Tripartite associations between Afrotropical 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 have to our knowledge never been tested in wild vertebrates. In this study, we use Afrotropical 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 identify 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 non-ectoparasitized bats. We also identify 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.

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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 have to our knowledge never been tested in wild vertebrates. In this study, we use Afrotropical 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 identify 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 non-ectoparasitized bats. We also identify 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.

Keywords: microbiome, bats, Chiroptera, Hippoboscoidea, malaria, Haemosporidia, Afrotropics

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 (1, 2), and mounting evidence suggests that bacterial symbionts may be responsible for mediating host-parasite interactions in ways that could ultimately shape host evolution (3). For example, studies of human and anthropophilic mosquito interactions have found that the human skin microbiome can influence mosquito feeding preference, thereby affecting transmission patterns of vector-borne pathogens (*e.g.* WNV, yellow fever, dengue, malaria, etc.), and potentially imposing selective pressures on human populations (4). Conversely, parasites may influence the relative abundance of host-associated microbes in ways that facilitate parasite transmission, as 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* (5-7). 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.

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 (8-11). Bats are also associated with microbial pathogens of importance for human health (*e.g.* *Bartonella* , *Pasteurella* , SARS coronaviruses, filaviruses, rabies, Hendra and Nipah viruses (12-15)), and serological surveys have supported the role of Afrotropical bats as reservoirs for a number of viruses (16-18), 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: Hippoboscoidea), which are obligate blood-feeding parasites of bats, are known to transmit several pathogens of human relevance (19, 20) and also are the primary hosts for bat-specific malarial parasites (Apicomplexa: Haemosporida). Bat flies are typically host-specific (21, 22) and prevalence can vary widely within a single species across different geographic locations (23). Malarial parasites vectored by bat flies are also typically host-specific but are observed less frequently in many bats relative to ectoparasitic bat flies.

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 (24). 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 (21, 25). The proximal mechanism by which parasites locate "preferred" hosts is also unknown, but as has been observed in other hematophagous parasites (e.g. mosquitoes (26-28), tse-tse flies (29)), may involve 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 (30, 31).

Here, we build on previous broad-scale studies of Afrotropical bat-associated microbes (32, 33) to characterize parasitism by bat flies and haemosporidia in four widespread lineages of bats. Using these data, we test the hypothesis that the bat microbiome (skin, oral, and gut) is linked to parasitism by two obligate, host-specific eukaryotic parasites.

2. METHODS & MATERIALS

2.1 Host and parasite sampling

Sampling was conducted at sixteen field sites in Kenya and Uganda from August to October 2016, using a combination of mist-netting and hand-netting. 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 3mm sterile disposable biopsy punches (Integra^{TM}Miltex®). Biopsy samples from each individual were combined and stored in sterile 95% ethanol. Whole tongues were collected (from apex to root) and stored in 95% ethanol for oral microbiome analysis. We collected ˜2-4mL 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 lN2. Following euthanasia, skin, and blood sampling, bats were fumigated in ethyl acetate for 15 minutes and then examined for ectoparasites. 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 using a Leica MZ16 stereozoom microscope. Collected specimens were compared to relevant taxonomic keys, to descriptions in the alpha-literature, and to reference collections 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. (2015). In brief, DNA was extracted from whole blood using Qiagen DNeasy (Qiagen, Valencua, CA) 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;S2).

2.2 Microbial DNA extraction, library preparation, and data generation

DNA was extracted using the MoBio PowerSoil 96 well soil DNA isolation kit (catalog no. 12955-4; MoBio, Carlsbad, CA, USA) following the standard Earth Microbiome Project protocol (http://www.earthmicrobiome.org/). PCR amplification and sequencing were performed as previously described in Lutz et al. (2019). Amplicon sequence variants (ASVs) were identified using Deblur following standard demultiplexing and quality filtering using the Quantitative Insights into Microbial Ecology pipeline (QIIME2) (34). 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 (35) and packages vegan2.4-2 (36), phyloseq (37), dplyr (38), and ggplot2 (39). Differences in mean alphadiversity measures (observed richness and Shannon index metrics) between ectoparasitized and non-parasitized bats within families were assessed using the Kruskal-Wallis test. PERMANOVA tests for differences in betadiversity were performed using the adonis2 function (R package vegan2.4-2 (36), with 1000 permutations.

