L. yerbabuenae* microbiome diversity that was associated with more diverse diets (Víquez-R et al., 2021).

One surprising result of this study was the lack of differentiation of individual bats' GIT microbiomes over time relative to the microbiome of other bats. Some *P. alecto* individuals demonstrated dramatic short-term changes in GIT microbiome alpha diversity, whereas others remain relatively constant. However, this depends on the alpha diversity metric used, which may indicate shifts in abundance of microbial taxa that may or may not be phylogenetically distant. A lack of differences of within- and between-bat distances between GIT microbiome community composition indicate that an individual *P. alecto* GIT microbiomes were not more similar to themselves over time relative to other bats at the same time. If individuals retained stable microbiomes, we would expect that individual microbiomes would look more similar to themselves over time (and, thus, have a lower Bray-Curtis distance), rather than more similar to other bats (or rather, just as different as other bats). However, we found that Bray-Curtis distances within and between bats were not different. By contrast, Kolodny et al. (2019) showed that captive individual bat GIT microbiomes were more similar in composition to their own sample, than other bats at concurrent time points, thus indicating that there was some stability in the *R. aegyptiacus* (which are frugivorous Pteropodidae) GIT microbiome. However, these bats were from captive colonies and bat GIT microbiomes converge (i.e., become more similar to each other) in captivity (Edenborough et al., 2020; Y. Xiao et al., 2019), which may explain why we see different patterns in our sampled, wild-caught *P. alecto.* It is possible that these rapid

shifts in individual GIT microbiome diversity may be related to individual diets and food availability. To our knowledge, this study is the first to sample the GIT microbiome of wild recaptured bats.

Most bacterial genera present in our samples were shared by only a few bats. The rapid decline of bacterial genera shared by *P. alecto* and the high proportion of singletons indicate that only a small portion of the GIT microbiome is shared among individuals. Many potential zoonotic pathogens (i.e., *Streptococcus, Gemella, Aerococcus*) in the core microbiome of *P. alecto* may contribute to nutrient and/or energy acquisition and are likely tolerated by hosts. Many bacteria within these frequently detected genera (e.g., *Streptococcus, Actinomyces, Fusobacterium*) have flexible metabolic capacities and are able to use a number of different substrates (Takahashi and Yamada, 1999, Rogers et al., 1991).

There were some limitations to our study. Because we used 16S sequencing (as opposed to whole genome sequencing), we were unable to distinguish further turnover within microbial taxa, which may or may not impact the microbiome function. Future studies should aim to assess functional changes via metabolomics in the GIT microbiome of wild recaptured bats. These studies could be used to identify thresholds for transitions to different states (i.e., microbial communities) that result in tipping points in health outcomes.

Because *P. alecto* GIT microbiomes change over a short period of time, it is important to identify the mechanisms underlying these shifts. These fluctuations could be normal and beneficial to the host. Plasticity in the GIT microbiome may be in response to external stimuli and may help animals cope with change in their environments (Alberdi et al., 2016). Stable and consistent changes in the GIT microbiome may be indicative of beneficial changes responding to

host physiology or natural environmental fluctuations (Risely et al., 2018). By contrast, rapid shifts in GIT microbiome composition and diversity may have negative implications such as increased susceptibility to environmental changes which may consequently push the GIT microbiome into a state of dysbiosis (Víquez-R et al., 2021; Risely et al., 2018). Furthermore, irregular changes in the GIT microbiome may be a result of stress or potentially leave hosts more susceptible to infection (Risely et al., 2018).

Alternatively, these changes may be neither beneficial nor detrimental, but rather stochastic. We show that *P. alecto* GIT microbiomes exhibit substantial variation across scales and high degree of inter- and intra-individual variation, indicating that *P. alecto* GIT microbiomes are highly dynamic over time. In combination with the lack of a high proportion of shared GIT microbes, these results support a theory of neutrality of the GIT microbiome as opposed to niche-assembly (Hubbell 2001). Future work should incorporate explicit tests of neutrality to determine if these microbial communities inhabiting bat GITs are random assortments or if they are functionally important. These tests will be crucial for understanding the extent to which the GIT microbiome functionally contributes to bat health.

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CHAPTER FOUR

NATIVE DIETS ARE ASSOCIATED WITH LOW ALPHA DIVERSITY AND COMPOSITIONAL CHANGES IN THE BLACK FLYING FOX (*PTEROPUS ALECTO*) GASTROINTESTINAL MICROBIOME

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Abstract

Gastrointestinal (GIT) microbiota contribute to host energy and nutrient acquisition. Food digested by the host becomes the substrate used by GIT microbes, thus diet can impact bacterial dynamics. Land-use change in Australia has altered habitat and resource availability for black flying foxes, *Pteropus alecto,* and consequently, their diet composition. We compared the microbiome of animals that consumed a diet of native plant species with those consuming a diet consisting of introduced plant species. We captured *P. alecto* across five sites in the greater Brisbane area. From each bat, we collected rectal swabs for microbiome analysis and feces for molecular dietary analyses. We found strong evidence for an association between the proportion of the diet that was identified as native and the Shannon diversity and composition of the *P. alecto* GIT microbiome. We identified six bacterial genera (*Mageeibacillus, Mannheimia, Enterococcus, Helococcus, Atopobium*, and *Clostridium*) that were more abundant in the GIT microbiome of *P. alecto* consuming a mostly native (>50%) diet relative to *P. alecto* whose diets consisted mostly of cultivated, introduced, and other broad dietary items that could not be specifically categorized. The GIT microbiomes of *P. alecto* that were not consuming a mostly native diet were enriched with 5 bacterial genera: *Aerococcus, Leptotrichia, Kocuria, Hymnobactre,* and *Methylocella*. These results indicate that the *P. alecto* GIT microbiome may be plastic in response to different diets. Plasticity in the GIT microbiome may help these bats cope with changing resource availability.

Introduction

Bats represent approximately 20% of all extant mammals (https://www.batnames.org) and are an interesting and useful system in which to investigate gastrointestinal (GIT) microbiome dynamics as they exhibit diverse feeding strategies including carnivory, insectivory, sanguivory, frugivory, nectarivory, and omnivory (Ingala et al., 2018a; Kunz et al., 2011). Patterns of alpha diversity within the bat GIT microbiome deviate from those found in other mammals. Generally, the GIT microbiomes of bats harbor lower diversity than other mammals (Nishida and Ochman, 2018) and are dominated by the bacterial phyla Proteobacteria and Firmicutes (Banskar et al., 2016; Carrillo-Araujo et al., 2015; Dietrich et al., 2017b; Fofanov et al., 2018; Ingala et al., 2018b; Li et al., 2018; Nishida and Ochman, 2018; Song et al., 2020; Vengust et al., 2018) regardless of diet, whereas most mammalian GITs are primarily colonized by Bacteroidetes and Firmicutes (Nishida and Ochman, 2018). Ley et al. (2008) found that GIT microbial diversity in mammals decreases as dietary strategies shift from herbivory to carnivory. By contrast, bats that primarily exhibit herbivorous feeding strategies have lower microbial diversity within the GIT than bats exhibiting insectivorous, sanguivorous, or omnivorous feeding strategies (Banskar et al., 2016; Carrillo-Araujo et al., 2015; Ingala et al., 2018b; Li et al., 2018) regardless of phylogenetic and geographic separation.

Gastrointestinal microbiomes of bats are associated with dietary guild (Carrillo-Araujo et al., 2015; Ingala et al., 2021, 2018b; Lutz et al., 2019) and have likely co-evolved with bats to contribute to nutrient and energy acquisition. For example, the vampire bat (*Desmodus rotundus*, family Phyllostomidae) GIT microbiome is enriched with bacterial genes with functions that help overcome the nutritional challenges of sanguivory, indicating that the vampire bat host and GIT

microbiome work together as a holobiont for optimal functioning (Zepeda Mendoza et al., 2018). Similarly, bacteria isolated from the frugivorous Indian flying fox, *Pteropus giganteus*, have cellulolytic and xylanolytic properties that may aid in the digestion of plant-based polysaccharides (Prem Anand and Sripathi, 2004). Frugivorous bats have significantly different predicted microbiome functions compared to insectivores, carnivores, and sanguivores (Ingala et al., 2021). Even bat species within the same dietary guild may have different GIT microbiome compositions, but the predicted function of those microbes is maintained (Phillips et al., 2017). However, it should be noted that predicted functions based on 16S sequences are notoriously inaccurate for non-human samples (Sun et al., 2020).

