# Parasite species identity and local community diversity mediate effects of habitat fragmentation on bacterial microbiomes.

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**Abstract**

Arthropod ectoparasites generally have nutrient-poor diets and are dependent on their bacterial symbionts for nutrient acquisition, development, and immune response initiation. As the body of research on parasite-microbiome interactions continues to grow, it is becoming more apparent that the parasite is not an island that physically and biologically constrains the microbiome. Suitable habitat fragment size, isolation, and distance from a source are important variables influencing community composition of plants and animals, but the role of the environment in determining composition and variation of host-associated microbial communities is poorly known. It is hypothesized that evolution and ecology of an arthropod parasite will influence its microbiome more than broader environmental factors, but this hypothesis has not yet been tested. To compare the relative influence of the broader environment to that of phylogenetic constraint on the microbiome, we applied high-throughput sequencing of the V4 region of 16S rRNA from 222 obligate ectoparasitic bat flies (Streblidae and Nycteribiidae) collected from 155 bats (representing six species) from ten habitat fragments in the Atlantic Forest of Brazil. We find that parasite species identity is the strongest driver of microbiome composition. To a lesser extent, reduction in habitat fragment area is associated with a reduction in connectance of microbial interaction networks and an increase in modularity, but size-independent measures of network topology and bacterial taxon richness do not show an impact of the environment. Instead, habitat fragments that support more diverse bat and bat fly communities also support more connected bacterial interaction networks.

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Running Title: Island biogeography of parasite microbiomes

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Abstract

Arthropod ectoparasites generally have nutrient-poor diets and are dependent on their bacterial symbionts for nutrient acquisition, development, and immune response initiation. As the body of research on parasite-microbiome interactions continues to grow, it is becoming more apparent that the parasite is not an island that physically and biologically constrains the microbiome. Suitable habitat fragment size, isolation, and distance from a source are important variables influencing community composition of plants and animals, but the role of the environment in determining composition and variation of host-associated microbial communities is poorly known. It is hypothesized that evolution and ecology of an arthropod parasite will influence its microbiome more than broader environmental factors, but this hypothesis has not yet been tested. To compare the relative influence of the broader environment to that of phylogenetic constraint on the microbiome, we applied high-throughput sequencing of the V4 region of 16S rRNA from 222 obligate ectoparasitic bat flies (Streblidae and Nycteribiidae) collected from 155 bats (representing six species) from ten habitat fragments in the Atlantic Forest of Brazil. We find that parasite species identity is the strongest driver of microbiome composition. To a lesser extent, reduction in habitat fragment area is associated with a reduction in connectance of microbial interaction networks and an increase in modularity, but size-independent measures of network topology and bacterial taxon richness do not show an impact of the environment. Instead, habitat fragments that support more diverse bat and bat fly communities also support more connected bacterial interaction networks.

**Keywords:** island biogeography, Streblidae, Nycteribiidae, Chiroptera, ecological interaction network, metabarcoding

Introduction

Island biogeography theory provides a null hypothesis for the way we expect communities to behave in mosaic landscapes. This theory suggests that small, isolated habitats will support low-diversity communities that

are a subset of the species found in larger source communities (MacArthur & Wilson, 1963), and has been applied to and supported by numerous empirical studies in landscapes beyond the classic case of islands separated by water (Warren et al., 2015). Examples include fragmented terrestrial habitats separated by inhospitable land (Bueno & Peres, 2019; Hanski, 2015), mountaintops separated by lowland habitat (Brown, 1971), rivers separated by dry land (Vanbergen et al., 2017), and many other island-like systems (Itescu, 2019). Deviations from the null hypothesis provide fundamental insights into the ways landscapes and biotic interactions shape the evolution of species and assembly of communities.

As a result of the recent explosion of molecular characterizations of microbiomes – communities of microorganisms associated with a site or host – there is mounting evidence that the presence and abundance of taxa in the microbiome is influenced by both host and environment, including environments that may behave like islands (Amato et al., 2013; Avena et al., 2016; Becker et al., 2017). In some systems, microbiomes follow expectations of the null hypothesis of island biogeography (Bell et al., 2005; Martiny et al., 2006; Zinger et al., 2014), but in others, there is no evidence that island area and isolation impact the microbiome (Carbonero et al., 2014; Martiny et al., 2006). Variation in the ability of host, environment, and geography to filter members of the microbiome community is a reflection of the complexity and diversity of microorganisms themselves (Martiny et al., 2006; van der Gast, 2015). Teasing apart the impacts of host, environmental, and geographic filtering on the microbiome community composition can be difficult due to this diversity.

Here, we examine the microbiome of host-specific ectoparasites as a model for testing the hypothesis that habitat island area, isolation, and distance from a source community (i.e., the mainland community in an island biogeography framework) will impact the composition of microbial communities in predictable ways. Obligate parasites represent a convenient system with which to test potential drivers of microbiome variation for several reasons. The number of factors influencing variation in the microbiome may be more limited in obligate parasites than in environmental microbiomes (e.g., soil or water) or microbiomes of free-living host species. Because obligate ectoparasites have extremely specialized diets, their movements in the broader environment beyond their host are constrained by their dependence on a host to survive. Ectoparasitic arthropods also have characteristically depauperate microbiome communities compared to arthropods with diverse diets (Weiss & Aksoy, 2011). We can take advantage of the hierarchical nature of the host-parasite-microbiome system to clearly delimit the microbiome community and restrict the sources of colonizing bacteria that may invade the parasite microbiome, hence providing a manageable system for testing hypotheses about community composition and factors governing assembly of the microbiome.

In this study, we used bat flies (Diptera: Streblidae and Nycteribiidae), which are obligate blood-feeding ectoparasites of bats, to assess community composition of insect-associated microorganisms across a fragmented landscape in the Atlantic Forest of Brazil (Table 1; Figure 1). Bat flies tend to be host-specific, generally occurring on one but up to three species of congeneric bats (Dick & Dittmar, 2014), and they are dependent on their hosts for dispersal (Speer et al., 2019). Bat flies belong to the superfamily Hippoboscoidea, which also contains tsetse flies (Glossinidae) and louse flies (Hippoboscidae). All Hippoboscoidea are adenotrophically viviparous, a condition in which a single egg hatches inside the female fly and feeds from milk glands until the larva is ready to pupate (Dick & Dittmar, 2014). In the case of bat flies, the female fly leaves the host bat to deposit the larva on the roost substrate (Dick & Dittmar, 2014), providing opportunities for both the host bat and environment to act as sources of bacteria for the microbiome of bat flies. The microbiome thus may be influenced by the bat fly (e.g., parasite), the host bat, and landscape factors. For example, habitat fragmentation impacts bat fly prevalence and abundance through changes in roost availability and quality (Hiller et al., 2020), and these changes in the local bat fly community may be reflected in associated microbiomes. Using patches of forest separated from a large, continuous segment of the Atlantic Forest by agricultural land, we can examine whether bat fly-associated microbiomes respond to environmental change following island biogeography theory.

