Pollen-feeding *Heliconius* butterflies host distinctive adult-stage microbiomes

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Abstract

Evolutionary transitions in animal diets often coincide with shifts in the microbiome, but the degree to which diet-microbiome feedbacks vary across host taxa and development is unresolved. We examined these potential feedbacks from the perspective of Lepidoptera (butterflies and moths), a diverse clade in which little is known about adult-stage microbial associations. With the exception of *Heliconius* butterflies, most lepidopteran adults are short-lived and either feed on simple substrates, like nectar, or do not feed at all. *Heliconius* consume pollen as adults, which provides amino acids and allows the butterflies to have an extended lifespan. Using 16S rRNA gene sequencing of 214 field-collected individuals, we found that adult passion-vine butterfly microbiomes exhibited a strong signal of host phylogeny, with a clear distinction between *Heliconius* and non-pollen-feeding relatives. This pattern was largely driven by differing relative abundances of bacterial phylotypes shared among host taxa, as opposed to the presence or absence of host-specific phylotypes. Using shotgun metagenomic sequencing, we also discovered trypanosomatids and microsporidia to be prevalent in butterfly guts, suggesting potential interactions with co-localized gut bacteria. Overall, we show that a major transition in adult-stage lepidopteran diet and life history coincides with a shift in microbiomes, and our work provides a foundation for future tests of microbiome function in adult butterflies.

Introduction

A major goal of contemporary microbial ecology is to identify the factors that influence the structure and function of host-associated microbiomes. In animals, diet has emerged as a consistently strong predictor of microbiome structure [1–3]. In some cases, microbes are also known to directly facilitate evolutionary transitions in host diet through their contributions to digestion, nutrient synthesis, or detoxification [4–6]. However, there are many animal taxa for which comparative microbiome data are either unavailable or only available for a specific phase of their development. This makes it difficult to generalize diet-microbiome interactions, as both microbiomes and diets often change radically during host development [7, 8].

Lepidoptera (butterflies and moths) are a diverse and ecologically important group in which microbiome roles are likely to be highly specific to certain host taxa and life stages. For lepidopteran larvae (caterpillars), even major shifts in larval diet do not coincide with functionally relevant shifts in the microbiome [9–11]. Lepidopteran larvae typically harbor a very low number of gut microbes, and most of these microbes are transient with the exception of pathogen infections [9]. However, the microbiomes of insects that undergo complete metamorphosis, such as Lepidoptera, often exhibit profound shifts in size, composition and function across host life stages [7]. A recent survey of various butterfly species found that adult-stage gut bacterial communities were generally abundant (median ~10^8 16S rRNA gene copies per gut) and distinct from diet-associated bacterial communities [12]. It is possible that evolutionary transitions in the diet of adult lepidopterans could cause, or be caused by, concomitant transitions in adult-stage microbiomes.

Arguably, the single most dramatic and consequential transition in adult diet among the 150,000+ described species of Lepidoptera [13] occurred in the ancestors of neotropical *Heliconius* butterflies (Nymphalidae: Heliconiini). While adults of other lepidopterans either do not feed at all, or feed on comparatively nitrogen-poor and/or inconsistently available substrates [14], *Heliconius* evolved the ability to consume pollen, an abundant resource rich in essential amino acids [15]. Directly or indirectly, pollen-feeding led to a suite of changes in *Heliconius* population structure, behavior, chemical ecology, mimicry, coevolutionary interactions with plants, and life history traits [15–18]. As an example of the latter, *Heliconius* are exceptional among butterflies in their adult longevity, with a potential lifespan of several months [18–20].

Heliconius have been an influential model system in evolutionary biology for over 150 years [17]. As with adult butterflies in general, however, very little is known about *Heliconius* microbiomes. Data for a handful of *Heliconius* individuals and species are available [12, 21, 22], but a comparative context with non-pollen-feeding members of the passion-vine butterfly tribe Heliconiini has been missing. Nectar- and fruit-feeding butterfly species were recently found to host distinct gut microbiomes [12], supporting the possibility that other kinds of shifts in adult butterfly diets could be linked to shifts in microbiomes.

Does the ability to feed on pollen coincide with the presence of novel symbionts, changes in community composition, or other microbiome differences? To answer this question, we collected 214 wild adult butterflies representing 23 species and subspecies across the Heliconiini

and characterized their microbiomes. We used amplicon sequencing targeting a portion of the 16S rRNA gene and assessed the distribution of exact sequence variants (ESVs) across host individuals, species, genera, and the heliconiine phylogeny. We evaluated the reliability of our amplicon sequencing approach by obtaining shotgun metagenomic data from a subset of samples. We also used the metagenomes to assemble near-full-length 16S rRNA genes and evaluate bacterial strain-level patterns that may not be evident from analyses of amplicon sequence data. Finally, we uncovered prevalent gut-associated trypanosomatids and microsporidia. These putatively parasitic microeukaryotes have been little-studied in butterflies, despite their potential interactions with gut bacteria and relevance to host fitness.

Materials and Methods

Field collections

The wild adult butterflies used for whole-body microbiome sequencing were collected from seven locations in Panama and Ecuador in May-August 2014 (more detail is provided in the supplemental file "Collection_localities.txt"). Butterflies were euthanized with ethyl acetate and stored in DMSO after removal of wings, following [23]. We also stored two DMSO-only blanks to use as negative controls. In June 2016, we collected additional adult butterflies for gut and head/thorax sequencing from Gamboa and Pipeline Road, Panama. For these specimens, we dissected the gut (hindgut, midgut, and the distal ~1/2 of the foregut) using sterilized tools prior to storage in DMSO. The whole head and thorax (including the proximal foregut) were stored separately. Species or subspecies were identified based on morphology. Butterflies were collected under permit # SC/A-7-11 from Panama's Autoridad Nacional del Ambiente and # 005-13 IC-FAU-DNB/MA from Ecuador's Ministerio del Ambiente.

Sample processing, PCR and sequencing

We removed whole bodies and head/thorax samples from DMSO and, after homogenization, used approximately 50 mg subsamples of homogenate for DNA extractions with the MoBio PowerSoil kit following the manufacturer's instructions. We added entire guts

