

# Associations between Afro tropical bats, eukaryotic parasites, and microbial symbionts

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## Abstract

Skin is the largest mammalian organ and the first defensive barrier against the external environment. The skin and fur of mammals can host a wide variety of ectoparasites, many of which are phylogenetically diverse, specialized, and specifically adapted to their hosts. Among hematophagous dipteran parasites, volatile organic compounds (VOCs) are known to serve as important attractants, leading parasites to compatible sources of blood meals. VOCs have been hypothesized to be mediated by host-associated bacteria, which may thereby indirectly influence parasitism. Host-associated bacteria may also influence parasitism directly, as has been observed in interactions between animal gut microbiota and malarial parasites. Hypotheses relating bacterial symbionts and eukaryotic parasitism have rarely been tested among humans and domestic animals, and to our knowledge have not been tested in wild vertebrates. In this study, we used Afro tropical bats, hematophagous ectoparasitic bat flies, and haemosporidian (malarial) parasites vectored by bat flies as a model to test the hypothesis that the vertebrate host microbiome is linked to parasitism in a wild system. We identified significant correlations between bacterial community composition of the skin and dipteran ectoparasite prevalence across four major bat lineages, as well as striking differences in skin microbial network characteristics between ectoparasitized and nonectoparasitized bats. We also identified links between the oral microbiome and presence of malarial parasites among miniopterid bats. Our results support the hypothesis that microbial symbionts may serve as indirect mediators of parasitism among eukaryotic hosts and parasites.

## KEY WORDS

Afrotropics, bat flies, bats, Chiroptera, Haemosporidia, Hippoboscoidea, malaria, microbiome

## 1 | INTRODUCTION

Animals are capable of hosting myriad biologically interdependent symbionts including viruses, bacteria, archaea, and eukarya. Many associations between eukaryotic parasites and hosts have ancient

origins (Larremore, 2015; Stevens et al., 1999), and mounting evidence suggests that bacterial symbionts may be responsible for mediating host-parasite interactions in ways that could ultimately shape host evolution (Ippolito et al., 2018; King & Bonsall, 2017; McFall-Ngai et al., 2013). For example, studies of human and anthropophilic

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mosquito interactions have found that the human skin microbiome can influence mosquito feeding preference (Busula, Takken, et al., 2017; Robinson et al., 2018; Verhulst et al., 2009, 2011). Such influence may affect the transmission patterns of vector-borne pathogens including West Nile virus (Campbell et al., 2002), yellow fever (Monath & Vasconcelos, 2015), dengue (Simmons et al., 2012), and malaria (Ippolito et al., 2018), potentially imposing indirect selective pressure on human populations (Kwiatkowski, 2005). Conversely, parasites may influence the relative abundance of host-associated microbes in ways that facilitate parasite transmission. This phenomenon has been observed in rodent models of *Plasmodium* transmission in which parasitism positively correlates with abundance of skin-associated microbes that produce volatile organic compound attractive to arthropod vectors of *Plasmodium* (Busula, Takken, et al., 2017; Busula, Verhulst, et al., 2017; Penn & Potts, 1998). Despite the potential evolutionary significance of interactions between animal hosts, microbial symbionts, and eukaryotic parasites, such interactions have not been well studied in wild vertebrates (although see Leung et al., 2018).

Bats (Mammalia: Chiroptera) are an ideal system for examining the interactions between microbial symbionts, eukaryotic parasites, and hosts. Bats are among the most speciose orders of mammals (second only to the Rodentia), providing a diverse comparative phylogenetic framework for hypothesis testing, and they harbor a great diversity of eukaryotic parasites such as dipteran insects, haemosporidia, and helminths (Dick & Patterson, 2006a, 2006b; Dittmar et al., 2015; Lutz et al., 2016; Schaer, 2013). Bats are also associated with microbial pathogens of importance for human health (e.g., *Bartonella*, *Pasteurella*, SARS coronaviruses, filoviruses, rhabdoviruses, Hendra and Nipah viruses) (Bausch & Schwarz, 2014; Billeter et al., 2012; Hayman et al., 2013; Sawatsky et al., 2008), and serological surveys have supported the role of Afrotropical bats as reservoirs for a number of viruses (Amman et al., 2015; Olival & Hayman, 2014; Towner et al., 2009), making our understanding of factors regulating parasite and pathogen transmission all the more relevant. Inter- and intraspecific transmission of pathogens among bats and other animals is an area of increasing concern in light of recent zoonotic pandemics. Bat flies (Arthropoda: Hippoboscidae), which are obligate blood-feeding parasites of bats, are known to harbor several pathogens of human relevance (Bennett et al., 2020; Goldberg et al., 2017) and also are the primary hosts for bat-specific malarial parasites (Apicomplexa: Haemosporida) (Garnham, 1966; Lutz et al., 2016). Bat flies are typically host-specific (Dick & Dittmar, 2014; Dick & Patterson, 2007) and prevalence can vary widely within a single species across different geographic locations (Urbietta et al., 2019).

Bat flies are nutritionally dependent on their hosts and maintain contact throughout most of their lives, living in fur and on skin membranes and leaving their hosts only for brief reproductive periods (Szentiványi et al., 2019). How these parasites maintain host-specificity with bats over evolutionary time is unknown, but blood protein and immunocompatibility between host and parasite are thought to play a role (Dick & Patterson, 2006a, 2006b; Dick &

Patterson, 2007). The proximal mechanism by which nycteribid bat flies locate "preferred" hosts in temperate regions include a combination of sensory mechanisms tuned to carbon dioxide, body heat, and host-associated odours (Lourenço & Palmeirim, 2008) as has been observed in other hematophagous parasites (e.g., mosquitoes (Verhulst, Andriessen, et al., 2010; Verhulst et al., 2009; Verhulst et al., 2011; Verhulst, Takken, et al., 2010), tse-tse flies (Soni et al., 2019)) that rely on host-associated chemical cues. Chemical cues can be produced in a number of ways, including via metabolic processes involving bacteria in the gut, oral cavity, or on the skin (Ezenwa et al., 2012; Klowden, 2013).