Bat GIT microbiomes can also change in association with shifts in ecology. When bats' diets change substantially (e.g., when the great evening bat, *Ia io*, transitions from insectivory to avivory seasonally), their GIT microbiomes change accordingly, both in composition and predicted function (Gong et al., 2021). Additionally, habitat fragmentation is associated with shifts in diet (from low to high livestock concentrations) and increased variability in the GIT microbiomes of vampire bats (*Desmodus rotundus*; Ingala et al. 2019)– indicating that increased consumption of non-native dietary taxa alters the GIT microbiome of these bats. Thus, diet likely plays a large role in shaping bat GIT microbiomes. As increasing land-use change continues to alter wildlife habitat (Ellis and Ramankutty, 2008), it is important that we understand the mechanisms and functional implications of altered GIT microbiome.

Australian *Pteropus* species feed on blossom (such as Eucalyptus, Angophora, Melaleuca, and Banksia), native and introduced fruits, and occasionally leaves and bark (Parry-Jones and Augee, 1991) (Ratcliffe 1932, Parry-Jones 1993). These *Pteropus* spp. eat a

nonrandom subset of fruits and some species, (i.e., *P. poliocephalus*) are considered to be "sequential specialists" that will favor certain plant species when many are available (Banack, 1998; Parry-Jones and Augee, 1991). For example, they prefer Myrtaceae and Banksia pollen, then over-ripe commercial crops like stone fruits (Parry-Jones and Augee, 1991). Samoan *Pteropus* spp. have been shown to prefer fruits from native forests rather than agroforest (Banack, 1998). Fruits from agroforest are consumed more frequently when resources in primary forest are low (Banack, 1998) and these non-native foods likely do not meet the nutritional needs of *Pteropus* (Pulscher et al., 2021). Because native trees have irregular and unpredictable flowering cycles (Nelson 1965) there is high variation in *Pteropus* diet within and among years (Parry-Jones and Augee, 1991). Consistent low availability of nectar in August and September coincides with peak availability of cultivated foods (Hawkins et al., 2018). Clearing of flying fox habitat has resulted in the extended occupation of urban camps (Connell et al., 2006; Parry-Jones and Augee, 1991; Puddicombe 1981; Eby et al. in review). As land use change is decreasing the amount of available habitat for flying foxes, and altering their dietary composition with increasing urbanization, we aim to determine how different diets affect the *P. alecto* GIT microbiome.

If *P. alecto* GIT microbiomes contribute to energy or nutrient acquisition, fluctuations in their GIT microbiome may be a result of a flexible response to different food sources. We determined whether there were associations between the proportion of the *P. alecto* diet that is native and the alpha and beta diversity (which respectively describe the within-bat diversity and the community composition relative to other bats) of the GIT microbiome. We hypothesized that *P. alecto* consuming a diet with a higher proportion of native plants will have decreased GIT

microbiome diversity, because their diet contains a lower diversity of plant species; and we hypothesize that *P. alecto* consuming a higher proportion of introduced species will have higher GIT microbiome diversity to reflect the higher diversity of their diet. We also predict that microbial composition is associated with the proportion of diet that is native.

Methods

Capture of P. alecto and field collection of samples

We captured *P. alecto* at five sites across the greater Brisbane area in southeast Queensland, Australia from December 2018 to July 2020 (MSU IACUC #201750 and Griffith University Animal Ethic Committee ENV/10/16/AEC). Bats were captured pre-dawn with mist nets and placed into pillowcases for holding until processing (within 6 hours of capture). Before processing, bats were anesthetized with an initial dose of gaseous 5% isoflurane then reduced to ~1.5% once fully anesthetized. We took rectal swabs using a sterile cotton swab collected into 500-750 μL of 70% ethanol for microbiome analysis. If the bat defecated during processing, we collected the feces into a small plastic resealable bag using a sterile swab. If no fresh feces produced under anesthesia, we checked the holding pillowcase for feces and used a sterile swab to collect fecal material into a small plastic resealable bag. All samples were placed on ice or in liquid nitrogen then stored in a -80ºC freezer until processing. All bats were given fluids (one part 2.5% glucose and 0.45% NaCl solution and one part Hartmann's solution) subcutaneously at 5-10% of total body weight and monitored for at least a half hour before release back into their roost.

Lab methods for microbial DNA extraction and sequencing

In preparation for DNA extraction from the rectal swabs, we evaporated the ethanol from the field tubes using an Eppendorf Concentrator 5301 at 60°C for approximately 30-45 minutes until dry. We extracted DNA from the rectal swabs using the ZymoBIOMICs DNA Miniprep Kit (D4300). ZR BashingBeads and ZymoBIOMICS Lysis Solution were added directly to the dried field tube containing the rectal swab. We then used a Mini-Beadbeater-16 (Biospec Products) at 3,500 oscillations per minute to bead-beat samples for 1 minute and a wait period of 5 minutes, repeating this beat-wait cycle for a total of five cycles. We followed the rest of the protocol for the extraction kit and used 100uL of DNase-free water to elute the DNA in the final step. We confirmed the presence of bacterial DNA in each sample with a polymerase chain reaction (PCR) using the primer set 27F/1492R (Frank et al., 2008) to amplify an ~1,500bp section of the microbial 16S rRNA gene and visualize the bands with gel electrophoresis. We sent samples to the University of Michigan Microbiome Core to sequence the V4 region of 16s rRNA gene with Illumina MiSeq according to (Kozich et al., 2013).

Lab methods for plant DNA extraction and sequencing

Field samples of feces were subsampled to 100 mg and frozen in a liquid nitrogen bath for 3 minutes (Wilson et al., 2021). Then, we added approximately 0.06 g of 0.1 mm silica beads and six to eight 2.3 mm silica beads to the tube with feces to assist with disruption and lysing (Wilson et al., 2021). Samples were then lysed (TissueLyser II, Qiagen) at 30 Hz for six minutes in two intervals (Wilson et al., 2021). We extracted DNA from the feces using the Qiagen DNeasy Plant Kit following the manufacturer's protocol except at step 5, eluting twice per sample in 50 μL of elution buffer.

We targeted the variable regions of the ITS gene using the primer pair ITS-S2F (Chen et al., 2010) and ITS4R (White et al. 1990), resulting in amplicons approximately 341 nucleotides in length. All primers were purchased from SIGMA Aldrich with HPLC purification. Polymerase chain reactions were used to amplify target regions of DNA using the following conditions: heat activation at 95˚C for 3 min, followed by 35 cycles of denaturation at 95˚C for 30 sec, annealing at 54˚C for 30 sec and elongation at 72˚C for 1 min, followed by a final extension at 72˚C for 10 min, holding at 10˚C until storage. Each reaction contained 5µL NEBNext® Ultra™ II Q5® Master Mix (New England BioLabs), 0.4 µL each of forward and reverse primer (from 10 µM aliquots), and 4.2 µL of DNA isolate, for a total volume of 10 µL. Polymerase Chain Reaction (PCR) product was visualized on a 1% agarose gel, using 2 µL of product and 4 µL of dye per sample. Each sample was amplified via PCR in triplicate then pooled to get the best representation of plant DNA and reduce PCR bias (Wilson et al., 2021). We then used the Invitrogen SequalPrep Normalization Kit to normalize amplicon concentrations between samples and remove any primer dimers in preparation for sequencing. After normalization, we combined all amplicons into a 2 mL tube prior to sequencing. We quantified pooled amplicons using a Qubit 3.0 and Agilent Bioanalyzer to determine the concentrations (ng/µL) and lengths (bp) of DNA. We denatured and diluted the resulting library and used the Illumina Miseq Reagent Kit v2 (500 cycle) for sequencing (Kozich et al., 2013).

Preliminary processing of 16S rRNA gene sequence data

We used R (v4.1.2, R Core Team 2021) for all bioinformatics and analyses. We used DADA2 (Callahan et al. 2016) to filter, trim (to 220bp size), assess sequencing error rates, remove chimeras, and form amplicon sequence variants (ASVs). To taxonomically assign ASVs,

we used SILVA (nr99_v1381.1, Quast et al. 2013) followed by *phyloseq* (McMurdie and Holmes 2013) and the frequency approach in *decontam* (Davis et al 2017) to identify and remove contaminants. After removing mitochondrial and chloroplast sequences, we calculated relative abundance of each ASV and we then used a 0.25% cutoff (Reitmeier et al., 2021) to remove any ASVs that were not present in at least 0.25% abundance in any sample.