93 directly to DNA extraction tubes, in which they were homogenized during the first bead-beating
94 step of the protocol. Two DMSO blanks and 30 DNA extraction blanks were also processed in
95 tandem with the butterfly samples and sequenced.
96 PCR amplifications (515F/806R primers, V4 region) and 2 X 150 bp Illumina MiSeq
97 sequencing of 16S rRNA genes followed standard Earth Microbiome Project protocols
98 (dx.doi.org/10.17504/protocols.io.nuudeww). For 29 butterfly individuals of nine species, gut
99 and head/thorax samples were also amplified using primers that target the ITS gene region of
100 fungi [24]. Amplification success with these fungal-specific primers (as estimated from gel
101 electrophoresis) was generally very low, suggesting a lack of abundant fungal DNA that was
102 later corroborated with the shotgun metagenomic data (see below). DNA extracts from a subset
103 of 15 amplicon-sequenced gut samples were used for shotgun metagenomic sequencing
104 following the approach described previously [25] with an input DNA concentration of 0.75
105 ng/ul, KAPA HiFi HotStart ReadyMix and bead cleanup with Ampure XP beads at a 0.9x ratio.
106
107 *Amplicon data processing*
108
109 Amplicons from the 2014 whole-body samples and 2016 gut and head/thorax samples
110 were sequenced on separate runs, demultiplexed using idemp (https://github.com/yhwu/idemp),
111 and combined for futher processing. Cutadapt [26] was used to remove primer sequences. We
112 then used the DADA2 pipeline [27] to quality-filter (max EE value = 1) and trim (150 bp
113 forward, 140 bp reverse) reads, infer exact sequence variants (ESVs), merge paired-end reads,
114 and remove chimeras. We classified ESVs using the RDP Naive Bayesian Classifier algorithm
115 [28] against the SILVA training set v. 132 [29].
116 Further data processing and analyses were conducted in R v. 3.6.0 [30]. We used
117 decontam [31] for prevalence-based identification of putative contaminant ESVs based on 34
118 negative controls (DMSO and DNA extraction blanks and PCR no-template controls). The
119 median percentage of contaminant sequences across butterfly samples was 0.09%, but two
120 samples with >10% contaminants were removed from further analysis. ESVs with < 100 total
121 sequences across all samples (out of a combined total of 5.6 million sequences) were removed,
122 as were ESVs classified as mitochondria or chloroplast, or bacteria lacking sub-domain

identification. These ESVs combined typically made up a low proportion of reads from the libraries (median of 2.6% across all samples).

As the resulting 16S rRNA gene amplicon datasets were highly variable in read depth across samples, we rarefied all libraries to 5,000 sequences, filtering out 13 samples with lower sequence depth. We also relabeled *Pantoea*, *Erwinia*, *Kluyvera*, *Citrobacter*, *Klebsiella*, and *Cronobacter* taxonomic assignments to *Enterobacter*. This step was taken as genera within Enterobacteriaceae are often polyphyletic and are difficult to resolve from short 16S rRNA gene regions (e.g., [32]), and we wanted to avoid spurious separation of ESVs among genera and resulting idiosyncracies in genus distributions across butterflies.

Amplicon data analysis

Beta diversity statistics and plots are based on Bray-Curtis dissimilarities and/or UniFrac distances (weighted and unweighted). To obtain a bacterial phylogeny for the latter, we used the fragment-insertion method [33] to place our ESV sequences into the Greengenes reference tree [34]. The butterfly phylogeny is from [35] (TreeBASE #Tr77496). [Note that although this phylogeny nests *aoede* within *Heliconius*, the phylogenetic position of this species is held by some to be uncertain (J. Mallet, pers. comm.)]. Four of the butterfly species in our sample set contained specimens from two distinct subspecies (e.g, *Heliconius sara sara* and *Heliconius sara magdalena*). To include these in the species-level host phylogeny, we inserted subspecies tips halfway along the terminal branches to their sister subspecies. Hereafter we refer to these subspecies as "species" for simplicity.

To test for host-phylogenetic signal in microbiomes, we used Mantel tests with 9999 permutations to calculate the correlation between microbial community dissimilarities/distances and host phylogenetic distances [36]. Intraspecific variation in microbiomes was handled by averaging the pairwise dissimilarities/distances between all individuals of one species and all individuals of another species. We used the phytools package [37] to visualize concordance between topologies of the host phylogeny and a dendrogram of bacterial community dissimilarities. Nodes were rotated with the "cophylo" function in phytools to maximize tip matching between the two trees.

Differences in overall community composition between host genera were tested with PERMANOVA as implemented in the vegan package [38]. Using the "betadisper" function we corroborated that significant test results were due to host genus-level differences in location and not dispersion [39]. We used a nonparametric statistical test (Wilcoxon rank-sum) to identify bacterial genera that differed in relative abundance between host taxa or between sample types (gut versus head/thorax) and applied a false discovery rate correction to the resulting p values.

Metagenome data processing and analysis

For 15 gut samples, we obtained shotgun metagenomic data to complement the bacterial 16S rRNA gene amplicon dataset. We quality-filtered these reads with sickle [40] and trimmed adapters with cutadapt [26]. We then used Bowtie 2 [41] to filter out reads matching a given sample's corresponding host species' genome, obtained from Lepbase [42]. The two *Dryadula phaetusa* metagenomes were mapped to a genome of the sister species *Dryas iulia* as no *Dryadula* genome was available. Since there was a high proportion of host-derived reads, we focused here on describing microbial diversity using ribosomal RNA gene reads present in the metagenomes. With the host-filtered reads, we used phyloFlash [43] to find and classify eukaryotic and bacterial SSU rRNA reads. Bacterial community composition was compared between the amplicon and shotgun metagenomic datasets using a Mantel test. We also used phyloFlash to assemble 16S rRNA genes from the 150 bp shotgun reads. These longer sequences allowed us to estimate the phylogeny of *Orbus*, the dominant bacterium in these 15 samples. *Orbus* sequences were aligned with MUSCLE [44], curated with Gblocks [45], and used for maximum likelihood reconstruction with the phylogeny.fr implementation [46] of PhyML [47].

Data availability

Amplicon data, metadata, and R code are available from figshare (figshare.com/projects/Heliconius_butterfly_microbiomes/70520). Metagenomes are available from MG-RAST (project no. MGP89563).

Results

Adult heliconiine butterfly microbiomes have a relatively low diversity, with a median of 26 bacterial ESVs per individual. A median of 11 ESVs accounted for 95% of the reads. These communities are also fairly consistent among conspecific butterflies. For example, within our most deeply sampled population (*H. erato petiverana* in Gamboa, Panama; N = 23), a median of 84% of the 16S rRNA gene reads obtained from a given individual's microbiome was assigned to 10 dominant bacterial genera (Fig. S1). Some of these genera are present in roughly similar relative abundances across individuals and even across our two sampling years.

Microbiomes from whole, homogenized butterfly bodies are mainly composed of gut-associated taxa. Isolated guts are similar to conspecific whole-body microbiomes in their bacterial community profiles (Fig. S1), with many bacterial genera occurring at similar relative abundances in guts and head/thorax tissue (Table S1). Some bacteria did differ between whole-body and gut samples, such as *Spiroplasma*, which is a known hemolymph (blood) associate [48] and which tends to be enriched in whole bodies (Fig. S1). Likewise, *Acinetobacter* was ~10-fold more relatively abundant in head/thorax samples than in guts (Table S1). *Orbus*, *Enterobacter*, *Asaia*, and some other dominant bacterial genera are clearly enriched in gut tissue (Table S1).