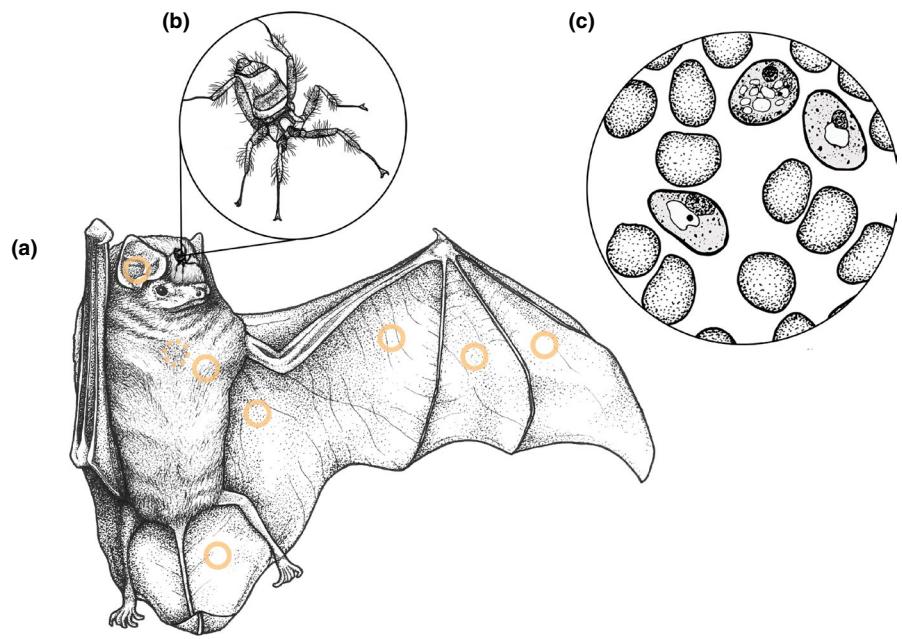
Here, we build on previous broad-scale studies of Afrotropical bat-associated microbes (Lutz et al., 2019; Song et al., 2020) to characterize parasitism by bat flies and haemosporidia in four widespread lineages of bats. Using these data, we tested the hypothesis that the bat microbiome (skin, oral, and gut) is linked to parasitism by two obligate, host-specific eukaryotic parasites.

## 2 | MATERIALS AND METHODS

### 2.1 | Host and parasite sampling

Sampling was conducted at 14 field sites in Kenya and Uganda from August to October 2016, using a combination of mist-netting and hand-netting. The taxonomy of collected individuals was verified upon accession at the Field Museum of Natural History based on morphological features and comparison to relevant taxonomic keys (Kingdon, 2013; Patterson & Webala, 2012). Samples were taken from bats collected as voucher specimens for biodiversity inventories, allowing for extensive post-mortem sampling of skin and fur from multiple points on the body. These included three biopsies from wing membrane, one from tail membrane, one from the ear, one from the interscapular region of the back, and one from the interclavicular region of the chest (Figure 1) using new 3 mm sterile disposable biopsy punches (Integra Miltex) for each individual. Biopsy samples from each individual were combined and stored in sterile 95% ethanol, the goal being to maximize representation of skin microbiota from individuals as a whole (as opposed to distinguishing between body sites within individuals). Whole tongues were collected (from apex to root) and stored in 95% ethanol for oral microbiome analysis. We collected ~2–4 ml of blood from euthanized bats via cardiac puncture. Blood was placed on Whatman FTA cards for nucleic acid extraction, and 2–3 blood films per individual were prepared for microscopic analyses. The remainder of each blood sample was stored in cryovials placed in IN2. Following euthanasia, skin, and blood sampling, bats were fumigated in ethyl acetate for 15 min and then examined for ectoparasites. Prior to any processing heretofore described, bats were held individually in individual sterile cloth bags to prevent cross-contamination of ectoparasites or skin microbiota. Presence or absence of dipteran parasites was noted, and parasites were collected into 95% ethanol for taxonomic identification. Bat flies were identified morphologically by examination under magnification

**FIGURE 1** Diagram of host-parasite associations and sampling. (a) Bat host, with orange circles indicating locations from which skin and fur samples were collected; dashed circle indicates interscapular sampling region on dorsal side of bat. (b) hippoboscoid bat fly, (c) bat red blood cells infected by malarial parasites. Illustration by Madison Erin Mayfield @MEMIllustration



using a Leica MZ16 stereozoom microscope, following keys and species descriptions of Theodor (1967) and Theodor (1968), and by comparison to reference specimens in the hippoboscoid collection of the Field Museum of Natural History, Chicago and the Bernice P. Bishop Museum, Honolulu. Malarial parasite presence and taxonomic identity were determined as described in Lutz et al. (2016). In brief, DNA was extracted from whole blood using Qiagen DNeasy and screened for the presence of malarial parasites using triplicate PCR and Sanger sequencing confirmation, followed by BLASTn to confirm parasite taxonomy. All sampling was conducted in accordance with the Field Museum of Natural History IACUC. Host and parasite vouchers are accessioned at the Field Museum of Natural History (Chicago, IL, USA) (Table S1). Collection permits and material transfer agreements were provided by the Uganda Wildlife Authority (UWA; Ref COD/96/02), Uganda National Council for Science and Technology (UNCST; Ref NS 417), the Kenya Wildlife Service (KWS; Ref KWS/BRM/5001; Ref KWS/4001), the Kenya Forestry Service (KFS; Ref RESEA/1/KFS/10) and the National Museums of Kenya (Ref NMK. Maml.2016.128). Permits to Import Infectious Biological Agents, Infectious Substances, and Vectors were provided by the United States Centers for Disease Control and Prevention (PHS Permit nos. 2017-11-057; 2018-05-086).