We evaluated differences among skin associated microbial ASVs between parasitized and non-parasitized 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 (40), 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 for 100,000 epochs with a batch size of 3, minimum feature count of 5, a learning rate of 1e-5, and a differential prior of 0.50. Ranked differentials were visualized using the program Qurro (41).

To examine whether skin microbial communities differ in stability and structure between parasitized and non-parasitized bats, we reconstructed skin microbial networks using the R package Sparse Inverse Covariance Estimation for Ecological Association Inference (SPIEC-EASI) (42). All network datasets were filtered to contain only ASVs that appeared in at least three individuals within each respective dataset and consisted of skin microbial libraries grouped by host family and ectoparasite status. Network results produced with SPIEC-EASI were summarized using the R packages CAVnet (43) and igraph (44). Network stability was assessed by sequentially removing network nodes (ordered by betweenness centrality and degree) 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 (Hipposideridae, 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 (3,361 ASVs, rarefied to 1000 reads), and 230 gut samples (5,771 ASVs, rarefied to 1000 reads) for microbiota profiling (Table 1). Hippoboscoid ectoparasites were recovered from all host taxa, with an average prevalence of 51% (SD±13%). Malarial parasitism was restricted almost entirely to the family Miniopteridae, within which prevalence ranged from 47-65% (mean 53%±11%). 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 (11). We observed only two non-miniopterid 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 were observed between skin, oral, or gut microbial alphadiversity and ectoparasitized or non-ectoparasitized bats at the host family or species levels ($p > 0.05$, Kruskal-Wallis) (Figure 2). However, subtle but significant differences in skin-associated bacterial betadiversity were observed between parasitized and non-parasitized bats for both weighted and unweighted UniFrac metrics ($p < 0.005$, PERMANOVA). Differences were only observed among the gut and oral microbiota using unweighted but not weighted UniFrac metric ($p < 0.002$, PERMANOVA) (Table 2), suggesting that differences were driven by the compositional variance of rare taxa.

Multinomial regression analysis of the skin microbiome 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 non-parasitized bats. Indeed, eight of fourteen 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 non-parasitized bats, revealing a significant decrease in cluster size ($p< 0.05$, Mann-Whitney-Wilcoxon rank sum test) and median node degree ($p < 0.05$, t test), as well as a significant reduction in network connectivity ($p < 0.05$, 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 non-malarial bats ($p < 0.002$, 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 Afrotropical 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 non-ectoparasitized bats, compared to relatively disconnected and apparently transient bacteria on the skin of bats harboring 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.

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. Bacteria positively correlating with ectoparasitism included Actinomycetales ASVs in the genera *Corynebacterium* ,*Dermacoccus* , *Janibacter* , and *Kocuria* . Some bacteria in these genera have been shown experimentally to produce VOCs that are attractive to other host-seeking hematophagus arthropods including anopheline mosquitoes (26, 45) and *Rhodnius prolixus* kissing bugs (the primary vector of Chagas disease) (46), 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 sulfur-containing compounds identified in the head space of*Corynebacterium minutissimum* (*e.g.* dimethylsulfide, dimethyltetrasulfide, octasulfur) associated with anopheline mosquito attraction to humans (45). Further validation is certainly needed.

Network analyses showed that presence of hippoboscoid parasites was significantly associated with a reduction in the size and stability of skin microbial clusters, with non-parasitized 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 (27). 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 (7, 27, 28). Similar mechanisms may be at play in the bat-ectoparasite system, particularly given the shared evolutionary history of dipterans (47).

As suggested by studies of human-mosquito interactions (7, 27, 48), 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. (27)). 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.