Host taxa	Phylum	Class	Order	Family	Genus	Mean prop. gut +/- SEM	Mean prop. head/thorax +/- SEM	p value
Heliconius	Bacteroidetes	Bacteroidia	Flavobacteriales	Weeksellaceae	*Apibacter*	0.036 +/- 0.009	0.002 +/- 0.001	< 0.001
					Chishuiella	0.007 +/- 0.003	0.095 +/- 0.027	0.001
					Chryseobacterium	0.011 +/- 0.004	0.096 +/- 0.024	0.001
	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	*Enterococcus*	0.031 +/- 0.008	0.021 +/- 0.006	0.117
				Streptococcaceae	*Lactococcus*	0.02 +/- 0.006	0.033 +/- 0.009	0.793
	Proteobacteria	Alphaproteobacteria	Acetobacterales	Acetobacteraceae	*Asaia*	0.024 +/- 0.007	0.004 +/- 0.002	0.001
					Commensalibacter	0.12 +/- 0.02	0.007 +/- 0.003	< 0.001
					Swaminathania	0.013 +/- 0.01	0.016 +/- 0.014	0.745
		Gammaproteobacteria	Betaproteobacteriales	Burkholderiaceae	*Variovorax*	0.01 +/- 0.004	0.021 +/- 0.006	0.017
			Enterobacteriales	Enterobacteriaceae	*Enterobacter*	0.233 +/- 0.034	0.031 +/- 0.007	< 0.001
					Serratia	0.037 +/- 0.01	0.027 +/- 0.017	0.013
			Orbales	Orbaceae	*Orbus*	0.163 +/- 0.024	0.021 +/- 0.01	< 0.001
			Pseudomonadales	Moraxellaceae	*Acinetobacter*	0.023 +/- 0.007	0.293 +/- 0.041	< 0.001
				Pseudomonadaceae	*Pseudomonas*	0.086 +/- 0.017	0.024 +/- 0.01	0.291
	Tenericutes	Mollicutes	Entomoplasmatales	Spiroplasmataceae	*Spiroplasma*	0.012 +/- 0.007	0.003 +/- 0.002	0.927
Other Heliconiini	Bacteroidetes	Bacteroidia	Flavobacteriales	Weeksellaceae	*Apibacter*	0.139 +/- 0.045	0.008 +/- 0.004	0.083
					Chishuiella	0.009 +/- 0.003	0.138 +/- 0.029	0.016
					Chryseobacterium	0 +/- 0	0.015 +/- 0.012	0.808
	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	*Enterococcus*	0.056 +/- 0.027	0.031 +/- 0.011	0.843
				Streptococcaceae	*Lactococcus*	0.036 +/- 0.026	0.012 +/- 0.011	0.098
	Proteobacteria	Alphaproteobacteria	Acetobacterales	Acetobacteraceae	*Asaia*	0.153 +/- 0.039	0.004 +/- 0.001	0.01
					Commensalibacter	0.031 +/- 0.015	0.002 +/- 0.002	0.106
					Swaminathania	0.007 +/- 0.004	0 +/- 0	0.226
		Gammaproteobacteria	Betaproteobacteriales	Burkholderiaceae	*Variovorax*	0.005 +/- 0.002	0.016 +/- 0.004	0.292
			Enterobacteriales	Enterobacteriaceae	*Enterobacter*	0.156 +/- 0.042	0.014 +/- 0.006	0.01
					Serratia	0.02 +/- 0.012	0.001 +/- 0	0.075
			Orbales	Orbaceae	*Orbus*	0.139 +/- 0.043	0.007 +/- 0.004	0.042
			Pseudomonadales	Moraxellaceae	*Acinetobacter*	0.046 +/- 0.018	0.45 +/- 0.054	< 0.001
				Pseudomonadaceae	*Pseudomonas*	0.02 +/- 0.017	0.011 +/- 0.004	0.393
	Tenericutes	Mollicutes	Entomoplasmatales	Spiroplasmataceae	*Spiroplasma*	0.068 +/- 0.038	0.052 +/- 0.052	0.843

Table S1. Relative abundances and within-body distribution of the top 15 bacterial genera (ranked by mean abundance) across butterflies collected from Gamboa, Panama in 2016. These individuals were dissected to compare microbiomes between isolated gut tissue and the combined head and thorax. Abundances are shown for *Heliconius* (top) and species belonging to other heliconiine genera (bottom) separately. P values are from a nonparametric statistical test of proportions in guts versus head/thorax samples, after FDR correction (see Methods).

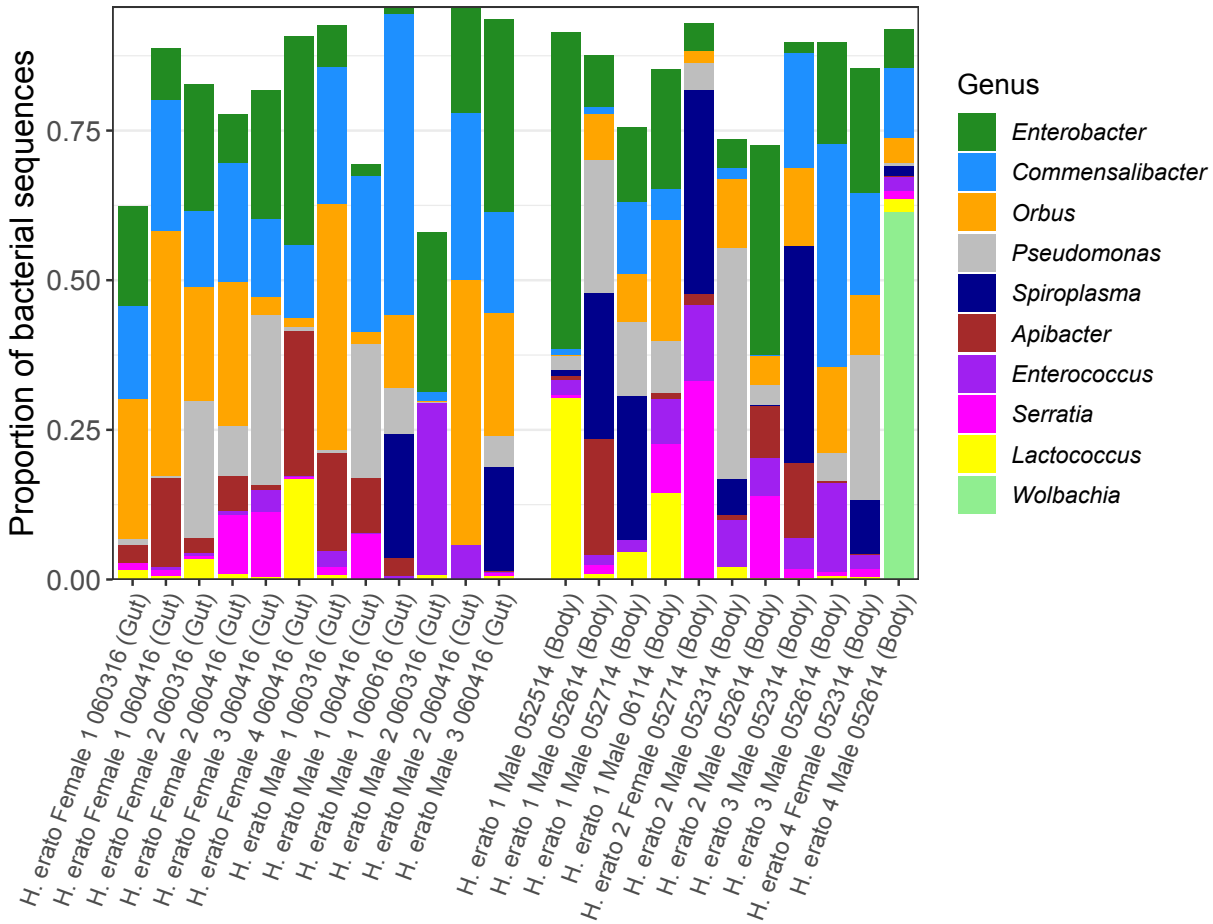

Figure S1. Whole-body bacterial communities are consistent among individuals and predominantly represent gut-associated taxa. Shown are the relative abundances of the top 10 bacterial genera (ranked by mean abundance) across *H. erato petiverana* collected from Gamboa, Panama. Remaining white space represents sequences belonging to other genera; these made up a median 16% of sequence libraries across individuals. Samples on the left are isolated guts from individuals collected in 2016, while samples on the right are whole-body homogenates from individuals collected in 2014. Note that *Enterobacter* here includes sequences originally assigned as *Klebsiella* and some other closely related Enterobacteriaceae genera (see Methods).