## 2.2 | Microbial DNA extraction, library preparation, and data generation

DNA was extracted using the MoBio PowerSoil 96 well soil DNA isolation kit (no. 12955-4; MoBio) following the standard Earth Microbiome Project protocol (<http://www.earthmicrobiome.org/>). Individual samples were randomly sorted and transferred to 96-well extraction plates. Two negative controls were included per extraction plate, which were carried through all downstream processing steps. PCR amplification and sequencing were performed

as previously described in Lutz et al. (2019). All amplicon libraries were pooled and sequenced on two lanes of a single Illumina HiSeq sequencing run at Argonne National Laboratory (Evanston). Amplicon sequence variants (ASVs) were identified using Deblur following standard demultiplexing and quality filtering using the Quantitative Insights into Microbial Ecology pipeline (QIIME2) (Bolyen et al., 2019). As described in our previous work (Lutz et al., 2019), libraries containing <1000 sequence reads were filtered out of the data set, at which point all negative controls were also removed as they contained <1000 reads/library. Skin, oral, and gut libraries were rarefied to an even read depth of 5000 reads, 1000 reads, and 1000 reads per library, respectively, based on rarefaction curve estimates. All 16S rRNA sequence data are publicly available via the QIITA platform (<https://qiita.ucsd.edu>) under the study identifier (ID) 11815 and the European Bioinformatics Institute (EBI) under accession number PRJEB32520; additional sequence library information is provided in Table S2. Code for sequence processing and analyses can be viewed at <https://github.com/hollylutz/BatMP>.

## 2.3 | Statistical analyses

Alphadiversity and betadiversity analyses were performed using the programming language R (R Core Team, 2019) and packages vegan2.4-2 (Oksanen et al., 2018), phyloseq (McMurdie & Holmes, 2013), dplyr (Wickham et al., 2018), and ggplot2 (Wickham, 2016). Differences in mean alphadiversity measures (observed richness and Shannon index metrics) between ectoparasitized and nonparasitized bats within families were assessed using the Kruskal-Wallis test. PERMANOVA tests for differences in betadiversity by host family, ectoparasite status, and locality were performed using the adonis2 function (R package vegan2.4-2; Oksanen et al., 2018), with 1000 permutations.

We evaluated differences among skin associated microbial ASVs between parasitized and nonparasitized bats grouped at the host family level by ranking multinomial regression coefficients (hereafter referred to as ranked differentials). This approach, implemented using the program Songbird (Morton et al., 2019), relies on estimated centered log ratios of features between sample groupings and thereby surpasses the need for absolute measures of feature differentiation between groupings. Songbird multinomial regressions were run on nonrarefied data for 100,000 epochs with a batch size of three, minimum feature count of five, a learning rate of 1e-5, and a differential prior of 0.50, with both ectoparasite status and sampling location included in our models to account for site-specific effects on the skin microbiome. Ranked differentials were visualized using the program Qurro (Fedarko, 2020).

To examine whether skin microbial communities differ in stability and structure between parasitized and nonparasitized bats, we reconstructed skin microbial networks using the R package Sparse Inverse Covariance Estimation for Ecological Association Inference (SIEC-EASI) (Kurtz et al., 2015). All network data sets were filtered to contain only ASVs that appeared in at least three individuals and all sites within each respective data set and consisted of skin microbial libraries grouped by host family and ectoparasite status. Network results produced with SIEC-EASI were summarized using the R packages CAVnet (Cardona, 2017) and igraph (Csardi & Nepusz, 2006). Network stability was assessed by sequentially removing network nodes (ordered by degree and betweenness centrality) and observing natural connectivity (i.e., eigenvalue of the graph adjacency matrix) as nodes are removed.

## 3 | RESULTS

### 3.1 | Microbiome, ectoparasite, and malarial parasite sampling and detection

We sampled 283 individuals representing eight species from four chiropteran families (Hippotideridae, Miniopteridae, Rhinolophidae,