Parasite-associated microbiomes impact aspects of parasite health, reproduction, and survival (Dheilly et al., 2015; Weiss & Aksoy, 2011). Blood-feeding insects such as bat flies are reliant on primary bacterial symbionts passed from mother to offspring to provision B vitamins that are missing from their diet (Feldhaar, 2011;

Weiss & Aksoy, 2011). In addition, the ability of blood-feeding parasites to vector pathogens is mediated by the composition of their microbiome (Cirimotich et al., 2011; Sassera et al., 2013; Weiss & Aksoy, 2011). Bat flies are vectors of bat-specific pathogens, including the haemosporidian parasite genus *Polychromophilus* (vectored by Nycteribiidae; Obame-Nkoghe et al., 2016), and the bacterial genus *Bartonella* , several members of which can infect humans (Morse et al., 2012; Regier et al., 2016). Changes in the microbiome in response to environmental factors may have consequences for the vector competence of bat flies.

Using island biogeography theory, we tested the hypothesis that parasitic insect microbiomes, which encounter fewer sources of variation than microbiomes associated with free-living hosts and environmental sites, exhibit a decrease in bacterial diversity in response to decreasing habitat fragment area and increasing isolation. Alternatively, other factors of the host-parasite system may better explain variation in the microbiome. We describe here the microbiomes of 14 bat fly species, including 4 genera whose associated microbes have never before been examined. In addition, we establish new protocols for generating and analyzing microbiome data from small-bodied arthropods, which can be extended to other low biomass samples.

## Methods

### *Sample collection and preservation*

Bat flies were collected from bats in 11 habitat fragments of the Atlantic forest of Brazil, State of Rio de Janeiro from 18 December 2015 to 19 January 2017 (Figure 1A and 1B), including a large protected area of pristine and secondary forest belonging to the Reserva Ecológica de Guapiaçu (REGUA). Samples were additionally collected from three geographically distant habitat fragments to reveal broad patterns in Neotropical bat fly microbiome composition (southern sites; Figure 1C). The REGUA protected area was sampled in three separate locations to capture more of the ecological variation from this large swath of continuous forest. Each site was sampled for 6 nights, 6 hours per night or at least 2 hours if there was heavy rain, and between 7 and 10 ground-level mist nets were used to capture bats each night (approximately 60m of nets were set per night; Teixeira, 2019). Bats were removed from mist nets and placed into freshly washed cloth bags for holding to minimize cross-contamination of ectoparasites. Each bat was searched for approximately 45s for ectoparasites, which were captured from the bats using featherweight forceps and immediately transferred to tubes containing 92% ethanol (stored at room temperature overnight and then transferred to -20°C). Bats were identified in the field following Emmons & Feer (1997) and Reis et al. (2013). All capture and handling methods followed recommendations in Sikes et al. (2016) and all work was conducted under Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis permit 19037-1. Because many bat species were only captured in a subset of sampled sites, we selected bat flies from the six most well-represented species for microbiome analysis.

Bat flies were identified to species morphologically following Wenzel (1976) and Graciolli & de Carvalho (2001a, 2001b) using a Leica S9i microscope (Leica Biosystems, Wetzlar, Germany; Table 1). Access to comparative morphological material was limited, so we barcoded all of our samples using *cytochrome oxidase I* (COI) (see supplemental methods) and confirmed that individual flies identified morphologically as conspecifics all belonged to the same genetic clade.

### *Environmental variables*

Summary variables describing habitat quality and fragmentation were calculated using ArcGIS 10.1 and Fragstats 3.1 with forest cover maps from the Instituto Brasileiro de Geografia and SOS Mata Atlântica (www.sosmataatlantica.com.br; (McGarigal et al., 2002). Variables considered included habitat fragment area (hectares), isolation (shortest distance between a fragment and its nearest neighboring habitat fragment), distance from source (shortest straight line distance from focal point of a fragment to the nearest point of REGUA forest), perimeter-area ratio, proximity index within a 500m and 1000m buffer (Gustafson & Parker, 1992), and forest cover within a 500m and 1000m buffer. Perimeter-area ratio, proximity index, and forest cover were correlated with habitat fragment area, isolation, and distance from source, so only these three landscape variables were used for downstream analyses. Landscape metrics were $\log_2$ transformed to prevent extremely large or extremely isolated fragments from unduly impacting correlation analyses. To examine

whether sampling sites were clustered by area, isolation, and distance from a source, we used sum of squares K-means clustering in the R package *NbClust* , v3.0 (Charrad et al., 2012).

*DNA extraction and 16S rRNA metabarcoding*

To extract DNA, 288 bat flies were separated into individual tubes and washed twice by suspending in 500µL phosphate-buffered-saline (1x) and vortexing to dilute exoskeletal bacterial contamination. Following washing, the abdomen of each fly was separated from the thorax using sterile forceps and proteinase K was used to digest soft tissue from the entire fly (digestion solution: 95uL Zymo Research Solid Tissue Buffer Blue, 95uL sterile water, 10uL Zymo Research proteinase K). Samples were digested overnight at 55°C. DNA from digested samples was then extracted using the Zymo Research ZymoBIOMICS DNA Miniprep Kit (Zymo Research, Irvine, California) in a Biosafety Cabinet, Class 2. Extractions followed manufacturer protocol with the following exceptions: samples were bead beat in a Disruptor Genie for 20 minutes at 3000 rpm (max speed); following bead beating, samples were stored at -80degC following manufacturer guidelines; sterile water used for elution of DNA from the filter was heated to 55degC prior to pipetting onto filter; the elution incubation step was increased from 1 to 5 minutes; and the elution step was repeated using the first eluate to re-hydrate the column filter. One negative control was used for each extraction kit to control for environmental and kit contamination and negative controls were pooled for amplification.