We used metagenomes (N = 15) to examine the accuracy of amplicon sequencing for describing bacterial community composition in our broader sample set. Interindividual microbiome variation was highly correlated between the amplicon-based dataset and the metagenomic dataset (Mantel $r = 0.63$, $p < 0.001$). Furthermore, for six of the most abundant bacterial genera, relative abundances in amplicon libraries were highly predictive of relative

abundances in metagenomes (Fig. S2). These results support the use of amplicon sequencing for estimating bacterial community composition in adult heliconiine butterflies.

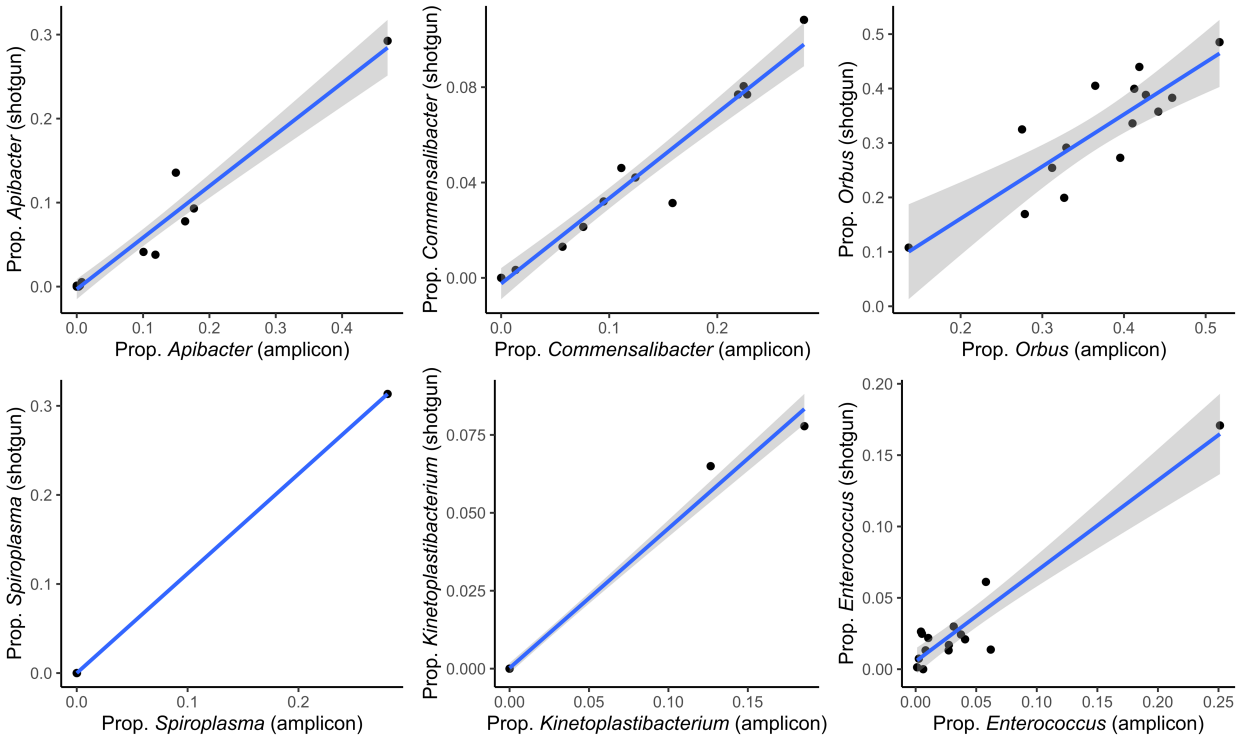

Figure S2. Bacterial genus-level relative abundances are highly correlated between the amplicon sequence libraries and metagenomes. Shown are relative abundances of six of the most abundant genera across the 15 butterfly gut samples for which we obtained both amplicon and shotgun sequence data.

Compared to other passion-vine butterflies, adult *Heliconius* harbor clearly distinct whole-body bacterial communities (Fig. 1). Microbiomes clustered by host genera to varying degrees depending on the distance metric used. The effect was strongest with taxonomic or phylogenetic metrics that incorporate information on the relative abundances of exact sequence variants (ESVs) (Bray-Curtis: $R^2 = 0.13$, $p = 0.001$; weighted UniFrac: $R^2 = 0.18$, $p = 0.001$) as opposed to purely presence/absence-based metrics (Jaccard: $R^2 = 0.11$, $p = 0.001$; unweighted UniFrac: $R^2 = 0.10$, $p = 0.001$). Six individuals of the non-pollen-feeding species *Heliconius aoede* (formerly *Neruda aeode*; see Methods) had bacterial communities more similar to those found in pollen-feeding *Heliconius* individuals, while two individuals had communities more similar to those found in other butterfly genera (Fig. 1).