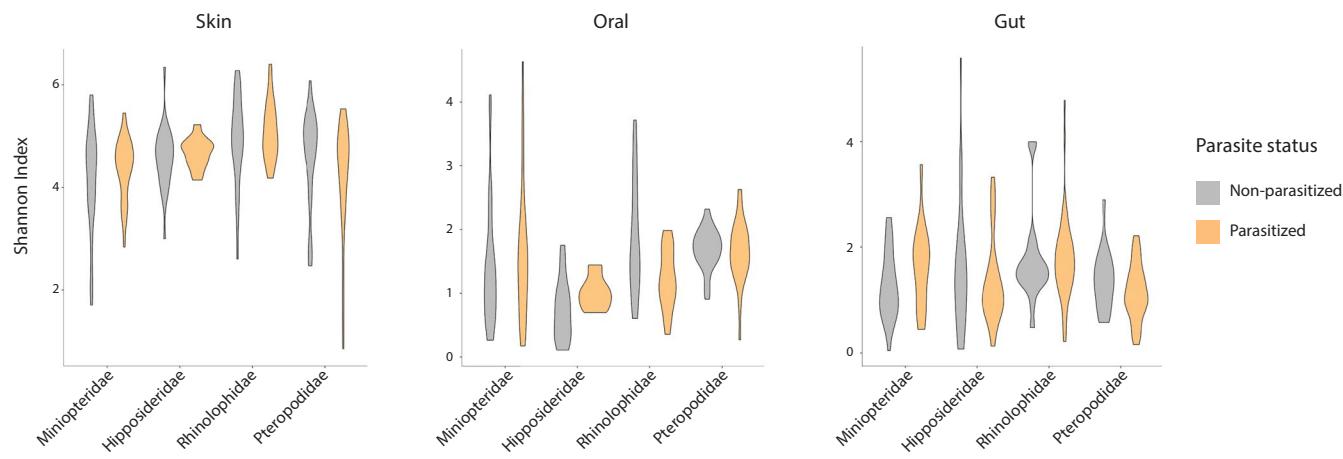
and Pteropodidae). Rarefaction and quality filtering of 16S rRNA libraries resulted in the retention of 237 skin samples (29,270 ASVs, rarefied to 5000 reads), 202 oral samples (3361 ASVs, rarefied to 1000 reads), and 230 gut samples (5771 ASVs, rarefied to 1000 reads) for microbiota profiling (Figure 1; Table 1). Hippoboscoid bat flies were recovered from all host taxa, with an average prevalence of 50.5% ( $SD \pm 13.0\%$ ). Malarial parasitism was restricted almost entirely to the family Miniopteridae, within which prevalence ranged from 47% to 65% (mean  $53.3\% \pm 10.5\%$ ). All malarial parasites observed in miniopterids belonged to the haemosporidian genus *Polychromophilus*, and shared 99%–100% sequence similarity to Cytochrome b lineages previously identified in Kenyan and Uganda miniopterid bats (Lutz et al., 2016). We observed only two individuals of the species *Rhinolophus clivosus acrotis* (Rhinolophidae) to be positive for malarial parasites, which belonged to the genus *Nycteria* and exhibited 98% sequence similarity to a Cytochrome b lineage previously identified in Uganda. All other bats were negative for haemosporidia by molecular and microscopic analyses (Table S1).

### 3.2 | Associations between the bat microbiota and ectoparasitism

No differences in alphadiversity measures were observed between parasitized and nonparasitized bats at the family or species levels for skin, oral, or gut microbiota ( $p > .05$ , Kruskal-Wallis) (Figure 2; Figure S1), nor were alphadiversity differences observed between host families, species, or sampling sites ( $p > .05$ , Kruskal-Wallis). Subtle but significant differences in skin-associated bacterial betadiversity were observed between parasitized and nonparasitized bats for both weighted and unweighted UniFrac metrics ( $p < .005$ , PERMANOVA). Differences among the gut and oral microbiota were only observed using unweighted but not weighted UniFrac metric ( $p < .002$ , PERMANOVA) (Table 2), suggesting that differences were driven by the compositional variance of rare taxa. We also observed significant differences between the skin, oral, and gut microbiota based on sampling locality and host family ( $p < .001$ , PERMANOVA). Subsequent

**TABLE 1** Bat skin, oral, and gut microbiome sampling ( $n$  = count of 16S rRNA libraries retained post-filtering), total ASVs recovered by sample type, and parasite prevalence ( $n_p/n$ ) of bat flies (Hippoboscidae) and malarial parasites (Haemosporidia)

Host taxonomy		16S rRNA libraries ( $n$ )			Parasite prevalence $n_p/n$ (%)	
Family	Species	Skin	Oral	Gut	Bat flies	Malarial parasites
Hippotideridae	<i>Hipposideros caffer</i>	39	37	43	17/47 (36%)	0/47 (0%)
Miniopteridae	<i>Miniopterus africanus</i>	22	20	22	9/22 (41%)	11/22 (50%)
	<i>Miniopterus minor</i>	16	15	15	11/17 (65%)	11/17 (65%)
	<i>Miniopterus natalensis</i>	41	31	31	20/47 (43%)	21/47 (45%)
Pteropodidae	<i>Rousettus aegyptiacus</i>	34	33	32	19/39 (60%)	0/39 (0%)
	<i>Stenonycteris lanosus</i>	32	24	37	13/37 (35%)	0/37 (0%)
Rhinolophidae	<i>Rhinolophus clivosus acrotis</i>	32	25	21	19/32 (59%)	2/32 (6%)
	<i>Rhinolophus eloquens</i>	21	18	24	17/26 (65%)	0/26 (0%)
Total ASVs		29,270	3361	5771		



**FIGURE 2** Alphadiversity among the skin, oral, and gut microbiota of bats grouped by family and ectoparasite status

**TABLE 2** PERMANOVA analyses of betadiversity bat skin, oral, and gut microbiota versus ectoparasite status (formula: betadiversity distance ~ host family/ectoparasite status, strata = host family, permutations = 1000; degrees of freedom = 4)

	β diversity metric	SumIfSqs	R <sup>2</sup>	F	Pr(>F)
Skin	Unweighted UniFrac	1.385	.022	1.399	.005*
	Weighted UniFrac	0.543	.033	2.288	.002*
Oral	Unweighted UniFrac	1.759	.031	1.750	.002*
	Weighted UniFrac	0.162	.012	0.918	.510
Gut	Unweighted UniFrac	1.771	.032	1.976	.002*
	Weighted UniFrac	0.201	.018	1.144	.265

\*Denotes significance of Pr(>F) < 0.05 with FDR correction

analyses therefore assessed taxonomic families independently, and locality was taken into account as described in the methods for differential abundance and network analyses (section 2.3).