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 (49). 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 (50). How and why this bacterium is associated with the oral microbiome of malarial bats requires more in-depth investigation. 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 (5-7, 48, 51, 52).

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 (53). Bat flies spend the majority of their lives living on the skin and fur of their chiropteran hosts, providing ample opportunity for the exchange of microbes between bat and bat fly. Moreover, even some host-species specific bat flies readily transfer between intraspecific host individuals (54) 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 non-parasitized 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 (33), 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 (55), and the bat skin microbiome has previously been documented to shift at the colony level over time (56). Thus, local conditions and bacterial composition of bat roosts are likely playing an important role in driving the composition of skin bacteria, and thereby potentially influencing which individuals become parasitized. Ecological and behavioral studies of bats have also observed that many species exhibit localized migration between caves, and it has been suggested that this behavior may be associated with the avoidance of parasites and pathogens (57). 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 CONTRIBUTIONS

HLL conceived and designed study, and performed field work, laboratory and data analyses. JAG guided statistical analyses and provided laboratory support. CWD provided taxonomic identification of hippoboscoid parasites. All authors contributed to writing of the manuscript.

REFERENCES

1. Stevens JR, Noyes HA, & Gibson WC (1999) The ancient and divergent origins of the human pathogenic trypanosomes, Trypanosoma brucei and T. cruzi. *Parasitology* 118:107-116.

2. Larremore DB*, et al.* (2015) Ape parasite origins of human malaria virulence genes. *Nature Communications* 6(1).

3. McFall-Ngai M*, et al.* (2013) Animals in a bacterial world, a new imperative for the life sciences. *Proc Natl Acad Sci U S A*110(9):3229-3236.

4. Kwiatkowski DP (2005) How malaria has affected the human genome and what human genetics can teach us about malaria. *Am. J. Hum. Genet.* 77:171-190.

5. Penn D & Potts WK (1998) How do major histocompatibility complex genes influence odor and mating preferences? *Advances in Immunology* 69:411-436.

6. Busula AO, Verhulst NO, Bousema T, Takken W, & de Boer JG (2017) Mechanisms of Plasmodium-Enhanced Attraction of Mosquito Vectors. *Trends in Parasitology* 33(12):961-973.

7. Busula AO, Takken W, JG DEB, Mukabana WR, & Verhulst NO (2017) Variation in host preferences of malaria mosquitoes is mediated by skin bacterial volatiles. *Med Vet Entomol* 31(3):320-326.

8. Dittmar K, Morse, Solo F., Dick, Carl W., Patterson, Bruce D. (2015) Bat fly evolution from the Eocene to the present (Hippoboscoidea, Streblidae and Nycteribiidae). *Parasite Diversity and Diversification: Evolutionary Ecology Meets Phylogenetics* , ed S. Morand BRK, D. T. J. Littlewood (Cambridge University Press, Cambridge, U. K.).

9. Dick CW, Patterson, B. D. (2006) *Micromammals and Macroparasites: From Evolutionary Ecology to Management* (Springer, Kato Bunmeisha, Japan).

10. Schaer J*, et al.* (2013) High diversity of West African bat malaria parasites and a tight link with rodent Plasmodium taxa.*Proceedings of the National Academy of Sciences* 100:17415-17419.

11. Lutz HL*, et al.* (2016) Diverse sampling of East African haemosporidians reveals chiropteran origin of malaria parasites in primates and rodents. *Mol Phylogenet Evol* 99:7-15.

12. Bausch DG, Schwarz, L. (2014) Outbreak of Ebola virus disease in Guinea: where ecology meets economy. *PLoS Negl Trop Dis*8(7):e3056.

13. Hayman DT, Bowen, R.A., Cryan, P.M., McCracken, G.F., O'Shea, T.J., Peel, A.J., Gilbert, A., Webb, C.T., Wood, J.L. (2013) Ecoogy of zoonotic infectious diseases in bats: current knowledge and future directions. *Zoonoses and Public Health* 60:2-21.