Extracted DNA was aliquoted into 96-well plates for amplification of the hypervariable region 4 (V4) of 16S rRNA following well-documented procedures outlined by the Earth Microbiome Project and the Illumina 16S Metagenomic Sequencing Library Preparation guidelines (see supplemental methods for details; (Apprill et al., 2015; Gilbert et al., 2010, 2014; Parada et al., 2016). Of 288 initial libraries, 77 libraries required an additional concentration step to reach the minimum 2nM concentration required for sequencing. These libraries were concentrated using SPRIselect magnetic beads to remove 10mM Tris pH 8.5 and vacufuged until dry. Libraries were re-hydrated with 4-6µL sterile water depending on initial concentration, and concentration and quality were assessed. Following concentration of low-yield samples, 206 equimolar libraries were combined into a 3.4nM "high concentration pool" and 23 libraries at a concentration of less than 3.4nM were combined into a single 1nM "low concentration pool", which was used to dilute the "high concentration pool" from 3.4nM to 2nM. The final pool contained 229 libraries at a concentration of 2nM and was sequenced using an Illumina MiSeq v3 Reagent Kit with 2x300bp reads and 18% PhiX spike-in on a MiSeq NGS platform (Illumina, San Diego, CA, USA) at the Bioinformatics and Computational Genomics Laboratory (Hunter College, City University of New York, New York, NY, USA).

*De-mulitplexing, Quality Filtering, and Phylogeny Reconstruction*

Samples were demultiplexed using the MiSeq Reporter Generate FASTQ workflow. Primer sequences were trimmed from forward and reverse sequence reads using cutadapt v.1.4.2 (Martin, 2011). Following demultiplexing, samples were processed using the QIIME2 v.2018.2 pipeline (https://docs.qiime2.org/2018.2/). DADA2 was used to filter out PhiX reads and chimeras, truncate the length of reads (forward reads cut at 200bp, reverse reads cut at 180bp), and cluster reads into unique amplicon sequence variants (ASVs) corrected for Illumina sequencing errors (Callahan et al., 2016). Reads were aligned using the MAFFT plugin in QIIME2 (FFT-NS-i; Katoh et al., 2002, 2005; Katoh & Toh, 2007). Default parameters were used to mask highly variable regions of the alignment and reconstruct a phylogeny using the FastTree2 plugin (Price et al., 2010), which was midpoint-rooted. The GreenGenes Database, v.13.5, trimmed to only the 16S rRNA V4 region, was used as a reference to train a naïve Bayes q2-feature-classifier, which was used to taxonomically identify ASVs in our data (DeSantis et al., 2006).

Contamination is ubiquitous in microbiome studies and especially problematic for low biomass samples (Eisenhofer et al., 2019; Salter et al., 2014; S. Weiss et al., 2014). To reduce the impact of contaminants, several filtering steps were performed. First, any bacterial taxon detected in the negative controls was removed from all other samples, with the exception of *Arsenophonus* . This genus of bacteria contains known symbionts of insects (Nováková et al., 2009) and is expected to be associated with bat flies (Wilkinson et al., 2016). As*Arsenophonus* is highly abundant in the samples sequenced for this study, it may be that its detection in the

extraction control (0.7% of 3,524 total reads) and PCR control (55% of 63 total reads) is due to index bleed, a known issue when multiplexing samples (Eisenhofer et al., 2019; Kircher et al., 2012; Mitra et al., 2015), and it is not treated as a contaminant here. Next, bacterial genera were removed that are known laboratory contaminants (Eisenhofer et al., 2019), as were reads that were classified as being derived from mitochondria, chloroplast, or Archaea, or those that could not be classified beyond phylum. Data were exported from QIIME2 and reformatted for import into the R package*phyloseq* v.1.26.1 (McMurdie & Holmes, 2012, 2013) for further decontamination and all downstream analyses. We used the R package*decontam* to identify ASVs whose frequency is inversely correlated with initial library concentration (Davis et al., 2018). Nine additional ASVs were identified as potential contaminants and eliminated from the dataset. *Arsenophonus* was not identified as a contaminant by*decontam* . Finally, the data were filtered by two coverage depths: 1) all ASVs present in a sample at <0.01% relative abundance were eliminated from that sample, and 2) all ASVs present at <0.1% relative abundance were eliminated from that sample. At a minimum relative abundance of 0.01%, spurious ASVs may remain in the dataset, but at a minimum relative abundance of 0.1%, rare ASVs may be incorrectly excluded (Alberdi et al., 2018; Bokulich et al., 2013). Analyses were performed on both datasets.

As low concentration libraries were used to dilute high concentration libraries prior to sequencing, sequencing effort across samples is not even. Low concentration samples may be more prone to contamination due to their low biomass or may not receive adequate sequencing depth to describe the diversity of the bacterial community. To assess the bacterial diversity captured by low concentration libraries compared to high concentration libraries, the *ggrare* function was used from the *phyloseq-extended* suite of tools, which wraps the function*rarefy* from the package *vegan* v.2.5.4 while maintaining the *phyloseq* data structure (*https://github.com/mahendra-mariadassou/phyloseq-extended/blob/master/R/graphical_methods.R* ; (Oksanen et al., 2010).

*Data visualization, Ordination, and PERMANOVA*

As a qualitative assessment of microbial communities, we constructed compositional bar plots of the relative abundance of bacterial genera in each bat fly species (*ggplot2* , v.3.1.0; (Wickham, 2011). Any genus with a relative abundance <1% of the total reads in a bat fly species were condensed into a "Low Abundance" group. To quantitatively assess variation between microbiome communities, principal coordinates analysis (PCoA), implemented in *phyloseq* , was used to visualize differences in microbial communities captured by Euclidean distance between *phylogenetic isometric log-ratio* -transformed relative abundances. Metabarcoding using high-throughput sequencing is compositional in nature – meaning the total observations (reads per ASV) for a sample contains no information about the total number of microbes and is dependent on the sequencing capacity of the instrument (Fernandes et al., 2014; Gloor et al., 2017; Gloor & Reid, 2016; Tsilimigras & Fodor, 2016; Xia & Sun, 2017). To correct for the compositional nature of 16S rRNA sequencing data, isometric log-ratio transformations were implemented in the R package*philr* v.1.8.1 (Silverman et al., 2017). This transformation utilizes a user-provided bacterial phylogeny to standardize the abundance of bacterial taxa in a sample by the abundance of its sister taxon, creating "balances" at each node on the phylogeny (Silverman et al., 2017). Euclidean distances between *philr* balances provide phylogenetic and abundance information about the bacteria in a sample, similar to weighted UniFrac, that can be used for ordination and down-stream statistical analysis (Gloor et al., 2017; Silverman et al., 2017).