Multinomial regression analysis of nonrarefied skin microbiome data using Songbird found models that included ectoparasite status of hosts significantly outperformed null models (Miniopteridae pseudo  $Q^2 = 0.12$ , Hipposideridae pseudo  $Q^2 = 0.28$ , Rhinolophidae pseudo  $Q^2 = 0.17$ , Pteropodidae pseudo  $Q^2 = 0.26$ ), allowing us to identify a number of ASVs potentially associated with ectoparasitism (Figure 3). The bacterial order Actinomycetales exhibited the greatest number of ASVs that were differentially abundant between parasitized and nonparasitized bats. Indeed, eight of 14 ASVs found to be consistently associated with the presence or absence of ectoparasites in all four host families belonged to the order Actinomycetales, with the remaining six belonging to the order Bacillales (phylum Firmicutes) and orders Burkholderiales, Pseudomonadales, Rhizobiales, and Sphingomonadales (phylum Proteobacteria) (Table 3).

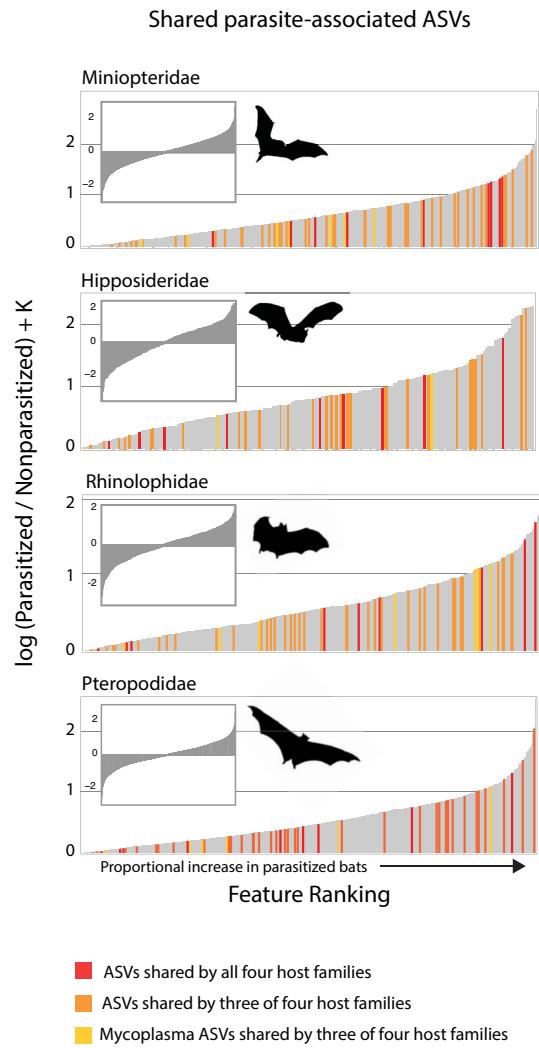
Network analyses revealed striking differences in the topology and stability of the skin microbiome in parasitized versus nonparasitized bats, with a significant decrease in cluster size ( $p < .05$ , Mann-Whitney-Wilcoxon rank sum test) and median node degree ( $p < .05$ , t-test), as well as a significant reduction in network connectivity ( $p < .05$ , t-test) for parasitized bats from three of the four bat families examined (pteropodids being the exception) (Figure 4).

### 3.3 | Host microbiome and haemosporidian parasitism

Of the bat taxa sampled, only species belonging to the family Miniopteridae exhibited malarial parasitism adequate for statistical analysis. We observed no differences in alphadiversity of the skin, oral, and gut microbiota of bats based on malarial infection status but identified significant differences between unweighted UniFrac betadiversity of the oral microbiota between malarial and nonmalarial bats ( $p < .002$ , PERMANOVA) (Table 4). Multinomial regression analyses identified a number of bacterial ASVs associated with malarial parasitism. Two ASVs exhibiting the greatest proportional increase in malaria positive bats belonged to the species *Pantoea agglomerans* and the genus *Acinetobacter*. ASVs most strongly associated with absence of malarial parasites belonged to the family Pasteurellaceae (Figure 5).

## 4 | DISCUSSION

In this study we identify associations between obligate ectoparasitic bat flies and the skin microbiome of four Afrotopical bat families, and limited association between rare taxa in the gut and oral microbiota. Network analyses identified consistent, stable, and taxonomically rich clusters of bacteria on the skin of nonectoparasitized bats,



**FIGURE 3** Ranked differential features associated with skin of ectoparasitized bats grouped by host family; full range of ranked features (including negatively associated features) shown in grey inset. Highlighted features include those observed in all four bat families (red), those observed in three of the four bat families (orange), and features belonging to the genus *Mycoplasma* that were also shared by three of four bat families. Bars of highlighted features have been enlarged for clarity

compared to relatively disconnected and apparently transient bacteria on the skin of bats harbouring ectoparasites. In addition to these links between ectoparasitism and the bat microbiome, we found a significant association between the oral microbiota and infection by malarial parasites among bats belonging to the family Miniopteridae. These results are the first to examine links between the microbiota and eukaryotic parasitism in wild bats and support the hypothesis that parasitism may be in part mediated by host-associated bacteria, or alternatively that the presence of ectoparasites may be modifying the host-associated skin microbiota.