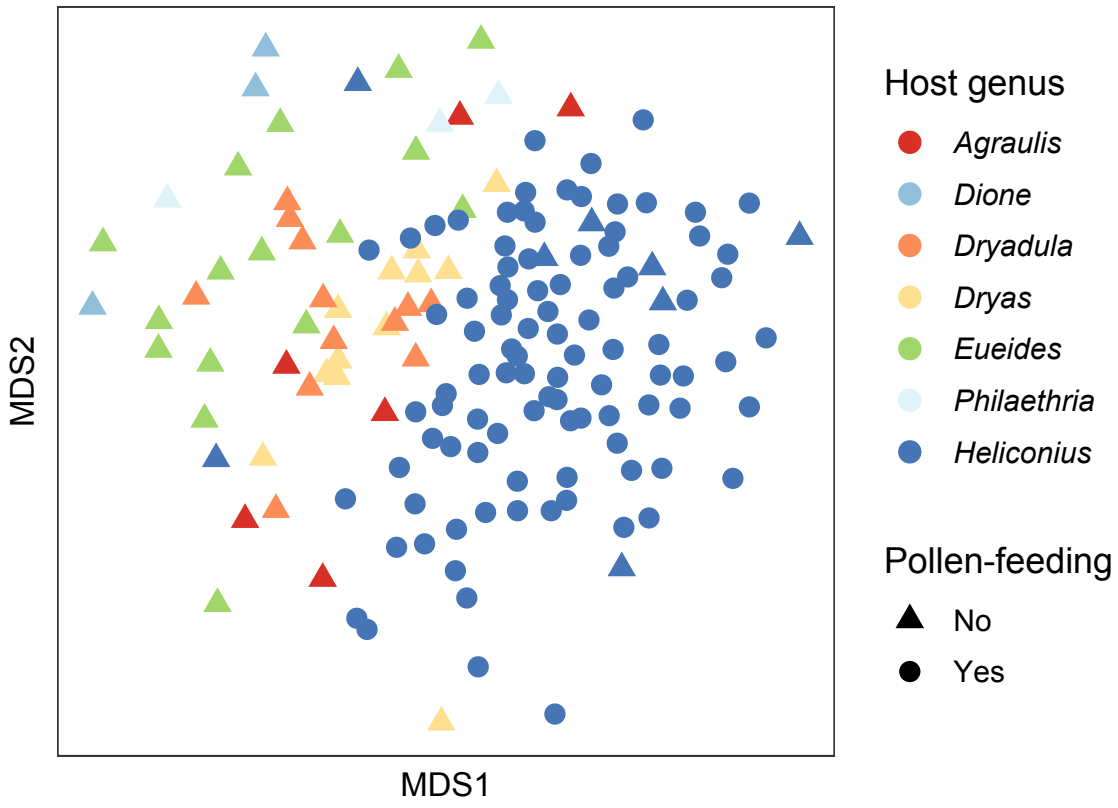

Figure 1. Adult *Heliconius* butterflies host distinct bacterial communities compared with related non-pollen-feeding genera. Shown is an ordination of microbiome variation (Bray-Curtis dissimilarities) among all whole-body samples (N = 104 *Heliconius*, 52 other Heliconiini). With the exception of *H. aoede* (dark blue triangles), pollen-feeding is exclusive to, and ubiquitous within *Heliconius*. However, the phylogenetic placement of *H. aoede* is not fully settled (see Methods).

As suggested by the host genus-level clustering, we found that variation in butterfly microbiome composition was associated with host relatedness (phylogenetic distance). More closely related butterfly lineages harbored more similar microbiomes (Bray-Curtis: Mantel r = 0.40, p = 0.01), and the topologies of the butterfly phylogeny and the dendrogram of microbiome similarity were moderately congruent (Fig. 2). This pattern was still statistically significant when microbiome variation was measured using weighted UniFrac distances (Mantel r = 0.31, p = 0.03), but not when using unweighted UniFrac (Mantel r = 0.03, p = 0.41) or Jaccard distances (Mantel r = 0.19, p = 0.08), which do not incorporate relative abundance information.

Figure 2. Heliconiine butterfly microbiomes exhibit a strong signal of host relatedness. Shown is the concordance between the host phylogeny (from [35]) and a dendrogram representation of microbiome variation among species (Bray-Curtis dissimilarities). Here, all nodes have been rotated to maximize tip matching. Note, however, that sets of parallel lines connecting tips do not always correspond to matching clades.

We then determined which bacterial taxa contribute to the observed taxonomic (Fig. 1) and phylogenetic (Fig. 2) structuring in overall community composition. The dominant bacterial genera *Enterobacter*, *Orbus*, and *Pseudomonas* (or *Serpens* [49]) were proportionally more abundant in *Heliconius* versus the non-pollen-feeding butterfly genera, while *Asaia* and *Apibacter* showed the opposite pattern (Fig. 3). None of these bacterial genera, however, were exclusively restricted to one host feeding guild, genus, or species, and their relative abundances were occasionally highly variable (Fig. 3). We also analyzed the data at the ESV-level, including all individual ESVs that were reasonably abundant within at least one host species (≥ 5% within-species average). We did not find evidence for prevalent host species- or genus-restricted ESVs (Fig. 4). Most ESVs are present, albeit with varying relative abundances, across host genus and species boundaries. However, ESV_22 (*Chryseobacterium*) is notable as being abundant in nearly all pollen-feeding species and rare or absent from non-pollen-feeding host species, including *H. aoede*.

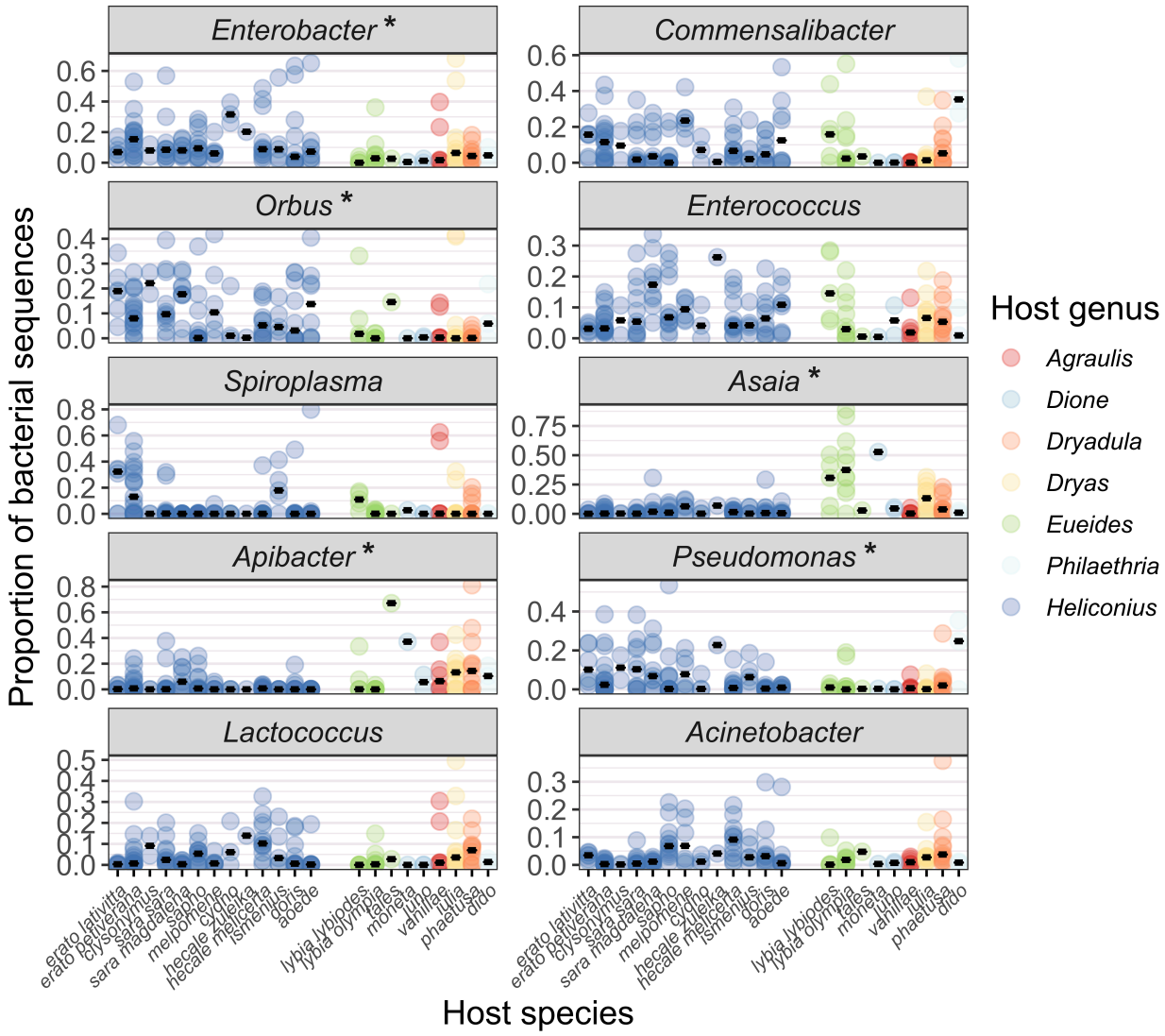

Figure 3. Dominant bacterial genera are largely shared among *Heliconius* and other butterflies, although some are differentially abundant. Shown are the relative abundances of the top 10 bacterial genera, ranked by mean abundance, in whole-body microbiomes. Dots indicate replicate individuals, and black bars indicate median proportions within a host species. Starred bacterial genera differed significantly in relative abundance between *Heliconius* and non-*Heliconius* butterflies ($p < 0.05$ after FDR correction). The arrangement of host species on the x axis corresponds to the phylogeny shown in Fig. 4. Note that *Enterobacter* here includes sequences originally assigned as *Klebsiella* and some other closely related Enterobacteriaceae genera (see Methods).