To test whether landscape variables, parasite variables, or host bat variables were correlated with microbial community composition, we used PERMANOVA with 9,999 permutations (*adonis* command in the R package *vegan* , v2.5.4; Anderson, 2014). When sampling is uneven between groups, PERMANOVA is sensitive to heteroscedasticity (Anderson & Walsh, 2013). Homogeneity of dispersion of each group of microbiomes was confirmed using *betadisper* permuted 999 times with*permutest* (R package *vegan* ). As this is a nested system (within each fragment, we expect to see a subset of bat species, and within each bat species, only a subset of bat flies occur, and within each bat fly only a subset of bacterial taxa occur), assessing each variable separately ignores the interactions that could impact our conclusions. Sequential (Type I) sum of squares was used to account for the nonindependence of variables in testing for significant differentiation between microbiome communities. In each test, parasite species was the first variable, followed by one additional variable, and the interaction between parasite species and the additional variable (e.g., pairwise sample distance matrix

~ parasite species + log-scaled area + the interaction between parasite species and log-scaled area). The additionally examined variables were bat species, bat sex, bat individual, region (REGUA area or southern sites), $\log_2$-scaled area, $\log_2$-scaled isolation, distance from source, protection status (within REGUA or outside of REGUA, excluding the southern sites), and sampling site. All PERMANOVA analyses were performed on a dataset containing all localities with taxa filtered at 0.01% relative abundance per sample, a dataset containing all localities with taxa filtered more strictly at 0.1% relative abundance per sample, only the REGUA area localities (no southern sites), and only unprotected REGUA area localities (no localities within REGUA).

When sampling is uneven, sequential sum of squares is sensitive to the order in which variables appear in the equation. To overcome this limitation, we examined the impact of landscape within the four most well-sampled bat fly species using only fly samples collected from the REGUA area with bacterial taxa filtered using a threshold of 0.01% relative abundance per sample. We ordinated samples within these species separately from the rest of the data and estimated variation explained by landscape variables using PERMANOVA on individual variables.

*Bacterial Richness and Interaction Network Analyses*

To examine the impact of landscape on taxon richness, we constructed boxplots of ASV richness in each sample by sampling site, mimicking standard island biogeography plots of richness by area, isolation, and distance to the source. We used Kruskal-Wallis to test whether mean richness was significantly different among sampling sites. Spearman correlation was used to examine ASV richness across continuous ranges of ranked area, isolation, and distance from a source.

Plotting ASV richness across habitat fragments does not account for changes that the landscape may induce in the relative abundance of bacteria. To estimate the impact of habitat fragmentation of the interactions between bacteria, networks were reconstructed using SPIEC-EASI (Kurtz et al., 2015). SPIEC-EASI uses the centered log-ratio transform of abundance to estimate an inverse covariance matrix between bacterial taxa, which means a connection between nodes cannot be better explained by a different network structure (Kurtz et al., 2015). For this analysis, interactions were estimated between ASVs detected in each habitat fragment. Southern fragments and REGUA area fragments F3 and F6 were excluded from the analysis, because they had fewer than 10 samples. Networks were estimated using the Meinshausen and Bühlmann method and parameters of network sparsity were adjusted until network stability was within 0.002 of the target 0.05 threshold (Kurtz et al., 2015; Liu et al., 2010). To account for differences in parasite sample size between sampling sites, we subsampled fragments with greater than 17 samples down to 10 samples and 15 samples, repeated 10 times, and reconstructed networks for each subsample.

Following network reconstruction, leading eigenvector modularity of each network and betweenness centrality of each node were estimated in the R package *igraph* , v.1.2.4 (Brandes, 2001; Csardi et al., 2006; Freeman, 1978; Newman, 2006). Modularity is a measure of the structure of a network, where higher modularity indicates nodes are grouped into tightly interacting neighborhoods with weak interactions occurring outside of this neighborhood (Delmas et al., 2019). Betweenness centrality measures the number of times the shortest path between all pairs of nodes in the network travel through a given node (bacterial ASV), giving an estimate of the influence of a node on the structure of the network (Delmas et al., 2019). Boxplots of network modularity with sampling sites grouped by protection status (within REGUA or not) and Mann-Whitney U tests were used to quantify differentiation between networks. Modularity and betweenness centrality are impacted by network size (number of edges) and shape (degree distribution; (Delmas et al., 2019), making comparisons of summary statistics between networks inaccurate. To account for variation in network size and shape, we created a null distribution for each network (i.e., each sampling site) of 100 randomly re-wired graphs with degree distribution preserved, shuffling ten times the total number of nodes in the network for each permutation. We centered the modularity of each measured network by the mean modularity of its corresponding null distribution. We also calculated the Z-score modularity using the mean and standard deviation of the measured networks (e.g., [modularity of F1 network-mean modularity of networks from other REGUA area sites]/standard deviation of modularity of networks from other REGUA area sites) and

the Z-score modularity using the mean and standard deviation of each null network (e.g., [modularity of F1 network-mean modularity of F1 null distribution]/standard deviation of F1 null distribution).

As a size-independent method of examining variation between networks, we used the graphlet correlation distance from Yaveroğlu et al. (2014) to ordinate the networks as individual points on a plot implemented in the R packages *pulsar* v.0.3.5 and *orca* v.1.1-1 (Hočevar et al., 2016; Müller et al., 2016). The graphlet correlation distance decomposes the network into up-to 4-node graphlets, counts the number of times each node matches one of 11 node types (called "orbits"), and estimates the Spearman correlation of these node types across all nodes. Following Mahana et al. (2016) and Ruiz et al. (2017), the Euclidean distance between these correlations can be used to ordinate the networks and more clearly visualize their differentiation. We compared ordination of the networks to a plot of the sampling sites ordinated solely by $\log_2$ area, $\log_2$ isolation, and distance to source as a null hypothesis about the placement of the networks in the graph if landscape variables are the primary determinant of network topology.