We found a number of ASVs belonging to the genus *Mycoplasma* that were associated with the presence of ectoparasites across multiple bat families, as well as ASVs that were positively associated with

ectoparasitism in all four bat families studied, suggesting possible convergence of bacterial associations with hippoboscoid ectoparasitism among these hosts. Mycoplasmas are widespread and have been associated with insect vectors of disease in the wild (Aguilar et al., 2019). It is possible that the *Mycoplasma* ASVs identified in this study are associated with or transmitted by ectoparasitic bat flies, but we are unable to determine the directionality of *Mycoplasma* (and other bacterial) associations within the scope of this study. Additional bacteria found to positively correlate with ectoparasitism included Actinomycetales ASVs in the genera *Corynebacterium*, *Dermacoccus*, *Janibacter*, and *Kocuria*. As suggested by studies of human-mosquito interactions (Busula, Takken, et al., 2017; Robinson et al., 2018; Verhulst et al., 2011), bacteria positively associated with increased rates of blood-feeding dipteran host selection may be producing VOCs on which the insects rely to identify their hosts. Bacteria that are negatively associated with such insects may be consuming the products of the former, or may be producing VOCs of their own that mask those of the former (suggested by Verhulst et al., 2011). Some bacteria in the genera we identified as positively correlated with ectoparasitism have been shown experimentally to produce VOCs that are attractive to other host-seeking hematophagous arthropods including anopheline mosquitoes (Verhulst, Andriessen, et al., 2010; Verhulst, Takken, et al., 2010) and *Rhodnius prolixus* kissing bugs (the primary vector of Chagas disease) (Tabares et al., 2018), and it is therefore interesting to find them consistently associating with blood-feeding hippoboscoid across divergent bat families. Although we did not quantify or characterize VOCs in this study, we hypothesize that the bacterial ASVs in these genera may be producing similar VOCs, such as sulphur-containing compounds identified in the head space of *Corynebacterium minutissimum* (e.g., dimethylsulphide, dimethyltetrasulphide, octasulphur) associated with anopheline mosquito attraction to humans (Verhulst, Andriessen, et al., 2010). To better understand the mechanisms underlying these correlations in wild populations, future experiments should consider including sampling and characterization of VOCs *in vivo* through mass spectrometry and other metabolomics approaches.

Network analyses showed that presence of hippoboscoid parasites was significantly associated with a reduction in the size and stability of skin microbial clusters, with nonparasitized bats exhibiting fewer clusters that contained greater microbial diversity. The differences in these network statistics were shared by all four bat families in the study and were significant for all but pteropodid fruit bats. Similar patterns have been observed in human-mosquito interactions, in which individuals with lower bacterial diversity on the skin are significantly more attractive to blood-seeking mosquitoes than individuals with higher diversity (Verhulst et al., 2011). In humans, skin bacteria play a known role in attracting mosquitoes via their production of VOCs and studies have shown that variation in skin microbial community composition can increase or decrease human attractiveness to blood-seeking mosquitoes (Busula, Takken, et al., 2017; Verhulst et al., 2009, 2011). Similar mechanisms may be at play in the bat-ectoparasite system, particularly given the shared evolutionary history of dipterans (Wiegmann, 2011).

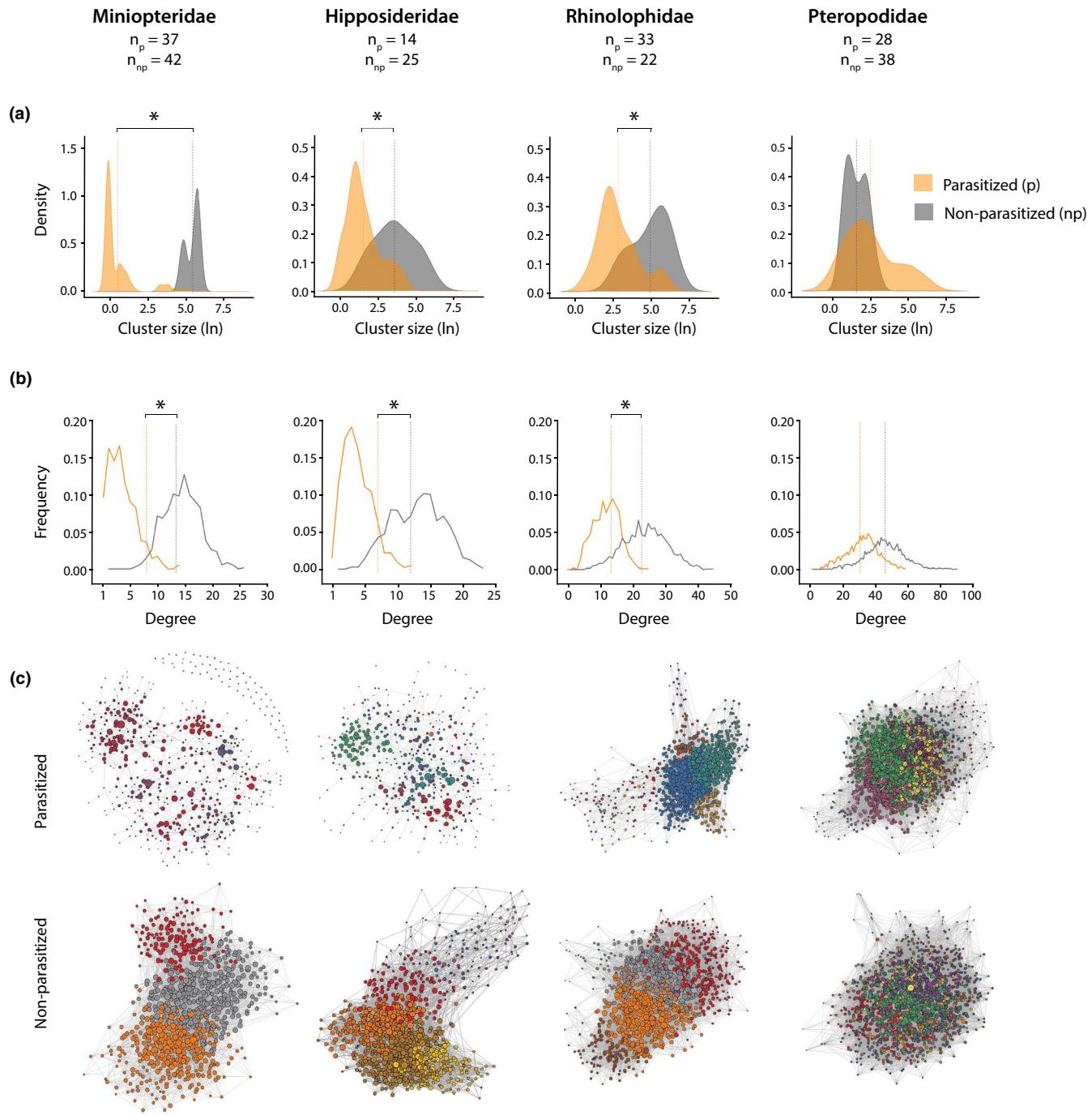
**TABLE 3** ASVs represented in all four host families as either positively (+) or negatively (−) associated with ectoparasitism