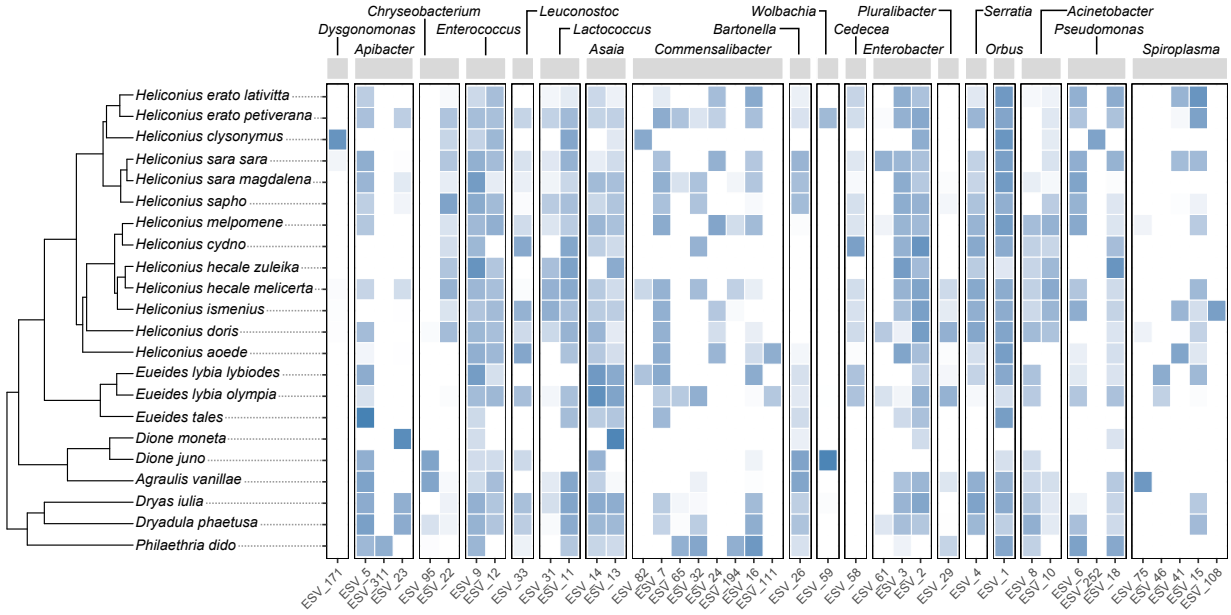

Figure 4. Most bacterial exact sequence variants (ESVs) are not host species- or host genus-specific. Shown are all ESVs in the dataset that had ≥ 5% mean relative abundance across conspecific individuals for one or more host species. For each ESV, the species-level mean relative abundance in whole-body samples, after log transformation, is indicated by the color of the cells (white = not detected in that species). Note that bacterial genera (labeled at the top) contained varying numbers of ESVs that met the aforementioned prevalence threshold.

Amplicon-derived ESVs are limited in their ability to resolve bacterial strains as they represent only a short fragment of the 16S rRNA gene (here, ~250 bp). To test for potential host specificity at a finer level of resolution, we obtained near-full-length 16S rRNA gene sequences from the bacterium *Orbus*, which is highly prevalent across gut and whole-body samples (Fig. 3, Fig. S1) and almost exclusively composed of a single ESV (Fig. 4). These sequences were reconstructed from the 12 metagenomes in which *Orbus* was sufficiently abundant. A phylogeny based on these sequences and other Orbaceae shows that butterfly-associated *Orbus* form a single, well-supported clade (Fig. 5). Host-phylogenetic or geographic structure was not evident within this clade. In fact, many of the Panamanian heliconiine butterflies harbored *Orbus* that have identical or nearly-identical 16S rRNA gene sequences to an *Orbus* strain isolated from an East Asian butterfly, *Sasakia charonda* [50].