Bioinformatics pipeline to process plant DNA sequences and identify species status

We processed metabarcoding sequences for quality using VSEARCH 2.14.2 (Rognes et al. 2016) following the pipeline of Wilson et al. (2021). We joined forward and reverse reads where possible to maximize accuracy plant identification while minimizing sequence errors. Otherwise, we used only the forward read, truncated at maxEE < 1. Sequences that were less than 200 bp in length or low quality (maxEE > 1) were removed. We then dereplicated, denoised, and clustered the remaining reads as ASVs. To classify ASVs, we used VSEARCH using global alignments and best hit selection (greater than or equal to 97% identity) against three reference databases: a local reference database of plants known to be in the Australian *Pteropus* diet (as compiled by M.K. Kessler, unpublished), a Queensland database of plants listed as occurring in Queensland according to the Atlas of Living Australia (https://www.ala.org.au), and global ITS2 plant database, all created with the bcdatabaser tool (Keller et al., 2020). Any sequences that were either classified as a hybrid or as taxa that are unlikely to be in the study area, were manually checked against the ALA database (https://www.ala.org.au/) and reassigned to the next highest taxonomic rank.

We mapped the quality filtered reads to ASVs to generate count data that was then analyzed using R (version 4.1.2) with the *phyloseq* package (McMurdie and Holmes, 2013). We

removed only one sample with a lower number of reads than the negative control (340 reads) and filtered out potential contamination based on negative controls from sequencing (Wilson et al., 2021). Any taxa that made up less than 1% of a sample were also filtered out. As a third filtering step, we manually checked and removed any plants that were highly suspect such as ground-dwelling weeds (where flying foxes would not likely be foraging) and were expected to represent contamination introduced during sample collection or processing. We calculated relative abundance for each ASV in each sample (Keller et al., 2020).

We classified each plant taxa as native (to Queensland and New South Wales), cultivated fruit, or introduced. If we could not identify the plant taxa to species level, we identified the taxa to the next lowest level possible. For some plant taxa, identification at the genus level or higher was not sufficient to be assigned to any of the three aforementioned categories. Therefore, we designated these taxa as "broad genus." For each fecal sample (i.e., each bat) we then determined the portion of the diet to each of these four categories.

Statistical methods

We used *vegan* (Oksanen et al. 2020) and *picante* (Kembel et al. 2010) to calculate two different alpha diversity metrics (Shannon's diversity and Faith's Phylogenetic Diversity; PD) of the bacterial ASVs for each rectal sample. Shannon's diversity metric takes the richness and evenness of each ASV into account, whereas Faith's PD takes phylogenetic relatedness of the bacterial taxa into account, but not abundances. To assess the effect of diet taxa composition on microbiome diversity, we used two separate multiple linear models (one for each alpha diversity metric) using microbiome alpha diversity as the response variable and proportion of the *P. alecto* diet that is identified as native. We then used the *vegdist* function to calculate the Bray-Curtis

distances among microbiome communities and used the ‘adonis’ function in *vegan* (Oksanen et al. 2020) to perform a PERMANOVA using the distance matrix of the ASVs as a response variable and the classifications of the diet proportions as independent variables. We repeated these methods to assess the strength of evidence of an association between the number of taxa detected and identified in the diet of *P. alecto* with their GIT microbiome alpha diversity and composition (beta diversity).

To determine which bacterial genera were differentially abundant between *P. alecto* consuming mostly (>50%) native versus those consuming mostly non-native foods, we fit a Bayesian regression model with a Bernoulli response distribution using Hamiltonian Monte Carlo as implemented in Stan (Stan Development Team 2022). Three chains were run for 4,000 iterations after 2,000 burn-in iterations. Convergence was checked using trace plots and R_hat values (RStan, Stan Development Team 2022). For all effect estimates a normal prior distribution with a mean of zero and standard deviation of 0.5 was used. All relative values were scaled to a mean of zero and a standard deviation of 1 before model fitting.

Results

Microbiome and diet results

From a total of 175 captured *P. alecto*, rectal swabs had a total of 6,377,434 reads with a mean of 27,607 reads per sample before filtering. After processing, we were left with a total of 5,366,110 reads across all samples and a mean of 23,229 reads per sample. This resulted in a total of 797 ASVs identified. Of these 175 bats, 101 *P. alecto* individuals, also had diet data from corresponding fecal samples. Before filtering, we detected a total of 360 plant taxa in the *P. alecto* fecal samples. After filtering, samples averaged 13,291 reads and we were left with 299

total plant taxa detected, with a minimum of one plant taxa found per sample up to 18 plant taxa per sample (mean = 6.26 plant taxa/sample). There was high variation in the proportion of each plant type consumed by individual *P. alecto* (Fig. 1).

The GIT microbiome relationship to diet

Shannon diversity of the GIT microbiome was negatively associated with the proportion of native diet (p=0.036, Fig. 2), but Faith's PD provided moderate to no evidence of an association with native diet (p= 0.11). There was no evidence to support that Shannon diversity (p=0.7) or Faith's PD (p=0.3) was associated with the number of plant dietary taxa. There is no evidence for a difference in the GIT microbiome composition with regard to the number of plant taxa in the diet (PERMANOVA; p=0.33), but there was strong evidence for a difference in the GIT microbiome composition with regard to the portion of the diet that is native (PERMANOVA; p=0.003).

There was strong evidence to support enrichment of *Aerococcus* and moderate evidence to support enrichment of *Leptotrichia, Kocuria, Hymnobactre*, and *Methylocella* in the GIT of *P. alecto* consuming non-native diets. By contrast, there was moderate evidence to support the enrichment of *Mageeibacillus, Mannheimia, Enterococcus, Helococcus, Atopobium*, and *Clostridium* in the GIT of *P. alecto* consuming mostly native diets.

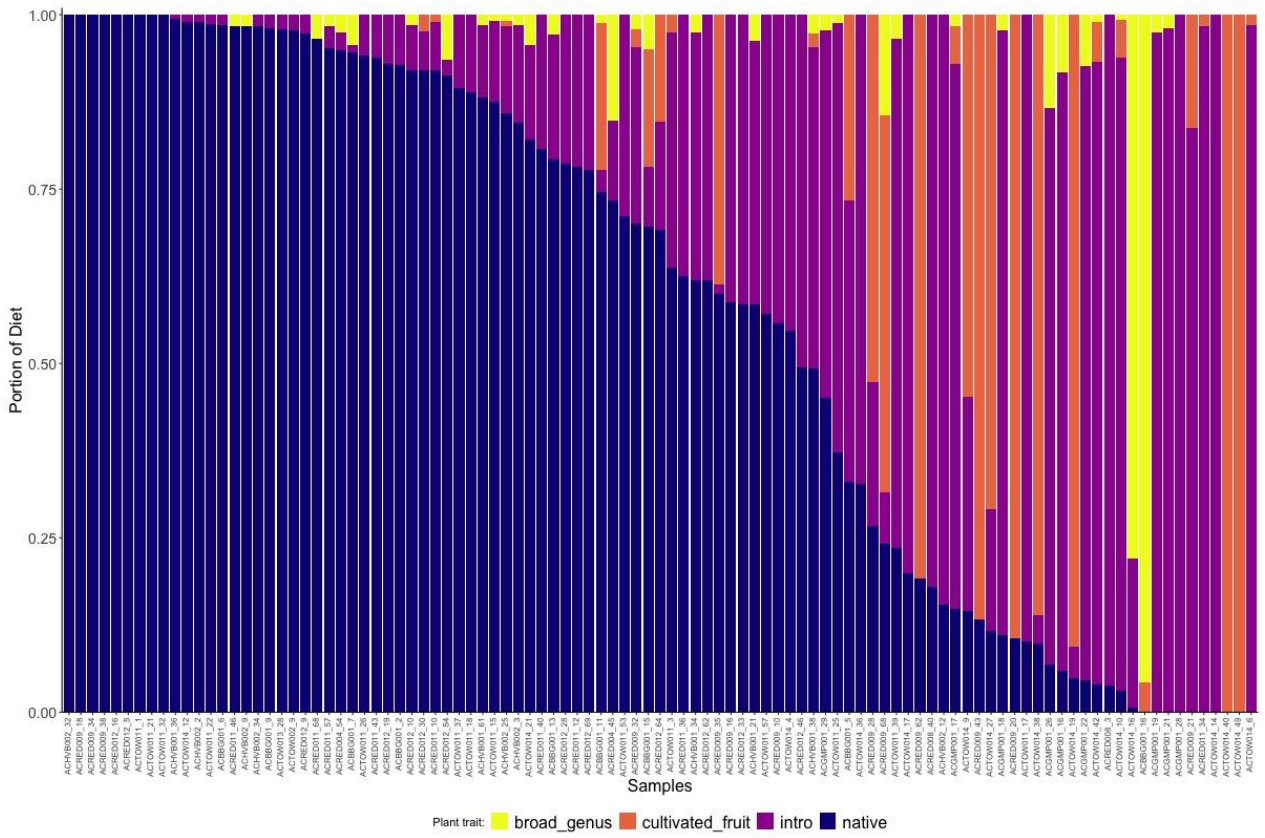

Figure 1: Stacked bar plot shows the proportion of plants identified as native, introduced (intro), cultivated (cultivated_fruit), or broad (broad_genus) in *P. alecto* fecal samples.