ASV taxonomy		Ectoparasite association
Actinobacteria		
Actinobacteria		
Actinomycetales		
Corynebacteriaceae	<i>Corynebacterium</i> sp.	+
Dermabacteraceae	<i>Brachybacterium</i> sp.	−
Dermacoccaceae	<i>Dermacoccus</i> sp.	+
Geodermatophilaceae	<i>Geodermaphilus</i> sp.	−
Intrasporangiaceae	<i>Janibacter</i> sp.	+
Microbacteriaceae	<i>Frondhabitans</i> sp.	−
Micrococcaceae	<i>Kocuria</i> sp.	+
Pseudonocardiaceae	<i>Actinomycetospora</i> sp.	−
Firmicutes		
Bacilli		
Bacillales		
Bacillaceae	<i>Lentibacillus</i> sp.	+
Proteobacteria		
Alphaproteobacteria		
Rhizobiales		
Rhizobiaceae	<i>Agrobacterium</i> sp.	+
Betaproteobacteria		
Sphingomonadales		
Sphingomonadaceae	<i>Kaistobacter</i> sp.	+
	<i>Sphingomonas</i> sp.	−
Burkholderiales		
Oxalobacteraceae	<i>Janthinobacterium lividum</i>	+
Gammaproteobacteria		
Pseudomonadales		
Moraxellaceae	<i>Acinetobacter</i> sp.	+

Associations between the oral microbiome and malarial parasitism were supported by unweighted UniFrac diversity metric analysis, suggesting that ASVs contributing to observed differences are relatively rare among the oral microbiota. Upon further investigation of differential microbiota abundances, we found a bacterial ASV belonging to the species *Pantoea agglomerans* to be most strongly associated with miniopterid bats infected with malaria. Interestingly, *P. agglomerans* has been the target of numerous paratransgenesis experiments aimed at controlling the transmission of malarial parasites (*Plasmodium* spp.) in anopheline and culicine mosquitoes (Dinparast Djadid, 2011). A common constituent of the dipteran midgut, *P. agglomerans* has been associated with the production of “immunopotentiator from *P. agglomerans* 1” (IP-PA1), a broad-spectrum antibiotic effective against bacterial, fungal, and viral pathogens (Dutkiewicz et al., 2016). How and why this bacterium is associated with the oral microbiome of malarial bats requires more in-depth investigation. It is possible, although speculative, that the ingestion of bat flies containing *P. agglomerans* symbionts is the underlying driver of *P. agglomerans* association with the oral microbiome of miniopterid

bats (Ramanantsalama et al., 2018). As no other bat groups experienced rates of malarial parasitism adequate for statistical analyses, we were unable to explore this relationship further. Future studies that incorporate greater sampling of malaria-positive species may reveal more robust microbial associations, as have been documented in numerous experiments with controlled rodent and human malaria infections (Busula, Takken, et al., 2017; Busula, Verhulst, et al., 2017; de Boer et al., 2017; De Moraes et al., 2014; Penn & Potts, 1998; Robinson et al., 2018).

Although we cannot ascertain causality of differences in the microbial composition of skin in this study, our results support the hypothesis that these differences may provide a mechanism by which ectoparasites can locate or distinguish hosts. Alternatively, observed differences in microbial composition could result from microbial transfer from parasites to hosts – indeed *Mycoplasma* bacteria, which were commonly associated with ectoparasitism in our study, are a common constituent of the hippoboscoid bat fly microbiome (Speer et al., 2020). Bat flies spend the majority of their lives living on the skin and fur of their chiropteran hosts, providing ample



**FIGURE 4** Network characteristics of the skin microbiome among ectoparasitized and nonparasitized bats grouped by host family, including (a) cluster size density (\* indicates  $p$ -value  $< .05$ , Mann-Whitney-Wilcoxon rank sum test), (b) degree distribution (\* indicates  $p$ -value  $< .05$ , t-test), and (c) Fruchterman-Reingold network topology coloured by individual network clusters

opportunity for the exchange of microbes between bat and bat fly. Moreover, even some host species-specific bat flies readily transfer between interspecific host individuals (Overall, 1980; Witsenburg et al., 2015) effectively utilizing the host population as habitat. Our analysis of the skin microbiota identified significant differences in microbial betadiversity as well as differentially abundant bacteria between parasitized and nonparasitized bats at the host family level, but we were unable to ascertain the origin of these bacteria. Bacteria