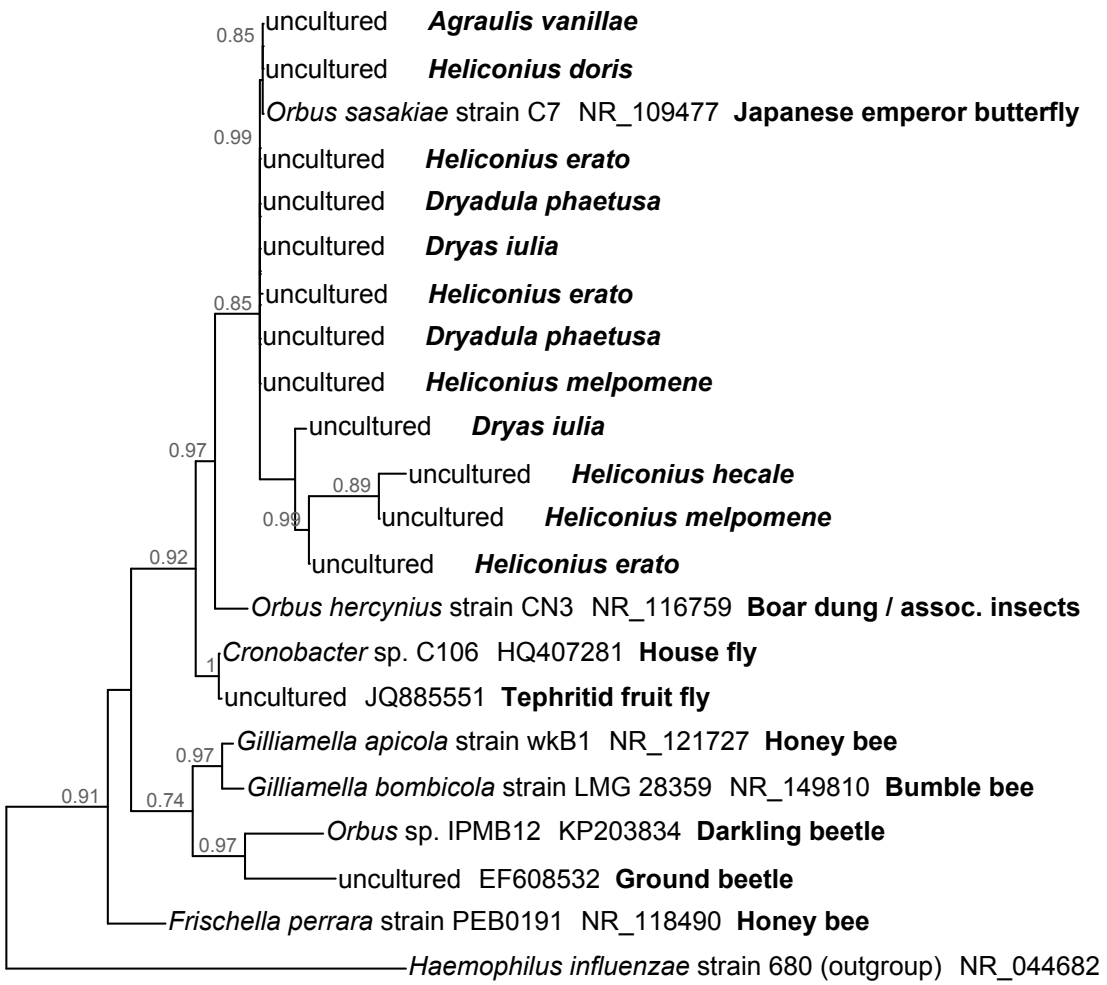

Figure 5. Maximum likelihood phylogenetic reconstruction of the bacterial family Orbaceae (Gammaproteobacteria: Pasteurellales). Some host taxonomic structure is apparent at the order level (i.e. Lepidoptera, beetles, bees, flies) but not within butterflies. 16S rRNA gene sequences from *Agraulis*, *Dryadula*, *Dryas*, and *Heliconius* were assembled from short metagenomic reads. Other 16S rRNA gene sequences are from GenBank. *Haemophilus influenzae* (Pasteurellaceae) was used as the outgroup.

We also used the shotgun metagenomes to search for microbial eukaryotes in butterfly gut samples, and found that microsporidia and trypanosomatids were prevalent (27% and 47% of the 15 individual samples analyzed, respectively) (Fig. S3). When trypanosomatids were detected in a given sample, we also sometimes detected 16S rRNA gene reads in the metagenomic data classified as *Kinetoplastibacterium*, an obligate bacterial endosymbiont of certain trypanosomatids [51]. *Kinetoplastibacterium* was not detected without its corresponding trypanosomatid host (Fig. S3). This observation led us to reexamine the larger, amplicon-based

bacterial dataset. We found that 9% of *Heliconius* individuals and 4% of individuals belonging to other heliconiine genera were infected with a trypanosomatid, as inferred by the presence of *Kinetoplastibacterium*. These proportions were not significantly different (Fisher's Exact Test, p = 0.28). They are also likely underestimates, given the aforementioned prevalence of trypanosomatids among metagenomes (47%) and the potential for false negatives—in five metagenomes, trypanosomatids were detected without *Kinetoplastibacterium* (Fig. S3). These results point to a need for more targeted analyses of butterfly-associated parasite diversity.

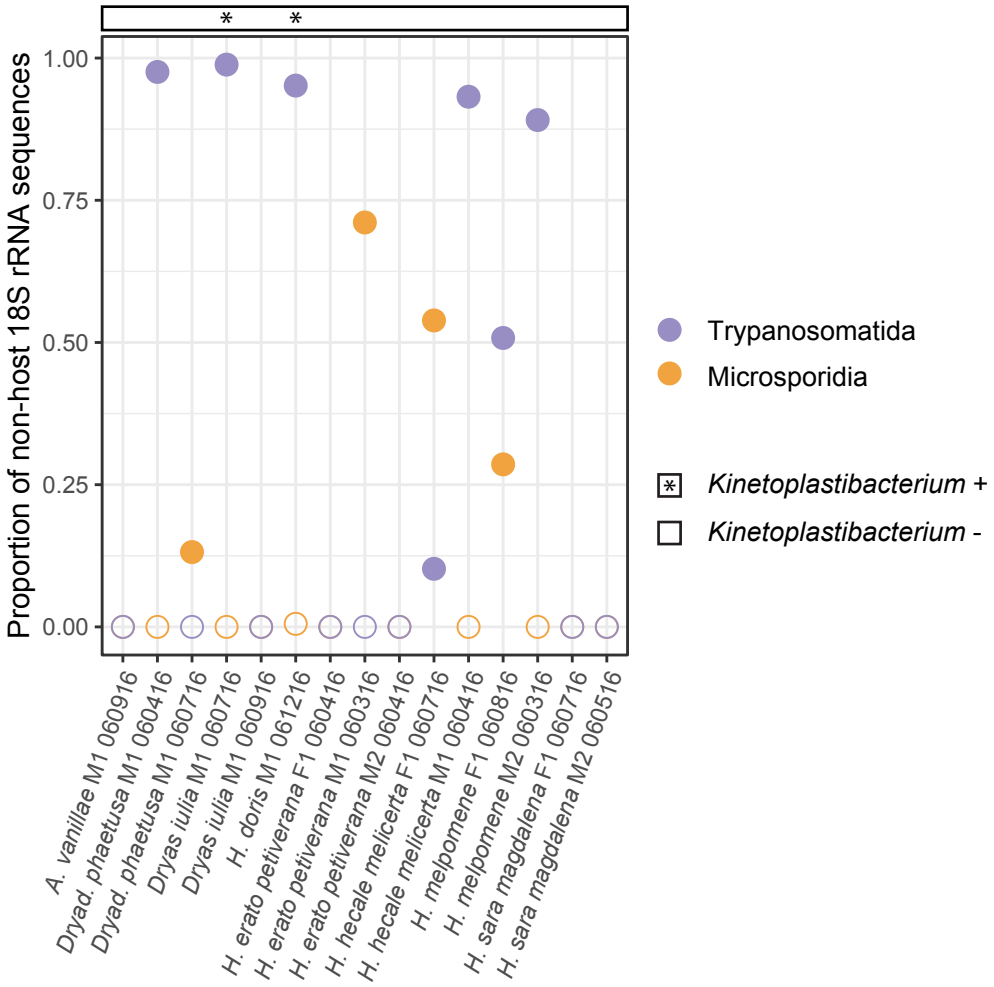

Figure S3. Microsporidia and Trypanosomatidae (related to *Crithidia*, *Leishmania*, and *Trypanosoma*) are prevalent in the 15 butterfly individuals with sequenced gut metagenomes. Points show the proportion of each microeukaryotic taxon out of all non-butterfly 18S rRNA sequences identified by phyloFlash. Circles are filled if the proportion of microsporidia or trypanosomatids exceeded 0.01. Asterisks indicate samples in which *Kinetoplastibacterium*, a bacterial endosymbiont of trypanosomatids, was detected. Other microeukaryotes not shown here comprise a variety of very low-abundance taxa (i.e. ≤ 30 total reads per metagenome) classified mainly as coccidia, ascomycete fungi, acanthamoeba, and algae.

Discussion

The evolution of pollen-feeding in *Heliconius* butterflies underlies a number of unique traits and likely facilitated the rapid diversification of this genus. Although *Heliconius* have been a major study system for decades, their microbiomes were unexplored beyond a few species [12, 21, 22]. In particular, it was unknown whether they evolved distinctive microbiomes concomitant with this major shift in diet and life history. This question is particularly interesting in butterflies, in which even major diet shifts in the larval stage (caterpillars) do not depend on microbiomes [9–11]. *Heliconius* is arguably the single lineage of Lepidoptera most reliant on adult-stage nutrition, and thus might be likely to engage in microbial symbioses as adults.