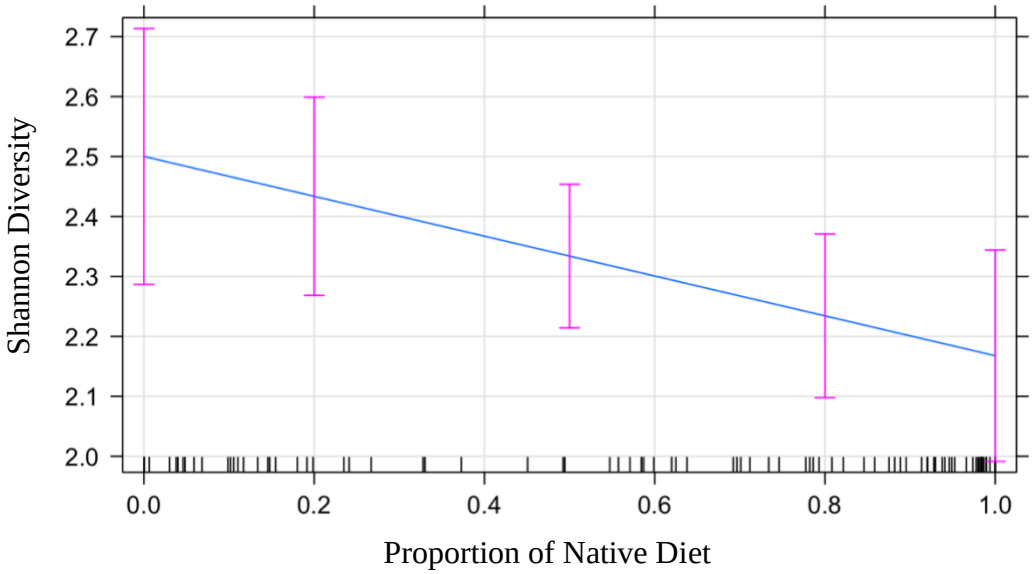

Figure 2: Effect of increasing proportions of native diet on the Shannon diversity of the GIT microbiome. The blue line shows a negative correlation between proportion native diet and GIT microbiome Shannon diversity. Tick marks along the bottom of the plot indicate the presence of samples with that portion of native diet.

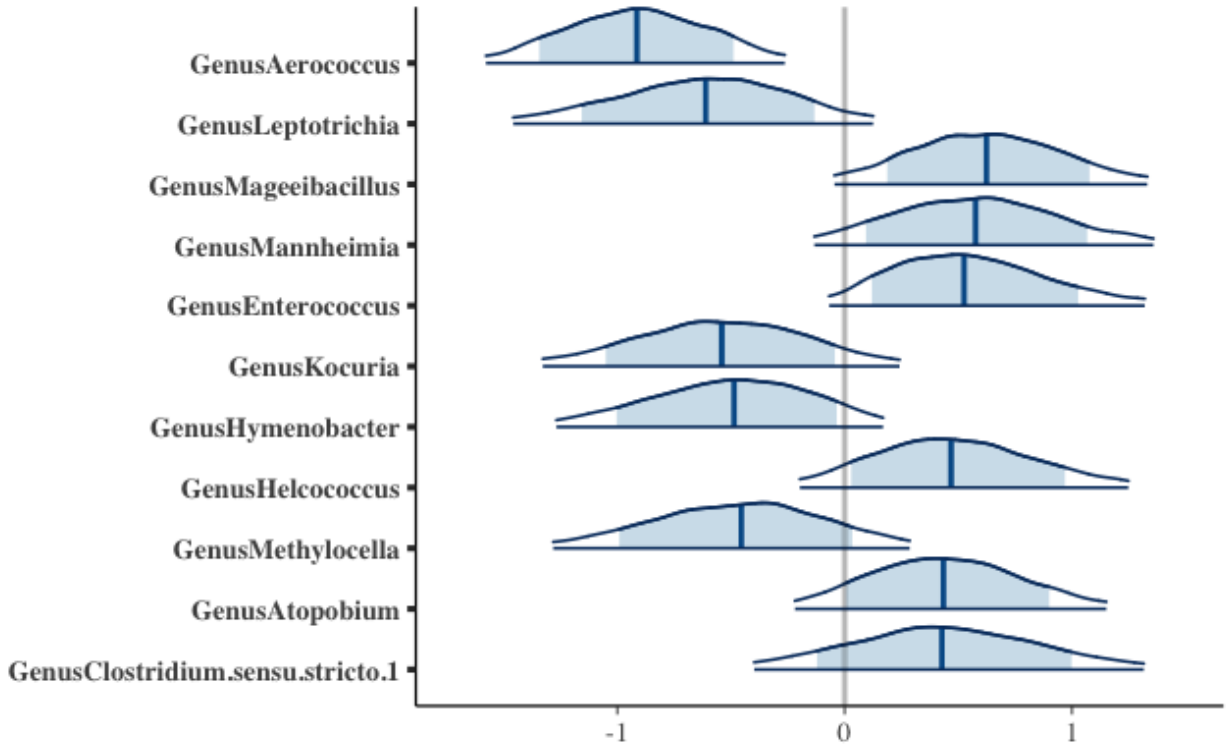

Figure 3: Posterior probability distributions for bacterial genera that are differentially abundant between bats consuming a mostly non-native diet (left) and bats consuming a mostly native diet (right). There is strong evidence for a difference in the abundance of *Aerococcus* in bats consuming a mostly non-native diet as the 95% interval around the mean of the distribution does not contain zero. For the other bacterial genera shown, there is moderate evidence of a difference in abundance.

Discussion

As GIT microbiota may contribute to host energy and nutrient acquisition, changes in diversity and composition of microbiota driven by changes in diet may impact the healthy functioning of individuals. Land-use change has altered habitat and food resource availability for Australian flying foxes, yet how flying fox GIT microbiomes respond to changes in diet is unknown. We demonstrate that Shannon (alpha) diversity of GIT microbiota decreased with increasing proportions of native plants in the *P. alecto* diet, along with changes in composition (beta diversity), potentially indicating that diet may play a role in shaping the GIT microbiome.

Some of the bacteria that are more abundant in bats consuming a mostly native diet may contribute to bat health. For example, *Enterococcus*, may aid in protecting the GIT microbiome through the production of bacteriocins– small peptides with antimicrobial properties (Silva et al., 2012). *Clostridium* is associated with substrate use, hydrogen production and the formation of soluble metabolites (Yang and Wang, 2019), which may also protect the GIT from invaders. Though some *Clostridium* can be pathogenic when overabundant, this bacterial genus is important for maintaining GIT function. Clostridium can produce butyrate (Pryde et al., 2002) which power colonocytes and can also inhibit NF-kB (an immunological biomarker of inflammation), thus leading to decreased expression of proinflammatory cytokines (Segain et al., 2000). *Mageeibacillu*s is in the family *Ruminococcaceae*, which are known to produce short chain fatty acids (SCFAs; Flint et al., 2008). *Mannheimia, Helococcus,* and *Atopobium* species have pathogenic potential (De Backer et al., 2010; Peel et al., 1997; Frank et al., 1996). Without whole genome sequencing, transcriptomics, metabolomics, or experimental approaches, it's difficult to pinpoint the role of each of these differentially abundant bacteria.

Decreasing Shannon diversity (alpha diversity) and changes in composition (beta diversity) associated with increasing proportions of native plants in the *P. alecto* diet indicate that diet may play a role in shaping the GIT microbiome. Perhaps this changing microbiome is helping the flying foxes adapt to introduced foods as their native food becomes less prevalent. However, we did not detect the same patterns with Faith's PD, indicating that microbial abundances and composition are shifting, but there are no major changes in the phylogenetic relatedness of these microbes or the number of microbial taxa in the GIT. *Pteropus* spp. have rapid GIT transit times and do not consume much fiber potentially negating the need for high microbial diversity to process their food (Jones et al., 2022; Kunz et al., 2011). In other mammals, GIT microbiome alpha diversity is strongly associated with diet. Western lowland gorilla (*Gorilla gorilla gorilla*) GIT microbiomes are distinguished by feeding behaviors and consumption of different food types (i.e., varying fiber content in fruits from dry and wet seasons; Gomez et al., 2015). In humans, decreased diversity of the GIT microbiome is associated with a western diet (high sugar and fat, low fiber, usually processed), whereas foods that are generally considered to be healthy (e.g., raw fruits and vegetables, fish) are associated with higher GIT microbiome alpha diversity (Partula et al., 2019). Given high alpha diversity is associated with increased fiber intake in humans and many other mammals, perhaps it is not surprising that low microbial Shannon diversity in *P. alecto* GIT microbiome is associated with high portions of native diet as nectar has a high sugar content and is devoid of fiber, whereas bats consuming fruits ingest small amounts of fiber and sometimes seeds. Perhaps for *P. alecto,* low GIT microbiome diversity is a "normal" or healthier state, though more research is needed.