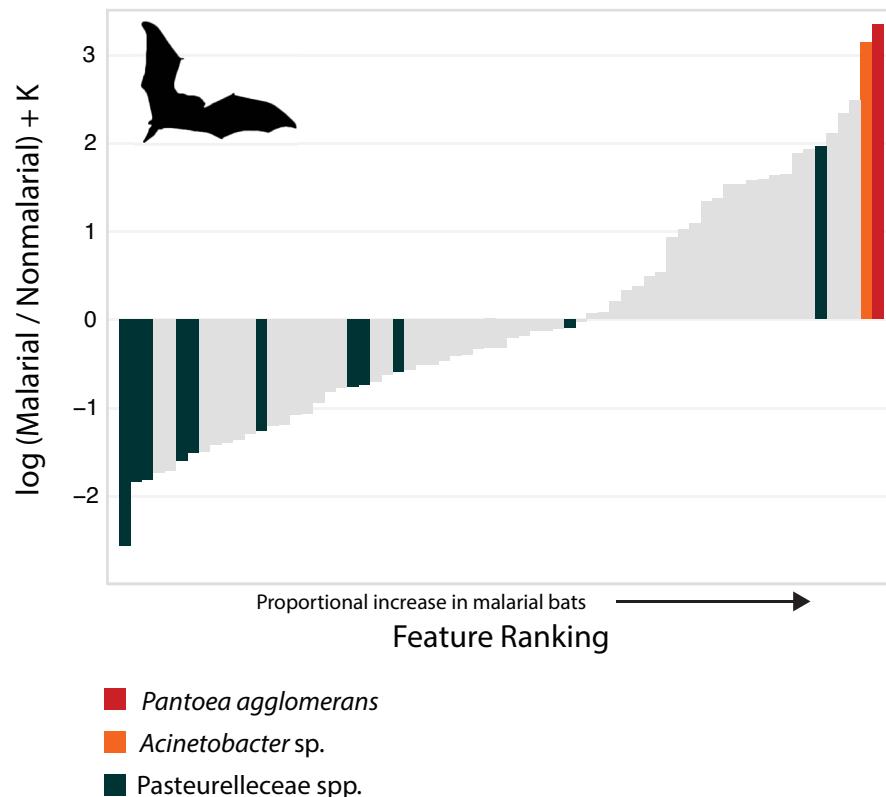
associated with parasitized bats may have originated in the bat flies themselves or may have been acquired from the environment. Given the known effect of locality and apparent absence of host phylogenetic signal in microbial community composition of skin (Lutz et al., 2019), one possible explanation is that local environmental variables play a greater role in determining host-bacteria associations in bats. Indeed, in North America, multiple bat species have been found to share many bacterial genera with soil and plant material (Avena et al.,

**TABLE 4** PERMANOVA analyses of betadiversity of miniopterid bat skin, oral, and gut microbiota and malarial status (formula: betadiversity distance ~ host species/malarial status, strata = host species, permutations = 1000; degrees of freedom = 3)

	$\beta$ diversity metric	SumOfSqs	R <sup>2</sup>	F	Pr(>F)
Skin	Unweighted UniFrac	0.762	.037	1.033	.37
	Weighted UniFrac	0.153	.026	0.927	.644
Oral	Unweighted UniFrac	1.317	.070	1.632	.002*
	Weighted UniFrac	0.260	.056	1.276	.237
Gut	Unweighted UniFrac	0.858	.045	0.998	.644
	Weighted UniFrac	0.242	.037	0.831	.682

\*Denotes significance of Pr(>F) < 0.05 with FDR correction.

**FIGURE 5** Ranked differential features associated with the oral microbiome of miniopterid bats. Positive and negative values are associated with parasitized and non-parasitized bats, respectively. Dark green bars indicate ASVs belonging to the bacterial family Pasteurellaceae; red and orange bars indicate top two ASVs associated with malarial parasitism, belonging to bacterial taxa *Pantoea agglomerans* and *Acinetobacter* sp



2016), and the bat skin microbiome has previously been documented to shift at the colony level over time (Kolodny et al., 2019). Thus, local conditions and bacterial composition of bat roosts probably play an important role in driving the composition of skin bacteria, thereby potentially influencing which individuals become parasitized. Ecological and behavioural studies of bats have also observed that many species exhibit localized migration between caves, and it has been suggested that this behaviour may be associated with the avoidance of ectoparasitic bat flies (Lewis, 1995; Reckardt & Kerth, 2007; Wilkinson, 1985). Longitudinal analyses of individuals will provide much-needed insight into the effect of local migration on skin microbial community composition and ectoparasite prevalence.

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#### AUTHOR CONTRIBUTION

H.L.L. conceived and designed the study, and performed field work, laboratory and data analyses. J.A.G. guided statistical analyses and provided laboratory support. C.W.D. provided taxonomic

identification of hippoboscoid parasites. All authors contributed to writing the manuscript.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## OPEN RESEARCH BADGES



This article has earned an Open Data Badge for making publicly available the digitally-shareable data necessary to reproduce the reported results. The data is available at <https://qiita.ucsd.edu/study/description/11815>.

## DATA AVAILABILITY STATEMENT

All 16S rRNA sequence data have been made publicly available via the QIITA platform (<https://qiita.ucsd.edu>) under the study identifier (ID) 11815 and the European Bioinformatics Institute (EBI) under accession number PRJEB32520. Code for sequence processing and analyses can be viewed at <https://github.com/hollylutz/BatMP>. Host and parasite vouchers are accessioned at the Field Museum of Natural History (Chicago, IL, USA).

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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