14. Billeter SA*, et al.* (2012) Bartonella species in bat flies (Diptera: Nycteribiidae) from western Africa. *Parasitology*139(3):324-329.

15. Sawatsky B RC, Weingartl HM, Czubet M. (2008) Hendra and Nipah Virus. *Animal Viruses: Molecular Biology.* , (Caister Academic Press, Norfolk, UK).

16. Towner JS*, et al.* (2009) Isolation of genetically diverse Marburg viruses from Egyptian fruit bats. *PLoS Pathog*5(7):e1000536.

17. Olival KJ & Hayman DT (2014) Filoviruses in bats: current knowledge and future directions. *Viruses* 6(4):1759-1788.

18. Amman BR*, et al.* (2015) A Recently Discovered Pathogenic Paramyxovirus, Sosuga Virus, is Present in Rousettus aegyptiacus Fruit Bats at Multiple Locations in Uganda. *J Wildl Dis* 51(3):774-779.

19. Goldberg TL, Bennett AJ, Kityo R, Kuhn JH, & Chapman CA (2017) Kanyawara Virus: A Novel Rhabdovirus Infecting Newly Discovered Nycteribiid Bat Flies Infesting Previously Unknown Pteropodid Bats in Uganda. *Scientific Reports* 7(1).

20. Bennett AJ, Paskey AC, Kuhn JH, Bishop-Lilly KA, & Goldberg TL (2020) Diversity, Transmission, and Cophylogeny of Ledanteviruses (Rhabdoviridae: Ledantevirus) and Nycteribiid Bat Flies Parasitizing Angolan Soft-Furred Fruit Bats in Bundibugyo District, Uganda.*Microorganisms* 8(5):750.

21. Dick CW & Patterson BD (2007) Against all odds: Explaining high host specificity in dispersal-prone parasites. *International Journal for Parasitology* 37:871-876.

22. Dick CW & Dittmar K (2014) Parasitic bat flies (Diptera: Streblidae and Nycteribiidae): host specificity and potential as vectors.*Bats (Chiroptera) as Vecotrs of Diseases and Parasities,*Parasitology Research Monographs, eds Klimpel S & Melhorn H (Springer-Verlag, Berlin Heidelberg), Vol 5, pp 131-155.

23. Urbieta GL, Torres JM, Anjos EACD, Carvalho CME, & Graciolli G (2019) Parasitism of Bat Flies (Nycteribiidae and Streblidae) on Bats in Urban Environments: Lower Prevalence, Infracommunities, and Specificity.*Acta Chiropterologica* 20(2):511.

24. Szentiványi T, Christe P, & Glaizot O (2019) Bat Flies and Their Microparasites: Current Knowledge and Distribution. *Frontiers in Veterinary Science* 6.

25. Dick CW & Patterson BD (2006) Obligate ectoparasites of bats.*Micromammals and Macroparasites* , eds Morand S, Krasnov B, & Poulin R (Springer, Tokyo), pp 179-194.

26. Verhulst NO, Takken W, Dicke M, Schraa G, & Smallegange RC (2010) Chemical ecology of interactions between human skin microbiota and mosquitoes. *FEMS Microbiol Ecol* 74(1):1-9.

27. Verhulst NO*, et al.* (2011) Composition of human skin microbiota affects attractiveness to malaria mosquitoes. *PLoS One*6(12):e28991.

28. Verhulst NO*, et al.* (2009) Cultured skin microbiota attracts malaria mosquitoes. *Malar J* 8:302.

29. Soni N, Chahda JS, & Carlson JR (2019) Odor coding in the antenna of the tsetse flyGlossina morsitans. *Proceedings of the National Academy of Sciences* 116(28):14300-14308.

30. Klowden MJ (2013) *Physiological Systems in Insects* (Academic Press, London) Third Edition Ed.

31. Ezenwa VO, Gerardo NM, Inouye DW, Medina M, & Xavier JB (2012) Animal behavior and the microbiome. *Science* 338(6104):198-199.