Low GIT microbiome diversity associated with a native diet may be due to anti-microbial properties of nectar (Sasu et al., 2010; Schmitt et al., 2021; Vannette and Fukami, 2016). Nectar is a nutrient-rich solution that attracts and rewards pollinators. To counteract unwanted microbial growth, nectars often contain antimicrobial compounds (i.e., proteins, secondary metabolites, and metals; Schmitt et al., 2021). We might expect that when *P. alecto* are nomadic and consume high portions of native diet (such as flowering *Eucalyptus*), their dietary breadth may be narrower as these bats may focus their foraging on large pulses of nectar. Similarly, howler monkeys (*Alouatta pigra*) consuming less diverse diets, had decreased fecal microbiome diversity and richness and altered composition (Amato et al., 2013). However, low diversity primate GIT microbiomes associated with low diversity diets are likely a result of suboptimal, fragmented habitat (Amato et al., 2013; Gomez et al., 2015), which is likely the reverse for *P. alecto*, as habitat degradation is resulting in fewer nectar sources and increasing *P. alecto* dietary diversity.

It is possible that with the rapid transit times of *P. alecto* that we were not able to detect all diet items consumed over the course of a night of feeding, potentially skewing our proportion of native and non-native diet. Because bats are the only mammals capable of powered flight, they experience strong physiological selection pressures. For example, bats have shorter GITs and more rapid digestive transit times relative to mammals of comparable size, likely to lighten the load for flight (Price et al., 2015). These rapid transit times may also limit the amount of time that GIT microbes have to interact with ingested substrates. Future work should assess the effect of food transit times on the flying fox GIT microbiome. Additionally, we did not distinguish plant taxa into nectar or fruit sources (flowering plants typically produce fruit) which may impact

these results. However, future work will incorporate these analyses to determine if nectar sources, specifically, are associated with decreased GIT microbiome diversity. As bats change their diet to more introduced foods to adapt to habitat loss, their microbiomes may change too. Future research should also examine the functional role of the GIT microbiome in association with the abundances of specific diet items.

Here, we demonstrated that high proportions of native dietary taxa in *P. alecto* feces correspond with low GIT microbiome Shannon diversity. As Australian flying foxes continue to lose habitat, it is important to understand how flying fox GIT microbiomes respond to changes in diet. If their GIT microbes are contributing to energy or nutrient acquisition, fluctuations in their GIT microbiome may either be a result of a plastic response to different food sources. Whether or not these changes are beneficial or detrimental to these bats, is unknown. Future studies examining the full breadth of *P. alecto* diet simultaneously with GIT microbiome samples will enable us to understand how these bats cope with a changing landscape.

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CHAPTER FIVE

BLACK FLYING FOX (*PTEROPUS ALECTO*) GASTROINTESTINAL MICROBIOME CANNOT PREDICT BODY COMPOSITION, INFLAMATION, OR VIRAL SHEDDING

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Animal Microbiome

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Biomed Central

Abstract

Bats are reservoirs of important zoonotic viruses, and it is imperative to analyze factors affecting bat health as these may consequently influence viral shedding dynamics. The gastrointestinal tract (GIT) microbiomes of a variety of mammalian species have been shown to impact and respond to mammalian host health in many ways; however, the influence of the bat GIT microbiome on host health and viral shedding dynamics remains to be determined. To improve our understanding of associations between bat health and their GIT microbiomes, we measured body composition with bioelectrical impedance analysis, quantified leukocyte ratios via blood smears, quantified Hendra virus (HeV) prevalence in captured and roosting black flying foxes (*Pteropus alecto*) and used 16S rRNA sequencing of the V4 region from rectal swabs to determine the *P. alecto* GIT microbiome. There was strong evidence for a difference in GIT microbiome composition in association with percent body fat and HeV shedding. Furthermore, HeV shedding was positively associated with one alpha diversity metric (Faith's PD). However, we observed no association between GIT microbiome alpha diversity and percent body fat or neutrophil:lymphocyte ratios (NLRs). Machine learning analyses indicate that the *P. alecto* GIT microbiome cannot accurately predict percent body fat, NLRs, or HeV shedding. Although there are associations between the *P. alecto* GIT microbiome alpha diversity and composition in relation to HeV shedding and body composition, 16S rRNA amplicon sequencing of the GIT microbiome may be insufficient as a biomarker of host health or viral shedding. Machine learning techniques enable us to go beyond looking at associations. The inability of our machine learning approaches to predict health outcomes strongly suggests the presence of other confounding factors that impact the bat GIT microbiome and health. It is still unclear if and how

the *P. alecto* GIT microbiome is functionally contributing to host health, though this may be an artifact of 16S rRNA profiling. Identification of potential biomarkers of bat health may ultimately enable us to improve bat health and better predict spillover events.

Introduction

In many mammals, gastrointestinal (GIT) microbes influence host physiology, behavior, nutrition, and immune function. Gastrointestinal microbiota affect host metabolic traits such as homeostasis, and the release of hormones, which is an important component of microbial regulation of host metabolism (Martin et al., 2019). GIT microbiota can also influence fat deposition. Obesity in humans and mice is associated with phylum-level changes in the GIT microbiome and reduced microbial diversity (Turnbaugh et al., 2009) and fecal microbiome transplants from obese mice into germ-free mice can promote greater fat deposition than transplants from lean donors (Turnbaugh et al., 2008).

In some species, microbiota inhabiting the GIT also influence host immune function and pathogen infection dynamics. GIT microbiota are important in educating the immune system (Zhao and Elson, 2018) since the GIT is constantly being exposed to environmental challenges. Gut associated lymphoid tissue—which contains both innate and adaptive immune cells—is located just beneath the epithelium of the GIT, thus making the GIT one of the largest immune organs in the body. Constant cross-talk between the GIT and microbes inhabiting the mucosa allows these microbes to act as mediators between host diet and immune function (Shi et al., 2022). For example, microbially-derived metabolites, such as butyrate, are involved in many cell signaling cascades and may either serve to increase or decrease inflammatory responses (as reviewed in Feng et al., 2018). Many bacteria can also stimulate anti-inflammatory responses by

driving IL-10 production or influencing Treg maturation (as reviewed in Cristofori et al., 2021). This phenomenon may explain why increased GIT microbial diversity in humans is associated with low neutrophil to lymphocyte ratios (i.e., biomarkers indicative of inflammation; Yoon et al., 2018). The relationship of the GIT microbiomes with host physiology and immune function can ultimately impact viral infection dynamics by affecting host susceptibility and pathogen colonization (Kau et al., 2011) and either facilitate or inhibit pathogen infections (Erickson et al., 2018; Wilks et al., 2015).

Because GIT microbes can have a large influence on host health through direct and indirect mechanisms, it is important to understand the role of the GIT microbiome in bat health and viral shedding. Bats are reservoirs of many emerging virulent zoonoses (Guth et al., 2021). Australian black flying foxes (*Pteropus alecto*) are natural reservoirs of Hendra virus (Halpin et al. 2000), which can have lethal results for humans and domestic animals that become infected. Consequently, it is crucial to understand factors that influence bat health and may ultimately affect bat-virus dynamics (Plowright et al., 2021). Microbes inhabiting bat GITs may be relevant to bat-virus dynamics (Jones et al., 2022). GIT microbes isolated from bats that have been transplanted into germ-free mice conferred tolerance to influenza virus (Liu et al., 2022). Viral infections in bats are associated with different compositions in bat GIT microbiomes, but this may depend on age and season (Dietrich et al., 2017a; Wasimuddin et al., 2018).