Results

Of 229 prepared DNA libraries, each representing one parasite individual, 222 libraries were used for downstream analysis following quality filtering (Table 1). Filtered libraries ranged in sequencing depth from 2,983 to 66,164 reads. A total of 1,155 ASVs were detected when a 0.01% filtering threshold was applied, while 526 ASVs were found under a 0.1% filtering threshold. Rarefaction curves showed a plateaued asymptote for each library and low concentration libraries fell within the range of ASVs detected in high concentration libraries generated from the same parasite species (Figures S1 and S2).

*Composition of bat fly microbiomes*

Plots of relative abundance of bacterial genera within each parasite species showed a stark difference between the microbiome communities in the parasite families Nycteribiidae and Streblidae (Figure 2; Figure S3). While nycteribiid bat flies had high relative abundances of *Wolbachia* and *Bartonella* , streblid bat flies were dominated by *Arsenophonus* . We found that *Arsenophonus* is mostly absent from nycteribiid bat flies and almost no *Wolbachia*was detected in streblid bat flies. *Bartonella* was present in some streblid bat flies, but at much lower relative abundance than in nycteribiid bat flies. *Mycoplasma* was also detected at higher relative abundances in streblid bat flies compared to nycteribiid flies.

Plots of the average microbiome composition in each fragment indicated a sharp contrast between the southern fragments and the REGUA area fragments (Figure S4). The flies in southern fragments were dominated by *Wolbachia* and *Bartonella* , likely due to the abundance of nycteribiid flies in these fragments. The REGUA area fragments were all dominated by *Arsenophonus* , and had varying relative abundances of *Wolbachia* and *Bartonella* .

*Variation in the microbiome in response to parasite, bat host, and environment*

Ordination and PERMANOVA of microbiome communities provide evidence that aspects of the parasite (i.e., parasite family and species), the host bat (i.e., bat family, bat sex, and bat individual), and landscape factors (i.e., region and sampling site) significantly contributed to bat fly microbiome variation (Table 2; Figure 3 and Figure S5). Other variables significantly contributed to microbiome community differentiation (i.e., bat feeding guild, bat species, protection status of sampling site, habitat fragment area, and isolation), but violated the assumption of homoscedasticity. Parasite species, parasite family, bat feeding guild, bat family, bat species, and sampling site had the largest effect sizes, however many of these variables are correlated with each other.

We used sequential sum of squares with free permutation to account for the hierarchical structure of the study system, and found that parasite species significantly impacted microbiome community structure (Table 3). Habitat fragment area, distance to source, and protection status also significantly contributed to microbiome variation, but violated the assumption of homoscedasticity. As the order of variables in sequential sum of squares can impact their significance when sample sizes are uneven, we also examined the impact of parasite,

host bat, and landscape variables on the four most well-sampled species (Table S1; Figure 4). None of the test variables significantly explained microbiome variation without violating PERMANOVA assumptions.

*Impact of area, isolation, and distance from source on bacterial taxon richness*

Median bacterial ASV richness fell between 6 and 11 for each sampled parasite individual, but the range of ASV richness per fragment varied dramatically (Figure 5). While the three sampled sites within REGUA had the highest ASV richness, there was no pattern of decreasing ASV richness with decreasing area, increasing isolation, and increasing distance from a source. A Kruskal-Wallis test confirmed that there was no significant difference between the mean ASV richness among the sampling sites (Kruskal-Wallis chi-squared statistic=17.856, p-value=0.1201). Treating area, isolation, and distance to source as continuous variables also showed no significant interaction between these variables as ASV richness (Area: rho=0.0613, p-value=0.4031; Isolation: rho=0.0020, p-value=0.9787; Distance to Source: rho=-0.0564, p-value=0.4421).

*Impact of area, isolation, and distance from source on bacterial interaction networks*

SPIEC-EASI networks of bacterial interactions changed in betweenness centrality and modularity associated with environment (Figures 6 and 7). In REGUA sampling sites, low-abundance bacteria and bacteria in the genera *Arsenophonus* , *Wolbachia* , and *Bartonella* had higher betweenness centrality than other bacteria in these networks, acting to connect graph neighborhoods (Figure 6). As networks became more modular (i.e. decreasing habitat fragment area), there were fewer bridge nodes with greater extremes of high and low betweenness centrality. In smaller habitat patches, low-abundance bacteria and*Mycoplasma* had much larger betweenness centrality compared to other bacteria within each network. *Arsenophonus* ,*Wolbachia* , and *Bartonella* did not have high betweenness centrality in the networks of smaller habitat fragments. The exception to this pattern is fragment F4, where betweenness centrality of bridge nodes was not extremely large compared to the betweenness centrality of the other bacteria in the graph and *Arsenophonus* ,*Wolbachia* , and *Bartonella* had high betweenness centrality. While fragment F4 had small habitat area, it was the only fragment outside of REGUA that supported all streblid bat fly species.

The raw modularity of each network was higher in fragments outside REGUA than in sites within the protected area, although this pattern was not statistically significant (Figure 7A; Wilcoxon Rank Sum test, p-value= 0.133). This is largely due to fragment F9, which showed much lower modularity than any other network. When we remove fragment F9, the difference in modularity between REGUA sites and fragments is significant (Wilcoxon Rank Sum test, p-value=0.033). Null distribution-centered modularity measures, which are standardized to account for variation in network size and shape, are highest in REGUA, the largest fragment (F10), and the fragment with the second highest bat fly diversity after the largest fragment (F4; Figure 7B). Smaller fragments and those distant from a source had low centered modularity, and fragment F9 was no longer an outlier. Standardizing modularity by comparison with the mean and standard deviation of the set of measured networks did not control for network size and shape variation, and mimicked the pattern exhibited by raw modularity. Calculation of the Z-score modularity using the null distribution for each site indicated that all measured networks have significantly higher modularity than a randomly connected network of the same size and shape.