32. Se Jin Song JGS, Frédéric Delsuc, Jessica Metcalf, Katherine Amato, Michael W. Taylor, Florent Mazel,, Holly L. Lutz KW, Gary R. Graves, Gregory Humphrey, Jack A. Gilbert, Shannon J. Hackett,, Kevin P. White HRS, Sarah M. Kurtis,mJack Withrow, Thomas Braile, Matthew Miller,, Kevin G. McCracken JMM,

Vanessa O. Ezenwa, Allison Williams, Jessica M. Blanton,, & Valerie J. McKenzie RK (2020) Comparative analyses of vertebrate gut microbiomes reveal convergence between birds and bats.*mBio* 11(1):e02901-02919.

33. Lutz HL*, et al.* (2019) Ecology and host identity outweigh evolutionary history in shaping the bat microbiome. *mSystems*4(6):e00511-00519.

34. Bolyen E*, et al.* (2019) Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nature Biotechnology* 37(8):852-857.

35. Team RC (2019) R: A language and environment for statistical computing (R Foundation for Statistical Computing, Vienna, Austria).

36. Oksanen JB, F. G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, P. R., O'Hara, R. B., Simpson, G. L., Solymos, P., Stevens, M. H. H., Szoecs, E., Wagner, H. (2018) vegan: Community Ecology Package), 2.5-1.

37. McMurdie PJ & Holmes S (2013) phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One* 8(4):e61217.

38. Wickham H, François R, Henry L, & Müller K (2018) dplyr: A Grammar of Data Manipulation), R package version 0.7.6.

39. Wickham H (2016) *ggplot2: Elegant Graphics for Data Analysis*(Springer-Verlag, New York, NY).

40. Morton JT*, et al.* (2019) Establishing microbial composition measurement standards with reference frames. *Nature Communications* 10(1).

41. Fedarko MW*, et al.* (2020) Visualizing 'omic feature rankings and log-ratios using Qurro. *NAR Genomics and Bioinformatics* 2(2).

42. Kurtz ZD*, et al.* (2015) Sparse and compositionally robust inference of microbial ecological networks. *PLoS Comput Biol*11(5):e1004226.

43. Cardona C (2017) CAVNet: Creation Analysis and Visualization of Networks).

44. Csardi G, Nepusz, T. (2006) The Igraph Software Package for Complex Network Research. *InterJournal, Complex Systems* 1695.

45. Verhulst NO*, et al.* (2010) Differential Attraction of Malaria Mosquitoes to Volatile Blends Produced by Human Skin Bacteria.*PLoS ONE* 5(12):e15829.

46. Tabares M*, et al.* (2018) Behavioral responses of Rhodnius prolixus to volatile organic compounds released in vitro by bacteria isolated from human facial skin. *PLOS Neglected Tropical Diseases*12(4):e0006423.

47. Wiegmann BM*, et al.* (2011) Episodic radiations in the fly tree of life. *PNAS* 108(14):5690-5695.

48. Robinson A*, et al.* (2018) Plasmodium-associated changes in human odor attract mosquitoes. *Proc Natl Acad Sci U S A*115(18):E4209-E4218.

49. Dinparast Djadid N*, et al.* (2011) Identification of the Midgut Microbiota of An. stephensi and An. maculipennis for Their Application as a Paratransgenic Tool against Malaria. *PLoS ONE*6(12):e28484.

50. Dutkiewicz J, Mackiewicz B, Lemieszek MK, Golec M, & Milanowski J (2016) Pantoea agglomerans: a mysterious bacterium of evil and good. Part IV. Beneficial effects. *Annals of Agricultural and Environmental Medicine* 23(2):206-222.

51. De Moraes CM*, et al.* (2014) Malaria-induced changes in host odors enhance mosquito attraction. *Proc Natl Acad Sci U S A*111(30):11079-11084.

52. de Boer JG*, et al.* (2017) Odours of Plasmodium falciparum-infected participants influence mosquito-host interactions.*Sci Rep* 7(1):9283.