Though mounting evidence indicates that bat GIT microbes contribute to nutrient acquisition in some systems (Dhivahar et al., 2019; Ingala et al., 2021; Phillips et al., 2017; Zepeda Mendoza et al., 2018), the role of the GIT microbiome in fat deposition, inflammation, and viral dynamics in bats is still unknown (Jones et al., 2022). Australian flying foxes are

experiencing significant habitat degradation, resulting in resource bottlenecks during winter (Eby et al. in prep). Consequent nutritional stress may impact the GIT microbiome and have cascading effects on host immune function, body composition, and viral shedding. Insight into these relationships will provide evidence as to whether GIT microbiomes can be used as biomarkers to assess health status of these bats. Measuring the associations between GIT microbiome diversity and composition may enable the identification of a potential mechanism linking nutritional stress to *P. alecto* health outcomes.

In this study, we characterized the *P. alecto* GIT microbiome using 16S amplicon sequencing of rectal swabs. We then measured the association of the *P. alecto* GIT microbiome alpha- and beta diversity with three potential proxies of health: percent body fat (where very low body fat may indicate nutritional stress), neutrophil:lymphocyte ratios (NLR; where high NLR may indicate inflammation), and HeV shedding status (where being positive for infection may indicate some underlying susceptibility to becoming infected or prolonged infection). Lastly, we used machine learning approaches to assess the ability of the *P. alecto* GIT microbiome to predict these three outcomes. To our knowledge, this study is the first to assess the *P. alecto* GIT microbiome in the context of body composition, immune function, and viral shedding.

Methods

Capture & sampling of bats

We captured *P. alecto* at five sites in the greater Brisbane area (MSU IACUC #201750 and Griffith University Animal Ethic Committee ENV/10/16/AEC) using mist nets put up at dawn as bats return from foraging. During processing (within 6 hours of capture), we anesthetized bats using isoflurane (starting at 5% then reducing to 1.5%) and used sterile

synthetic cotton swabs to collect rectal swabs into 70% ethanol for microbiome analysis. Samples were stored on ice or in liquid nitrogen until they could be stored at -80C. For each individual, we collected forearm length (mm), mass (g), and identified the sex, age class, and reproductive status. Upon capture, all bats were checked for a Passive Integrated Transponder (PIT) tag. If no PIT tag was present, from May 2019 onwards, we inserted a RFID PIT tag (ZD Tech Group China) under the skin between the scapulae while the bat was anesthetized. All bats were given subcutaneous fluids (one part 2.5% glucose and 0.45% NaCl solution and one part Hartmann's solution) at 5-10% of body mass and monitored for at least a half hour before release back into their roost. Additional samples were collected from captured flying foxes and are described below.

Microbiome profiling

Rectal microbiome samples were analyzed as described in Jones et al. (Chapter 2, in prep). Briefly, ethanol was dried from the tubes and microbial DNA was extracted from the swabs using the ZymoBIOMICS DNA miniprep kit (Irvine, CA). We followed the manufacturer's protocol except we increased the bead beating step to 5 total cycles of 1 minute of beating the swab, beads, and lysis solution, with a 5-minute wait in between beat cycles. Presence of DNA was confirmed with polymerase chain reaction (PCR) using the 27F/1492R primer pairs (Frank et al., 2008) and visualized on a 1.5% agarose gel. All rectal DNA was sent to University of Michigan Microbiome Core to amplify and sequence the hypervariable region 4 of the 16S rRNA gene using Illumina Miseq according to Kozich et al. (2013).

Leukocyte profiles

Leukocyte profiles were determined as described in Hansen and Hunt et al. (2022). Briefly, we collected a maximum of 2.5 mL of blood from each bat (not exceeding 0.6% of body mass). We then prepared blood smears on-site from blood taken directly from the syringe (sans anticoagulant agents). Blood was smeared onto glass slides, allowed to air dry, then fixed in 100% methanol. Slides were stored at room temperature and out of light for up to 2 years then stained with Romanowsky stain variant DipQuick (Jorgensen Laboratories, Loveland, CO, USA). Only high-quality blood smears were analyzed. Standard leukocyte differentials were performed manually by counting 100 different leukocytes in the monolayer of each smear. Neutrophil:lymphocyte ratios (NLR) was calculated by dividing the number of neutrophils by the number of lymphocytes.

Determination of percent body fat

Here, we briefly describe the methods to determine percent body fat as in Dale et al. (in prep). While *P. alecto* individuals were under anesthesia and in a supine position on a non-conductive table, we positioned four bent one-inch 25 gauge needles in the skin along the midline of the body, with one placed in the skin of the cheek, in the skin at the sternum, in the skin by the pubic bone, and in the skin between the knee and ankle. We used a tetrapolar electrical bioimpedance spectrometer (IMP SFB7, ImpediMed Limited) to measure impedance (Z, ohm), resistance (R, ohm), and reactance (Xc, ohm) at 256 frequencies between 3 and 1,000 kHz. Five replicate measures were taken with each scan and each bat was scanned twice consecutively for a minimum of 10 measurements per individual. We used calipers to measure the distance between the two proximal electrodes.

We analyzed impedance spectrum data according to the Cole model (Cornish et al. 1993) using Biomp software (v4.5.0.0, Impedimed Limited). The resistance at 50 kHz (R50), the impedance at the characteristic frequency (Zc), resistance at zero (R0), and infinite (Rinf) were obtained from the best-fit semi-circle to a graphical plot of measured reactance versus resistance. We tested the goodness of fit with the standard error of the estimate. In all cases, the standard error of the estimate was less than one percent.

Hendra virus detection

We collected urine from bats either under anesthesia (where urine was given freely or by gently palpating the abdomen) or from the bats' plastic-line holding bags funneled into plastic baggies. Urine samples were collected into sterilized microcentrifuge tubes with either viral transport medium (VTM), viral lysis buffer (Buffer AVL, Qiagen), or sans buffer. We sent urine samples to Rocky Mountain Laboratories in Hamilton, MT for HeV detection via quantitative polymerase chain reaction (qPCR; protocol is further described in Peel and Yinda et al. (Peel et al., 2022)). We used the qPCR cycle threshold (ct) values (≤40 cycles) to determine if bats were either positive or negative for HeV at time of sampling.

Statistical Methods

To determine the strength of association of each of the three health metrics with GIT microbiome alpha diversity metrics (Shannon's diversity and Faith's Phylogenetic Diversity, PD) we fit two linear regression models (one for each type of alpha diversity) for two of the response variables (percent body fat and NLRs). Shannon's diversity and Faith's PD were calculated using the R packages vegan package (Oksanen et al. 2022) and picante (Kembel et al. 2010). For HeV shedding, since there were only seven individuals that tested positive, we used a

Mann-Whitney U test (non-parametric t-test using either of the alpha diversity metrics and HeV infection as a binary status) to test for a difference in alpha diversity among HeV positive and negative bats.

Additionally, we calculated the Bray-Curtis distance among bacterial amplicon sequence variants (ASVs) among all sampled bats and used the 'adonis' function in the *vegan* R package (Oksanen et al. 2022) to perform three permutational multivariate analyses of variance (PERMANOVA) with 999 permutations to calculate a difference between the beta diversity (i.e., microbial community composition) among bats with varying health outcomes (i.e., variation in percent body fat, NLRs, and HeV shedding).

To determine which bacterial genera were differentially abundant between *P. alecto* associated with HeV shedding, NLR, and percent body fat, we fit three separate Bayesian regression models with a Bernoulli (for the binary variable HeV infection status; logit link function), Gaussian (for the log-transformed continuous variable NLR; log link function) and Beta (for continuous variable percent body fat; log link function) response distribution using Hamiltonian Monte Carlo as implemented in Stan (Stan Development Team 2022), using R package *rstan* (Stan Development Team 2022). Three chains were run for 4,000 iterations after 2,000 burn-in iterations. Convergence was checked using trace plots and R_hat values (RStan, Stan Development Team 2022). For all effect estimates weakly informative normal priors with a mean of zero and standard deviation of 0.5 was used, which has a regularizing effect that is suitable for dealing with many variables. All relative abundance values were scaled to a mean of zero and a standard deviation of 1 before model fitting. For all our models, we reduced the microbiome dataset to include bacterial genera that were detected in at least 10 bats as rare

microbes would need a substantial effect to be detected since the dataset contained a large number of genera (n=235) and many genera contain a large number of zeros.

To test whether HeV status, percent body fat or NLR could be predicted using differences in microbiome relative abundances, we used 10-fold cross validation where the dataset is randomly divided into 10 subsets, and each subset in turn is being predicted using a model that has been fit using only the remaining subsets (i.e., out-of-sample prediction). Cross validation performance of the real data was compared with that of a random 'null model' dataset to assess how much better than random the model predictions are. The null model dataset was constructed using row-wise permutation, where the relative abundance values for each individual are randomly reassigned to different genera. Cross validation metrics kfoldic (calculated using the kfold function in package brms; Bürkner et al. 2022), proportion correctly predicted (for HeV status) and root mean square error (for percent body fat and NLR) were used to compare performances. In addition to the regression models, random forest models were used because these are better able to model non-linear effects and interactions between variables (genera). The function train of R package caret (Kuhn 2022) was used. All data handling and analyses were performed in R (v4.1.2, R Core Team).