Sample size (i.e., number of parasite individuals) was lowest in small fragments and highest in large fragments, with the exception of F4 which has intermediate area, isolation, and distance to source measurements. While there was no impact of sample size on ASV richness in each network, a greater number of samples may allow detection of more edges between nodes (more interactions between ASVs). Efforts to reconstruct SPIEC-EASI networks using subsampling of the sites with the largest sample sizes failed because SPIEC-EASI networks on subsamples never reached stability. To examine the topology of the network independent of size (number of connections), we decomposed the graph into 4-node graphlets and examined correlations between the incidences of graphlet orbits and ordinated the networks using PCoA (Mahana et al., 2016; Ruiz et al., 2017; Yaveroğlu et al., 2014). The position of networks in the ordination differed from what we would expect if landscape variables were the primary driver of network topology (Figure 7D). The REGUA2 sampling site was found to be quite different from the other REGUA sampling sites which were instead closest to fragment

F9, the second largest fragment. Outside of the REGUA sites, there was no pattern of differentiation with decreasing area, increasing isolation, and increasing distance to source.

## Discussion

### *Parasite species drives microbiome composition*

Our results suggest that the bat fly families Streblidae and Nycteribiidae may have different primary symbionts in Brazil (Figure 2).*Arsenophonus* has been previously identified as a primary symbiont of bat flies (Morse, Dick, et al., 2012; Morse et al., 2013; Trowbridge et al., 2006; Wilkinson et al., 2016). The high relative abundance and prevalence of *Arsenophonus* in all streblid bat flies sampled in our study is consistent with the hypothesis that *Arsenophonus*acts as the primary symbiont in the family Streblidae. However, it is unlikely that *Arsenophonus* is acting as the primary symbiont of the nycteribiid species that we sampled, given its absence or low relative abundance. Only one previous study which examined*Arsenophonus* as the primary symbiont of bat flies included representatives of New World Nycteribiidae (Morse et al., 2013). Using cloning and Sanger sequencing, those authors detected an*Arsenophonus* variant in two individuals of *Basilia boardmani* (Nycteribiinae), which is restricted to North America. The strain detected was distantly related to the *Arsenophonus*symbionts found in all other examined members of the subfamily Nycteribiinae and similar to those found in triatomine bugs. In combination with the evidence presented here, these findings suggest that Brazilian *Basilia* spp. rely on a different endosymbiont than both the Streblidae and North American and Old World members of Nycteribiidae.

In contrast to what we found in streblids, nycteribiid species were dominated by *Wolbachia* and *Bartonella* (Figure 2), both of which have been previously detected in bat flies (Morse, et al., 2012; Wilkinson et al., 2016). *Wolbachia* is a primary symbiont in some insects (Dedeine et al., 2005; Hosokawa et al., 2010; Nikoh et al., 2014), but acts facultatively in others (Pontes & Dale, 2006), and also sometimes functions as a reproductive parasite, causing mitochondrial DNA sweeps as it increases its own prevalence in a population (Cariou et al., 2017; Jiggins, 2003; Lack et al., 2011; Speer et al., 2019).*Bartonella* is an intracellular pathogen found in many mammalian groups, including humans (Breitschwerdt & Kordick, 2000).*Bartonella* is common in bats and bat flies (Stuckey et al., 2017), with bat flies potentially acting as a vector to bats (Morse, et al., 2012). It may be that *Wolbachia* and *Bartonella* both have high relative abundance in nycteribiid bat flies because they are acting as primary or facultative symbionts, or because they are acting as a reproductive parasite and pathogen, respectively. If the latter is true, it may be that one of the less abundant bacteria, like*Candidatus* Phlomobacter11Standard nomenclature for a candidate genus., *Mycoplasma* , or *Rickettsia* , is the primary symbiont or another microbe not detected by 16S rRNA sequencing (e.g. fungi; (Gibson & Hunter, 2010).

In addition to family-level differences between bat flies, microbiome composition was parasite species-specific (Table 2; Figure 3). Host-specificity of arthropod microbiomes has been found previously in tsetse flies (Glossinidae; Aksoy et al., 2014), which are also members of the Hippoboscoidea. That microbiome composition shows such a strong signal of parasite species identity indicates that even non-maternally inherited bacteria may be maintained through life history traits (e.g., host bat associations, microclimatic preference). The impact of bat species identity on the parasite microbiome is difficult to parse given the hierarchical nature of the association of bat fly species with specific bats species. Where bat species and bat sex seem to have a large impact on the bat fly microbiome when considered as individual variables (Table 2), these effects disappear when the interaction between parasite species and each host bat variable is taken into account (Table 3). We did not have information on bat roosts, but this has been previously shown to have a large impact on bat fly abundance and prevalence (Hiller et al., 2020). As indicated by our network analyses of landscape variables, it may be that PERMANOVA is good at identifying variables that have a strong impact on variation (i.e., parasite species), but is not the right approach for teasing apart the impact of variables of small effect in this type of complex system (i.e., host bat and landscape).

### *Island biogeography theory applied to the microbiomes of parasites*

Habitat fragment area and protection status, but not degree of isolation or distance to source, have a

measurable but lesser effect on the microbiome of bat flies compared to parasite species (Tables 2 and 3). As bat flies travel with their host bat, it is not surprising that isolation and distance to source do not explain bat fly microbiome variation in these narrowly-separated habitat patches. These stretches of inhospitable agricultural landscape between forest patches may be more of a barrier for parasites of other vertebrates that cannot fly. Mean and median bacterial ASV richness does not decrease with decreasing habitat fragment area, increasing isolation, and increasing distance from a source, contradicting expectations of the island biogeography model (Figure 5). Given the host-specificity of the bat fly microbiome, it is unrealistic to expect ASV richness to change with environment, especially if bacterial ASVs in a bat fly are selected for or maternally transmitted. Instead, variation of the microbiome in response to environment may be better reflected by variation in relative abundance of ASVs. Examining both relative abundance and diversity of bacteria using ecological interaction networks provided a clear statistical signal that habitat fragment area impacts microbiome composition (Figures 6 and 7).

Habitat fragmentation resulting in decreasing habitat patch area was associated with the loss of nodes that act as bridges between modules in bacterial interaction networks (Figures 6, 7A). There were fewer ASVs with high betweenness centrality in small fragments and more ASVs with small betweenness centrality, consistent with the loss of connectivity between disparate portions of the network (Figure 6). Large fragments had lower raw modularity scores than small fragments, indicating more connectivity between neighborhoods than in small fragments (Figure 7A). ASVs that acted as bridge nodes in large sites, are present in smaller sites (Figure 5), but these ASVs no longer perform the function of connecting modules in the network in smaller fragments.