53. Kelly A. Speer TT, Alexis Brown, et al. (Parasite species identity and local community diversity mediate effects of habitat fragmentation on bacterial microbiomes. *Authorea* .

54. Overal WL (1980) Host-relations of the batfly Megistopoda aranea (Diptera: Streblidae) in Panama. 52: 1–20. *University of Kansas Science Bulletin* 52:1-20.

55. Avena CV*, et al.* (2016) Deconstructing the Bat Skin Microbiome: Influences of the Host and the Environment. *Front Microbiol* 7:1753.

56. Kolodny O*, et al.* (2019) Coordinated change at the colony level in fruit bat fur microbiomes through time. *Nat Ecol Evol*3(1):116-124.

57. Lewis SE (1995) Roost Fidelity of Bats: A Review. *Journal of Mammalogy* 76(2):481-496.

FIGURE LEGENDS

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 haemosporidian parasites. Illustration by Madison Erin Mayfield @MEMIllustration.

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

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 gray 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.

Figure 4. Network characteristics of the skin microbiome among ectoparasitized and non-parasitized bats grouped by host family, including A) cluster size density (* indicates p -value < 0.05, Mann-Whitney-Wilcoxon rank sum test), B) degree distribution (* indicates p -value < 0.05, t-test), and C) Fruchterman-Reingold network topology colored by individual network clusters.

Figure 5. Ranked differential features associated with oral of malarial miniopterid bats. Highlighted are the two highest-ranked features associated with presence of malarial parasites (*P. agglomerans*(red), *Acinetobacter* sp. (orange)), and the most abundant features associated with absence of malarial parasitism (ASVs in the Pasteurellaceae family (dark green)).

DATA AVAILABILITY STATEMENT

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. 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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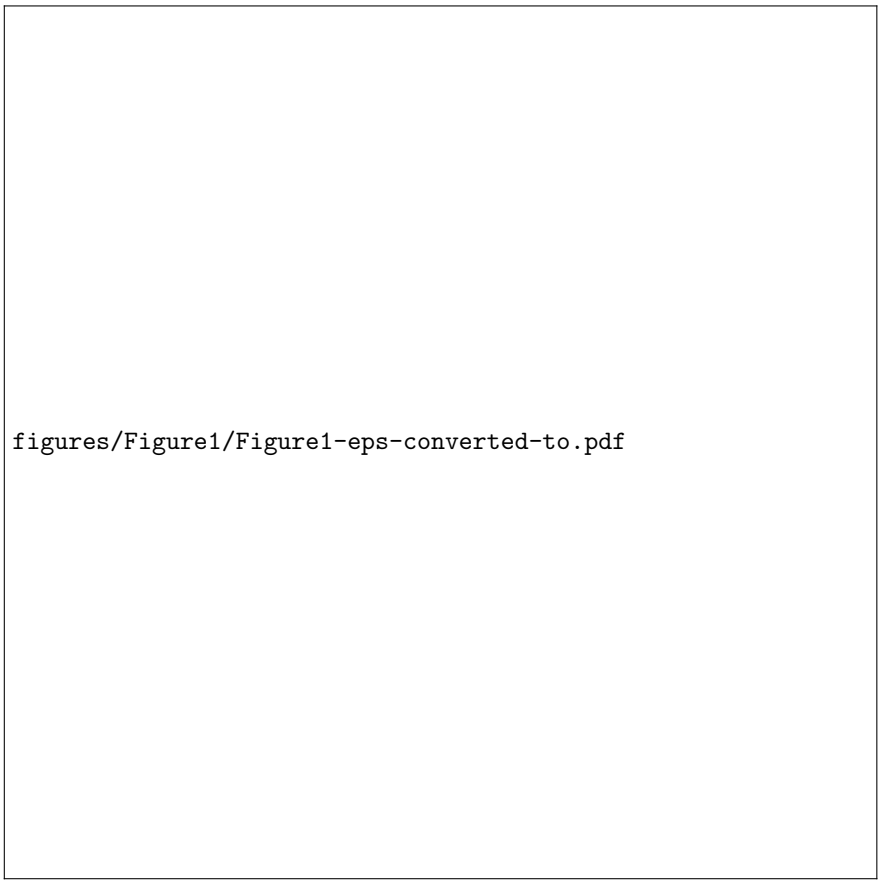