Results

HeV associations with the GIT microbiome

Out of 195 captured bats, we obtained HeV infection status for 166 *P. alecto.* There was no evidence for a difference in Shannon diversity (p=0.92) of the GIT microbiome between HeV infected and uninfected bats. However, there was strong evidence for a difference in both Faith's PD (p=0.0439) and composition (p=0.007) of the GIT microbiome between infected and

uninfected bats. Bats infected with HeV had greater microbial phylogenetic diversity (i.e., Faith's PD) (Fig. 1). There was strong evidence for increased abundance of *Aquabacterium*, *Rothia*, *Cyanobacterium* PCC6307 and *Fructobacillus* in HeV positive bats (Figure 2).

Ten-fold cross-validation using our true microbiome data set did a slightly better job predicting HeV outcome (kfoldic=87.6, SE =22.7) compared to the null data set (kfoldic=65.3, SE=16.5), though we do not have strong enough evidence for a real difference between the null and true model abilities to predict HeV infection status. These results are further supported by cross-validation, which showed that although our true microbiome data can predict HeV infection status 95.2% (SE=0.015) of the time, the null model can predict HeV status nearly identically (95.3%, SE=0.012).

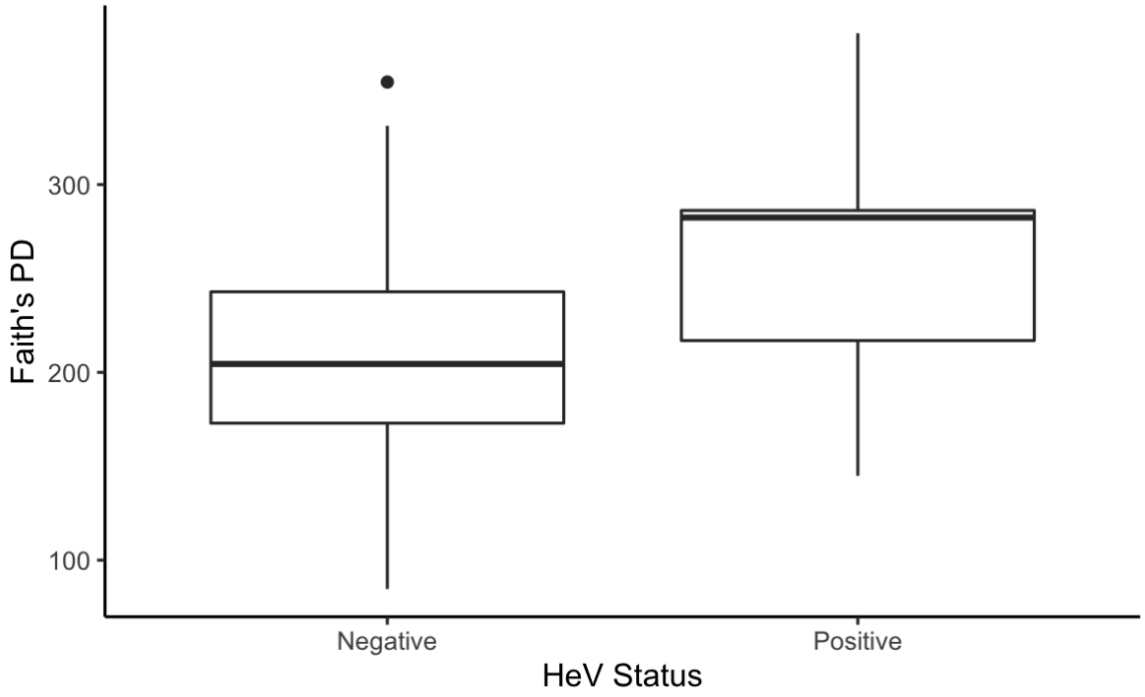

Figure 1: Bats that tested positive for HeV had greater Faith's PD of their GIT microbiome.

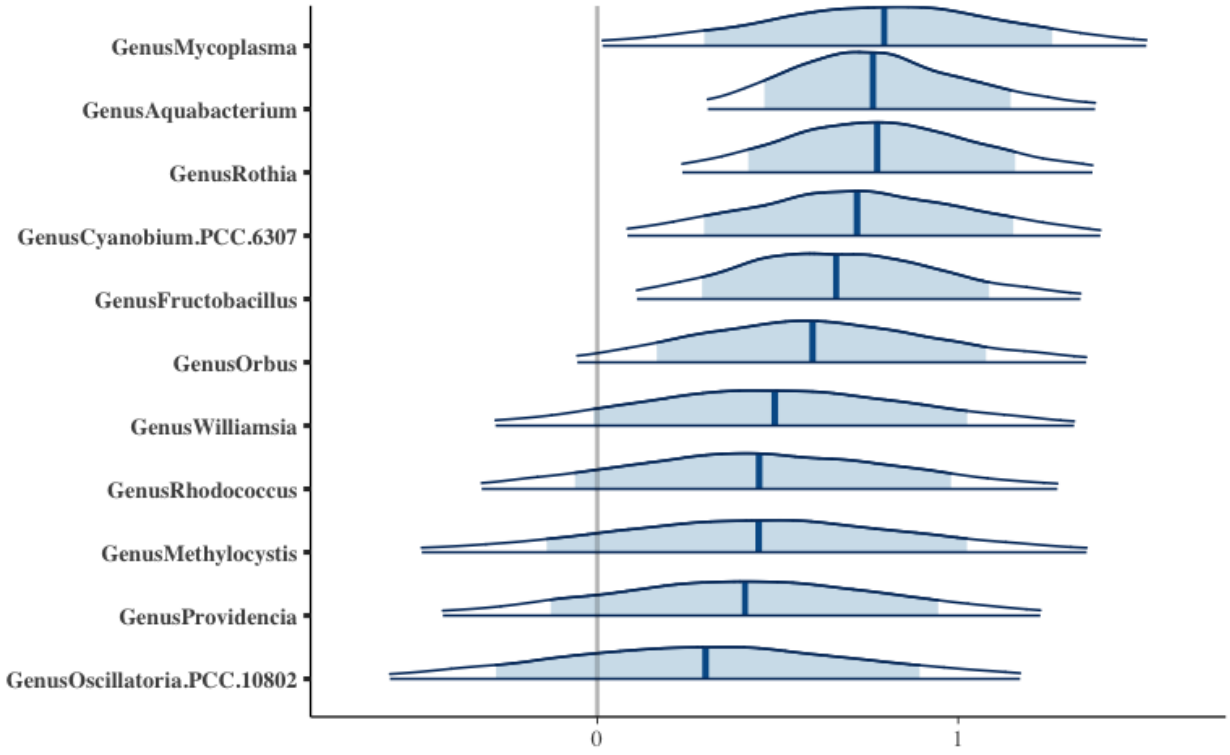

Figure 2: The bacterial genera that are more abundant in bats that test positive for HeV. There was strong evidence that the bacterial genera that are greater than zero (and whose distributions do not cross zero) are more abundant in bats that are positive for HeV infection. For the bacterial genera that are mostly greater than zero (and whose distributions do touch or cross zero), there is moderate evidence that these taxa are more abundant in bats that test positive for HeV.

Prediction of Neutrophil:Lymphocyte ratio with the P. alecto GIT microbiome

For the 77 bats with associated NLR data, there was no evidence of a difference in GIT microbiome Shannon diversity (p=0.838), Faith's PD (p=0.363), or microbial community composition (p=0.291) among bats with varying NLRs. For the 77 *P. alecto* with NLR data, ten-fold cross-validation using our true microbiome data set did slightly worse in predicting NLRs (kfoldic=314.6, SE=9.0) compared to our null dataset (kfoldic=336.5, SE=6.3). These results are further supported by random forest prediction and cross-validation where our true data (mean RMSE= 0.752, SE= 0.061) was not able to discriminate NLRs better than our null model (mean RMSE=0.77, SE=0.048).