Site F4 is an outlier to this pattern in that it is intermediate in area, isolation, and distance to a source, but has similar modularity and betweenness centrality to the sites within REGUA and the largest site (F10). This is likely driven by the high sample size at F4 compared to other fragments of similar area, isolation, and distance to a source. While we standardized the field collecting method across sampling sites, we sequenced bat fly microbiomes only from the most prevalent bat fly species, corresponding to the most prevalent bat species, across all of the sampling sites, which may have introduced sampling bias into measures of network size. Generally, sites with higher parasite microbiome sample sizes are also the sites with a higher number of captured bat fly species. Considering only the bat fly species that were selected for sequencing, fragments F10 (the largest fragment outside of REGUA, 9 bat fly species) and F4 (8 bat fly species) have the greatest parasite richness outside of REGUA. The three REGUA sites, F10, and F4 are the only sampling sites where the bat species *Artibeus lituratus* and *Desmodus rotundus* and their respectively associated bat flies *Paratrichobius longicrus* and *Strebla wiedemanni* were collected; all other sites are missing one or more of these species. Bat species and bat fly species responses to habitat disturbance are highly species specific, and may not always be negative (Hiller et al., 2020; Pilosof et al., 2012; Saldaña-Vázquez et al., 2013). Anecdotally, site F4 had many large trees, suitable for roosts, compared to other patches of similar size. It is possible that factors of the habitat at F4 which we did not evaluate, such as availability of permanent tree roosts (Patterson et al. 2007) or access to particular foraging resources (Pilosof et al. 2012), may mediate the effects of habitat fragment size in some cases, allowing relatively smaller fragments to support more bat species, and hence more parasite species, which in turn may affect structure and connectivity of parasite microbiomes.

The loss of bridge nodes as sampling sites decrease in area and parasite species richness is driven by network size (i.e., number of interactions), which corresponds to sampling size at each site (Figures 6 and 7). When we attempted to control for variation in sample size by using subsampling, SPIEC-EASI networks built from subsampling sites with large sample sizes never reached stability. Stability is assessed by subsampling, so that when we provided SPIEC-EASI with a reduced dataset, it was not able to infer the more complex networks from the REGUA sites, F10, and F4. This may indicate that higher network size in these sites is not an artifact of sample size. PCoA between correlations of orbit incidences were used as a size-independent examination of the topology of each network. There is no evidence that the environment alters the underlying topology of the network, but it may drive network size through increasing parasite richness (Figures 7). Regardless, sample size cannot be controlled for when examining network size, so the correlation may be spurious.

The finding that large fragments have higher null-adjusted modularity scores than small fragments may appear contradictory to our conclusion that bridge nodes are absent from fragmented habitats (Figure 7B). However, this pattern is consistent with our finding that habitat fragmentation causes changes in network size and not topology. Randomizations of edges to create null distributions preserves the degree distribution of the measured network, so measured networks with many nodes of high degree will have null networks that have low modularity. Large fragments have many ASVs with high degree (i.e., many connections) and these are mostly distinct from ASVs with high betweenness centrality (i.e., more shortest paths between nodes traverse through these ASVs). The ASVs with high degree form dense subgraphs rather than nodes connecting many modules in large, near to source fragments, leading to greater disparity in modularity between the null and measured networks than in small, distant fragments. More samples in a site lead to more detected connections (i.e., larger network size), but do not change the shape of the network by detecting more connections outside of densely connected neighborhoods.

*Implications of changes in network size in response to environment*

Bacteria with high betweenness centrality may act as hub species that maintain the stability of a network (Röttjers & Faust, 2018). In large fragments, *Arsenophonus* , *Wolbachia* , and *Bartonella*had high betweenness centrality, but these bacterial taxa were less central to the networks from small fragments despite maintaining high relative abundance in flies at these sites (Figures 2 and 6). If*Arsenophonus* and *Wolbachia* are acting as primary symbionts, decreasing betweenness centrality may be indicative of changing symbiont-host interactions in response to microbiome community perturbations. The primary symbionts of blood-feeding insects play an important role in vector competence in insects (Weiss & Aksoy, 2011). For example, in tsetse flies, primary bacterial endosymbionts in the genus *Wigglesworthia* impede the invasion of trypanosome parasites by assisting host defenses and subsequently decrease the competence of tsetse flies to vector these harmful parasites to downstream hosts including humans (Weiss et al. 2013). As bat flies are important arthropod vectors of bat pathogens, changes in the structure of their microbiomes in response to habitat fragmentation may have implications for the disease ecology of arthropod vectored pathogens in bats.

Modules may delimit groups of bacteria with specific functional specializations and/or groups that respond in similar ways to environmental variables (Röttjers & Faust, 2018). Higher modularity may protect a community of free-living organisms from invading pathogens, because a pathogen would be isolated to one module within the community (i.e., diversity-stability debate; (Krause et al., 2003; Stouffer & Bascompte, 2010)). This hypothesis may be applicable to bacterial networks if pathogens are limited in transmission by direct competition with endogenous bacteria. However, high modularity in bacterial interaction networks may also reflect the absence of microbiome-mediated host defenses against pathogen invasion. If we revisit the tsetse fly example above, *Wigglesworthia* and trypanosome parasites would share an edge in a microbe interaction network because*Wigglesworthia* abundance and prevalence is associated with low trypanosome abundance and prevalence mediated by host defenses. The absence of this interaction in a network might indicate that*Wigglesworthia* -induced host response to trypanosome invasion is impaired by other microbes in the network (e.g., priority effects) or other aspects of host health. Trypanosome transmission would be less regulated in this instance, but modularity would be high as long as trypanosome abundance and prevalence did not lead to changes in the abundance or prevalence of other microbes. In the case of bat flies, the consequences of more isolated modules for the functionality and stability of the microbiome are unclear and merit future investigation.

Our research builds upon previous evidence that the environment influences microbiome composition in addition to host factors (Amato et al., 2013; Avena et al., 2016; Becker et al., 2017; Ingala et al., 2019). However, all previous studies on the impact of the environment on microbiome composition have been conducted on free-living organisms. The interactions of bat flies with the broader environment are filtered through their obligate associations with host bats, yet the signal of environmental change is detected in the composition of bat fly microbiomes. This indicates that environmental degradation may have community-wide implications for the composition of microbiomes in various organisms, possibly through microclimatic

changes that alter the pool of bacteria available in habitat patches.