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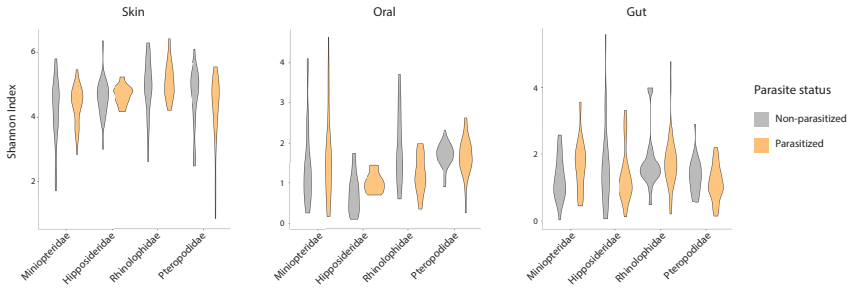

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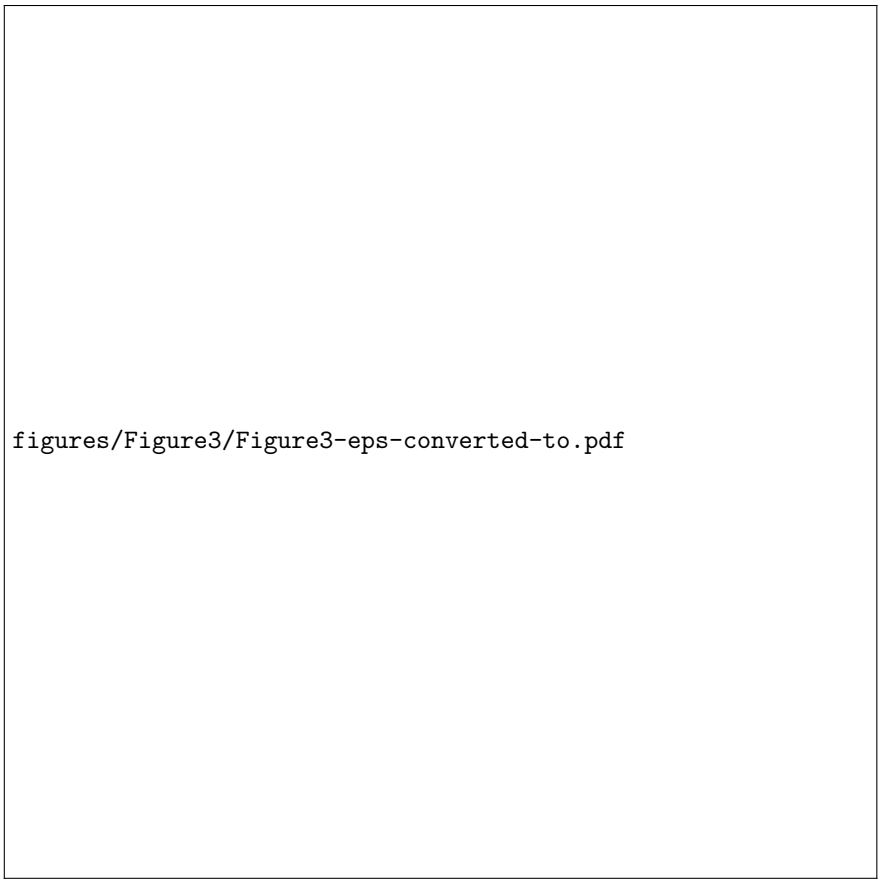