Percent body fat K-fold models with cross-validation and Random Forest

We had 77 bats with BIA data ranging from 4.8% to 22.6% body fat, but most of these bats had more than 11% body fat. There was no evidence for a difference in Shannon diversity (p=0.849) or Faith's PD (p=0.273) of the GIT microbiome in association with percent body fat. By contrast, there was strong evidence for a difference in composition of the GIT microbiome based on percent body fat (p=0.048). Despite the differences in microbiome composition, for the 85 *P. alecto* with percent body fat data, the GIT microbiome was not able to predict percent body fat (mean RMSE=-65.8, SE=9.4) better than a null model (mean RMSE=-49.1, SE=9.4). The random forest models support the ten-fold cross validation approach and show that the true dataset cannot accurately predict the percent body fat of *P. alecto* (mean RMSE=0.037, SE=0.003) any better than the null data set (mean RMSE = 0.038, SE=0.004).

Discussion

The role of the GIT microbiome in bat health is still being investigated. However, as zoonotic pathogens continue to spillover at unprecedented rates (Jones et al., 2008), it is important to explore host-associated factors that may ultimately impact pathogen dynamics. While the GIT microbiome may be reflective of health (either directly or indirectly), our results indicate that *P. alecto* GIT microbiome samples may not be useful as biomarkers of health status.

The lack of association between GIT microbiome alpha- and beta diversity with NLRs as well as the high RMSE scores from the random forest approach, indicate that there is no detectable association between NLRs and the GIT microbiome and that the GIT microbiome cannot accurately predict NLRs. It is possible that other biomarkers of immune function, such as pro- or anti-inflammatory cytokines, may be better indicators of inflammation status and future work can examine such associations.

Differences in GIT microbiome composition (but not alpha diversity) in association with percent body fat, indicate that the GIT microbiome may be associated with differences in percent body fat. However, results from machine learning indicate that despite these compositional differences, the GIT microbiome is not able to predict body composition better than a null model. This inconsistency of results between approaches suggests that there may be other factors (potentially undetected confounders) linking body fat and the GIT microbiome. The inclusion of more bats with low body fat, may enable the machine learning techniques to differentiate percent body fat using the GIT microbiome.

Increased Faith's PD and microbial community composition in bats that tested positive for HeV indicate that there may be an increase in the phylogenetic diversity of the microbes

present in the *P. alecto* GIT microbiome. The increased microbial diversity may either be a result of viral infection or if this diversity is not beneficial, may enable hosts to become infected. However, lack of detection of differences in Shannon diversity may be a result of low sample size of bats that test positive for HeV or could be due to a lack of change in relative richness and evenness among samples. Similarly, it is likely that our machine learning methods had high success rates of determining HeV status because almost all bats tested negative for HeV. Larger sample sizes and experiments will be necessary to determine if viral infection may influence GIT microbiome or vice versa.

Despite differences in microbiome composition, but not alpha diversity, with regard to percent body fat, and differences in Faith's PD and composition in relation to HeV infection status, machine learning techniques indicated that the *P. alecto* GIT microbiome was not able to predict either of these outcomes. It is likely that there are other factors (e.g., diet) not included in this study that impact either the GIT microbiome, the health metrics, or both. These results suggest no clear link between the *P. alecto* GIT microbiome and these three metrics of health.

Our results have important limitations. We were only able to use leukocyte differentials as opposed to counts. These data may also limit our understanding of how the GIT microbiome is associated with leukocyte profiles. Additionally, it is likely that our true and null model performed equally well (>95% accuracy) due to the small sample size of HeV positive bats (seven out of 224 bats sampled for this study tested positive for HeV). Though bats have highly variable GIT microbiomes as determined by 16S profiling, the functions of those different microbes may be redundant or conserved (Phillips et al. 2017). Thus, 16S profiles of the GIT microbiome may not be sufficient to detect important changes related to health, but whole-

genome sequencing and metabolomics (to identify specific microbial functions within the GIT) approaches may be more suitable as biomarkers of health. Future research should incorporate whole genome sequencing, transcriptomics, or metabolomics to enable researchers to determine which bacteria are actively transcribing at the time of sample collection and understand which microbially derived metabolites may be present and useful to the host. Additional molecular analyses and incorporation of additional health metrics will elucidate mechanisms underpinning bat health.

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CHAPTER SIX

CONCLUSION

To our knowledge, these studies are the first to show the temporal dynamics of the *P. alecto* GIT microbiome in addition to its associations with diet and health. Moreover, we applied machine learning techniques to assess the ability of the GIT microbiome to predict these health outcomes.

In chapter two, we showed that *P. alecto* GIT microbiomes were highly dynamic over time (annually and seasonally), and were affected by sex, age, reproductive status and urban residency. Additionally, we showed that the GIT microbiomes of wild recaptured *P. alecto* demonstrated high intra-individual variation that is not different from inter-individual variation. These results indicated a lack of stability in the *P. alecto* GIT microbiome. These frequent shifts could be beneficial for bats adapting to a dynamic environment, detrimental to bats if they rely on the presence of particular microbial contributions, or these dynamics may be neutral, meaning that the microbiome is functionally neither beneficial nor detrimental, but rather is stochastic. We propose that future research should incorporate tests of neutrality to determine if the *P. alecto* GIT microbiome is somewhat random, or if there is structure that is important for the bat host.

In chapter three, we showed that diet played a role in shaping the *P. alecto* GIT microbiome. Here, we showed that the dietary richness (the number of dietary species consumed) was not, but the type of food was associated with GIT microbiome alpha and beta diversity. *P. alecto* that primarily consume native plants had lower alpha diversity and the microbiome composition differed substantially from bats eating introduced and cultivated foods.

These results could indicate that the *P. alecto* GIT microbiome fluctuates in response to their environment and resource availability. Similar to our results in chapter one, these fluctuations may be beneficial and contribute to nutrient or energy acquisition, or these fluctuations could simply indicate an influx of different bacteria associated with the environment.

Our combination of linear regression and machine learning approaches in chapter four showed that despite associations with three different health metrics and GIT microbiome diversity and composition, we were not able to use the microbiome (as determined by 16S amplicon sequencing) to predict any of our measured health outcomes. There were three potential reasons why the GIT microbiome cannot predict these three outcomes: 1) There were other confounding factors (such as diet) that we did not account for in these models; 2) 16S amplicon sequencing did not adequately represent the microbial diversity and therefore the approaches were not sensitive enough to predict outcomes; and 3) The *P. alecto* GIT microbiome follows neutral theory as opposed to niche-assembly and is therefore random and cannot predict health outcomes.

Taken together, results from these chapters indicate that the GIT microbiome of *P. alecto* may only play a small role in health. We propose that the *P. alecto* GIT microbiome is a somewhat random assortment of microbes. This is supported by previous work that has described the bat GIT microbiome as dominated by ‘weedy’ species like those within the phylum Proteobacteria, that are able to grow quickly and utilize numerous substrates (Hammer et al., 2019; Le Bouguénec and Schouler, 2011). Furthermore, recent evidence (Lutz et al. 2019) shows a lack of phylosymbiosis (alignment between the host and microbial phylogenies) between bats and their GIT microbiomes. These results indicate that ecology may be more important in

shaping the GIT microbiome than a shared evolutionary history among bat hosts. More simply put, our results contribute to the evidence supporting the theory that bats and their GIT microbiomes did not co-evolve together.

Current research on bat GIT microbiome function has relied heavily on functional predictions based on 16S amplicons, which is likely insufficient for identifying the functions of bacteria inhabiting bat GITs. The incorporation of whole genome sequencing, transcriptomics, and metabolomics will offer more insights into microbially derived functions (as opposed to solely the bacterial identity) that impact the host and may lead to the identification of more useful biomarkers of bat health. Additionally, experimental studies will be crucial in elucidating the role of the GIT microbiome in bat health and in their unique roles as viral reservoirs. Critical experiments include feeding trials to assess the impact of diet to the use of antibiotics to determine successional changes in the GIT microbiome and its associated changes in a variety of health metrics. Most importantly, future work should incorporate tests of neutrality to determine if the bacteria present are, in fact, random or if there is some structure and as a likely result, some benefit, that we are not detecting with alpha and beta diversity metrics.

Continued research into bat GIT microbiomes that goes beyond 16S identification of bacteria and associations using alpha and beta diversity metrics will enable researchers to identify mechanisms underpinning bat health. These analyses are increasingly important as land-use change continues to affect resource availability for wildlife with possible implications for the spillover of zoonotic viruses. This work suggests that GIT microbiomes may not be useful as a biomarker of zoonotic virus infection but further work on the functions of GIT microbes is needed before it can be concluded that there is not an association between GIT microbiomes and

health in flying foxes. These results serve as first pass at developing biomarkers of viral infection in bats, though further analyses are required. Biomarkers of health and viral shedding are important for prediction and prevention of zoonotic spillovers.

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