*Future directions and summation*

Bat flies are a largely understudied group of blood-feeding arthropods that play an important role in transmission of bat pathogens. This study poses several hypotheses to be tested in future research:

1. New World nycteribiid bat flies apparently have different primary symbionts than streblids and Old World nycteribiids. While it is unlikely that *Arsenophonus* acts as the primary symbiont in the nycteribiid flies sequenced for the study, the inferences that we can make from relative abundances based on metabarcoding are limited. Future studies examining signatures of gene loss using metagenomic sequencing or testing which bacteria are present in the bacteriome of new world nycteribiid flies will provide more information about which bacterium may be functioning as the primary symbiont.
2. The implications of decreasing microbial diversity or changes in the relative abundance of bacteria on the emergent properties of the microbiome are unclear (Shade, 2017). Through comparison with network analysis, we hypothesize that decreasing parasite species richness contributes to greater microbial network modularity and fewer central bacteria. However, methods used to generate interaction networks from bacteria suffer from low precision and accuracy, and results are not robust to the parameters and data used to construct networks (Röttjers & Faust, 2018). Establishing null expectations for a healthy microbiome may help improve assessment of the underlying network and estimation of emergent properties.
3. The downstream impact of habitat fragmentation on mitigating vector competence of bat flies is not tested here. Further research is needed to examine how these changes are reflected in the prevalence of bat pathogens, like *Bartonella* . This requires more complete sampling of the bat and bat fly community.

In this study, we tested whether habitat fragment area, isolation, and distance to a source impact microbiome composition. We found that parasite species identity explains the majority of microbiome variation with habitat fragment area explaining less of the variation, but nevertheless significantly impacting microbiome community composition. Specifically, decreasing habitat patch size led to a decrease in the species richness of parasites that caused a decrease in the size of bacterial interaction networks, leading to highly modular microbiomes in parasites collected from small habitat patches. We did not find evidence that the microbiome changes in accordance with island biogeography theory in response to habitat fragmentation. Instead, it appears that environmental change impacts the microbiome of parasites though cascading community-wide effects on relative abundance of bacteria. Taken together, there is not currently a good null hypothesis for how microbiome communities change in response to environmental gradients. Establishing baseline expectations about variation in diversity and abundance of bacteria has inherent importance to understanding how pathogen transmission may be impacted by environmental degradation, especially in arthropod vectors of disease.

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Data Accessibility Statement

All metabarcoding data was uploaded to QIITA (doi to follow), COI sequences of bat flies were added to GenBank (Accession numbers to follow), and an R Markdown of all analyses is on Github (link to follow).

Author Contributions

KAS, TSMT, MRI, NBS, and ELB designed the study; TSMT collected samples in the field; KAS, AMB, KD, CW, KK, CD, MVV, ACD collected lab-based data; KAS, TSMT, AMB, MVV, ACD, and SCG analyzed the data; KAS, SLP, and ELC funded the research; KAS wrote the manuscript; and all authors contributed to review and editing of the manuscript.

Table 1: Sampling of bats and their corresponding flies used for sequencing. Columns labelled "Total" include female and m

**Bat Family**

Phyllostomidae

Vespertilionidae

Unknown host

Table 2: Univariate PERMANOVA results indicating the p-value (top; *=p<0.05, **=p<0.005, ***=p<0.0005), R2 (middl

**Parasite Variables**

**Bat Variables**

Table 2: Univariate PERMANOVA results indicating the p-value (top; *=p<0.05, **=p<0.005, ***=p<0.0005), R2 (middl

**Landscape Variables**

Table 3: Sequential Sum-of-Squares where the impact of each variable is considered after the impact of parasite species is ac

**Parasite Species**

**Bat Species**

**Bat Sex**

**Individual Bat**

Table 3: Sequential Sum-of-Squares where the impact of each variable is considered after the impact of parasite species is ac

**Region**

**$Log_2$ Area**

**$Log_2$ Isolation**

**Distance to Source ($Log_2$-scaled for REGUA Area unprotected)**

**Protection Status**

**Sampling Site**

Figure 1: Sampling map constructed in QGIS v3.12 of REGUA area sites with fragments outside of REGUA labelled with the prefix "F" and ordered from smallest (1) to largest (10; A), the extent of the sampled area (B), and the southern sites in relation to REGUA (C). Green area (A) indicates forested habitat based on imagery from SOS Mata Atlántica, while white areas are all non-forested habitat types.

Figure 2: Relative abundance of each bacterial genus summed across repeated samples of each parasite species. Colors indicate different bacterial genera and bars represent each parasite species. The black box surrounds the nycteribiid bat flies. Low abundance bacteria were those comprising less than 1% relative abundance in each species. Unknown bacteria could not be identified to genus using the Greengenes database.

Figure 3: Principal Coordinates Analysis on the Euclidean distances between philr-transformed microbial abundances of the complete dataset. Colors represent parasite species.

Figure 4: Principal Coordinates Analysis on the Euclidean distances between philr-transformed microbial abundances of each of the four most well-sampled bat fly species (A-D). Each species is plotted with distance to source colored from white (near) to red (far) and separately with habitat fragment area colored white (large) to red (small).

Figure 5: Box and whisker plot of the bacterial ASV richness in each sampling site in order of decreasing area (A), increasing distance from a source (B), and increasing isolation (C).

Figure 6: Interaction networks constructed for bacterial ASV in each habitat fragment. Sampling sites are ordered by decreasing habitat fragment area, with the largest sites in the top left and the smallest sites in the bottom right. The size of the nodes in the networks corresponds to the z-score betweenness centrality of that node scaled by the range of betweenness centralities detected within the network. A bold black box is placed around figure F4, which has similar network structure to REGUA sites, despite an intermediate habitat patch area.

Figure 7: Plots of summary statistics from SPIEC-EASI networks showing raw modularity between protected (REGUA) and unprotected fragments (A), null-centered modularity for each fragment decreasing by area (B), vertex degree and betweenness centrality by fragment decreasing by sample size (C), and Principal

Coordinate Analysis of graphlet correlation distances (D). Dark green corresponds to REGUA sites and lime green corresponds to sites outside of REGUA for which networks were reconstructed.