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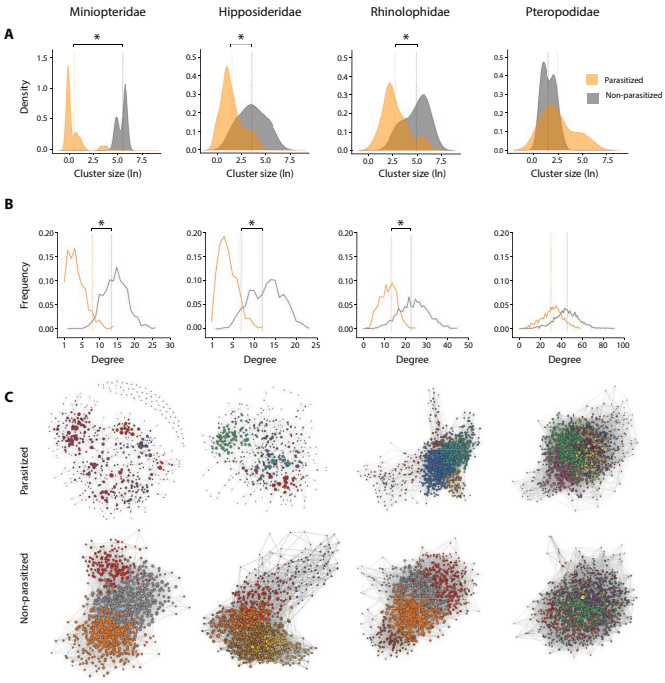

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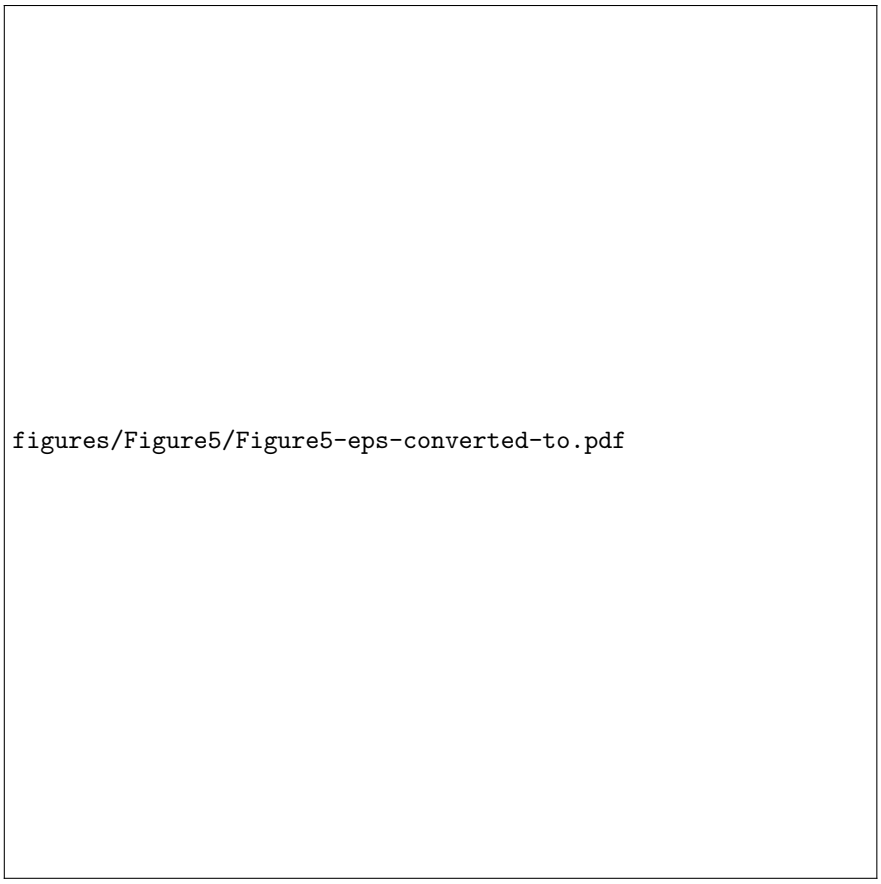

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