Using extensive field collections of 21 species and subspecies of heliconiine butterflies, we found that adult *Heliconius* have relatively low-diversity bacterial communities that are compositionally distinct from those found in their non-pollen-feeding relatives (Fig. 1, Fig. 3). Most of the dominant bacterial taxa were present in isolated gut tissue, but others were enriched elsewhere in the body, such as the hemolymph or structures in the head and thorax (Fig. S1, Table S1). Beyond the clear separation of *Heliconius* from other heliconiine genera, there was also strong a phylogenetic signal in adult heliconiine butterfly microbiomes (Fig. 2). The strength of this signal was comparable to that observed in mammalian gut microbiomes [3] and other host groups [36].

Host-phylogenetic structure in microbiomes can arise from host-microbe co-diversification and/or contemporary host filtering of environmental microbes [4, 36]. Given that the dominant bacterial taxa appear to be largely shared among host lineages (discussed below), we suggest that host filtering is likely to be particularly important in heliconiine butterflies. For example, the shift to pollen-feeding in adult *Heliconius* represents a major change in nutrient inputs, potentially altering which resident bacteria are able to proliferate in the gut. Pollen itself also harbors microbes [52], some of which may colonize the gut. Community assembly in the adult stage could also be influenced, in part, by dynamics occurring in earlier life stages [7]. Some bacterial taxa present in mature, adult *H. erato* are already present in freshly emerged (unfed) adults, pupae and larvae, and are potentially carried over from the larval host plant through metamorphosis [22]. Moreover, Heliconiini larvae show marked variation in the species

identity and the age (young vs. old foliage) of host plants they consume [17], traits reported to structure leaf microbiomes [53–55].

Certain bacterial taxa were enriched in *Heliconius* butterflies and their abundances may be associated with with the evolution of pollen-feeding. For example, *Orbus* was the most dominant bacterial genus among *Heliconius* and was more than twice as proportionally abundant in *Heliconius* as compared with other heliconiine butterflies. The biology of the Orbaceae is largely unknown, excepting the specialized bee gut symbionts *Gilliamella* and *Frischella* [56, 57]. In honey bees, which also consume pollen, some *Gilliamella* strains can degrade and ferment structural compounds in pollen walls [58, 59]. Adding pollen to honey bee diets also increases gut colonization by *Gilliamella* and *Frischella* [60]. However, a key difference is that pollen digestion in *Heliconius*, unlike bees, occurs extra-orally. Pollen grains collected on the proboscis are steeped in exuded saliva, and amino acids and other compounds released from the grains are then ingested [61]. Host-encoded enzymes that likely play a role in pollen digestion have been identified [62, 63]. Whether microbes contribute to this process is still unclear, but we identified candidate bacterial taxa that are overrepresented in the head and thorax (Table S1), structures that contain the proboscis and salivary gland. The exact localization and role of these microbes is worth more detailed investigation.

Based on the amplicon-based data, we found that microbiome differences across heliconiine butterflies were largely driven by shifts in the relative abundance of shared bacterial genera and ESVs, as opposed to the presence or absence of host-specific bacterial taxa. These bacteria might be widely distributed among larval host plants or adult nectar sources, and independently acquired by different butterflies. Sympatric butterfly species might also exchange bacteria, given their frequent co-occurrence at nectar sources. Flowers can act as hubs for the horizontal transmission of microbes among pollinators [64, 65].

The amplicon data were based on a short region of the 16S rRNA gene and might obscure finer-scale diversity and bacterial distribution patterns among hosts. We examined this possibility using near-full-length 16S rRNA gene sequences from the bacterium *Orbus*. As inferred from the amplicon data, this genus was represented by a single ESV that was nearly ubiquitous across all butterflies (Fig. 4). We found additional diversity among the longer sequences, but within butterflies, different *Orbus* phylotypes did not segregate by butterfly phylogeny or geography (Fig. 5). At a broader scale, the Orbaceae do appear to exhibit some

host-taxonomic specificity (Fig. 5; also see [49]). Potentially, the Orbaceae have diversified into loosely insect-order-specific lineages which at finer scales (e.g. within butterflies) frequently disperse among host taxa and regions. However, we note that the 16S rRNA gene evolves slowly, making it difficult to differentiate environmental transmission from co-diversification in a young host radiation such as the Heliconiini [35] without additional information [4].

Adult butterflies clearly structure the composition of their bacterial communities, but the reciprocal effect of these microbes on adult butterfly biology is completely unknown. A recent study found nectar- and fruit-feeding butterflies to host gut microbes with different abilities to metabolize substrates *in vitro* [12], but whether these differences are expressed *in vivo* and whether they would matter for host nutrition remains unclear. A subsequent experiment on the butterfly *Speyeria mormonia* using antibiotics did not find a strong relationship between gut bacterial loads and measures of adult fitness [66]. However, antibiotic-based studies could fail to test a role of the microbiome in resistance to parasites and pathogens if the latter are also suppressed by antibiotics.

Colonization resistance is a common feature of many animal gut microbiomes [4, 67, 68] and could be important in adult butterflies. Here, we discovered a high prevalence of putative eukaryotic parasites (trypanosomatids and microsporidia) across adult heliconiine butterflies. Butterfly-associated microeukaryotes are almost unknown, and there is very little research on any adult-stage pathogens or parasites (with the exception of the neogregarine *Ophryocystis elektroscirrha* in monarch butterflies [69]). *Serratia* were also widespread among heliconiine butterflies and are frequently pathogenic in insects [70, 71]. Given their co-localization in butterfly guts, we expect direct interactions and potential antagonism between these putative parasites and other gut microbes. For example, in some pyrrhocorid bugs, tsetse flies and social bees, gut bacteria provide an important layer of defense against trypanosomatid parasites and *Serratia* [72–75].

Conclusions

This study provides the first in-depth characterization of *Heliconius* and other passion-vine butterfly microbiomes, adding a new dimension to a classic model system in evolutionary biology. Our finding that a major diet shift (pollen-feeding) coincides with a shift in adult-stage

microbiomes advances previous work on Lepidoptera feeding ecology and microbial interactions, most of which has focused on the larval stage. The ecological dynamics of microbiomes appear to be very different between larval and adult life stages, at least for lepidopteran groups that feed as adults. We also identified a strong host phylogenetic signal in passion-vine butterfly microbiomes, and speculate that it arises from host filtering of environmental microbes with conserved dietary or behavioral traits, as opposed to co-diversification.

The unique microbiome in *Heliconius* likely represents a response to the nutritional and microbial inputs from pollen, but may also represent a functional contribution to host pollen digestion. However, despite these microbial community-level differences, individual bacterial taxa were typically shared across host species and diet guilds. We suggest there is also overlap in microbiome function among hosts. In particular, the common occurrence of putative parasites in guts of various host species points to colonization resistance as a candidate function of adult microbiomes in passion-vine butterflies. Manipulative experiments can now be performed, informed by our characterization of natural microbial community structure across butterfly hosts. Such studies would fill a critical gap in our understanding of butterflies, a diverse group of considerable ecological, societal, and scientific importance [76].

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