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Abstract

Gut microbial communities have important roles in reproduction, digestion, and pathogen protection of their insect hosts. Given the importance of these endosymbiotic communities to their host, research on the diversity and ecology of microbiomes is receiving increasing attention. I wanted to test the relative importance of host species and geography in shaping microbiome composition. Using the V4 region of the 16S gene, we compared microbiome communities of two species of butterflies across two geographic locations. I used 14 individuals from two species, *Heliconius cydno* and *Heliconius clysonymus,* from forest reserves in Manizales, Caldas and Filandia, Quindío, in the Central Range of the Colombian Andes. Alpha diversity indices, including Shannon and Inverse Simpson, demonstrated similar amounts of taxonomic diversity across species and sites but with changes in abundance between butterfly species. Principal Coordinate Analysis (PCoA) of the microbial communities of individuals showed that the variability in microbiomes was decoupled from species identity and site. Proteobacteria was the most abundant phylum across all samples and *Commensalibacter* was the most common bacterial genus. In addition, we found the presence of intracellular symbiont *Spiroplasma* and *Wolbachia* in our samples.

Introduction

The microbiome is defined as a "characteristic microbial community occupying a reasonably well-defined habitat which has distinct physio-chemical properties" (Whipps et al., 1988). This term not only refers to the microorganisms involved, but also their theatre of activity, which results in the formation of specific ecological niches prone to change in time and space (Berg et al., 2020). The study of the microbiome of insects has gained attention in recent years mainly because of its importance in host-plant adaptation. Research has demonstrated that these communities of microorganisms can provide information on life history, behavior, digestion, and immune response of hosts (Majumder, 2019), and even may help to protect hosts against pathogens (Krishnan et al., 2014). But also, their gut-associated bacteria can acquire new genetic material (e.g., like detoxifying genes) via horizontal gene transfer from external bacteria; or can be replaced by new bacteria with different metabolic potential (Hansen et al., 2014). Santos and colleagues (2020) found that certain gut-associated bacteria of the whitefly (i.e., phloem-feeding hemipteran tabaco pest), *Bemisia tabaci* (Gennadius, 1889) increased the ability to feed from different types of host plants.

It has been suggested that diet is a crucial factor in shaping insect gut microbial communities (Hammer et al., 2014). Minard et al. (2019) demonstrated that individual caterpillars of the butterfly species, *Melitaea cyxia* (Linnaeus, 1758), that feed on the same host plant can developed distinct microbiota in their guts. This suggests the need for studying not only dietary-related factors, but also other factors, including microbiome acquisition by maternal and horizontal transmission, and other unexplored phenomena, including nuances across taxonomy.

Long-wing butterflies are members of the genus *Heliconius* and are one of the best-known insect diversifications, due to their high degree of wing pattern divergence (Joron et al., 2006). They are members of the family Nymphalidae, commonly known as the brush-footed butterflies (Turner, 1968). At present there are 48 described species, but within that small number of species, there are hundreds of wing color variants across their geographical distribution (Willmott, n.d.). These are determined by combinations of alleles of a few sets of genetic loci with a large phenotypic effect (Joron et al., 2006); for example, three of the major loci affecting wing patterning genes are: optix, cortex, and WntA (Morris et al., 2019). These butterflies have been subject of many studies including important evolutionary processes like, aposematism, Müllerian mimicry in local populations, convergence, speciation, and sexual selection, among others (Kronforst & Papa, 2015, Morris et al., 2019, Baxter et al., 2008). *Heliconius* butterflies are also famed for their innovative ability to collect and utilize pollen as a protein source (Gilbert, 1972). Because of the wealth of knowledge of the genus and their unique utilization of pollen as a food source, several recent studies have examined gut microbiota in *Heliconius* species (Hammer et al., 2014, 2020; Van Shooten et al., 2018).

Hammer et al., (2014) explored the microbiome diversity of *Heliconius erato* throughout their ontogeny. This study showed that the change in diet accompanying the shift from caterpillar to adult resulted in significant differences in microbiome composition of the two life stages. Other studies have explored the variation between *Heliconius* species that share similar trophic niches. Van Shooten (2018) showed that the microbiome composition differs between individuals within and across *Heliconius* species living in Gamboa, Panama. While these recent studies have increased our understanding of how the

microbiome can vary within these charismatic butterflies, information is still biased to specific locations (primarily Gamboa, Panama), few species, and, in some studies, life cycle stages.

In this study I explored the gut microbiome bacteria diversity and composition of two *Heliconius* species from two populations located in the Colombian Central Cordillera (Fig. 1). I included *Heliconius cydno*, with nine described subspecies, and *Heliconius clysonymus,* which has two subspecies within Colombia (Rueda comms. pers). In the case of *Heliconius cydno*, their wing polymorphism is observed for band color (yellow- white), pattern (triangle or band) and hindwing bandwidth (Kapan, 1998). Studies have demonstrated there is a strong male preference to court their same color-pattern females, which can explain the presence of different partially isolated subspecies across the distribution for this species (Chamberlain et al., 2009). Whereas *H. clysonymus* is significantly less polymorphic and displays little variation across its geographical range. I evaluated the following research questions: 1) Is the diversity and composition of abdominal microbiome bacteria different for both species, *Heliconius cydno* and *Heliconius clysonymus*? 2) Is diversity and composition of microbiomes at both sampling sites different or similar considering the conservation state (e.g.; fragmented vs. non-fragmented) of each location? And finally, 3) Does the Colombian butterfly samples show similar gut-microbiome bacteria diversity and composition than the ones previously assessed from other countries like Panama and Ecuador? Overall, this small assay will provide new information in regard to wing diversity, local and broad geographical scales of the gut-microbiome diversity for this butterfly genus.

Materials and Methods

Study area

My collaborators (Emily Khazan field crew) sampled *Heliconius cydno cydnides* and *H. clysonymus clysonymus* at two sites in the Colombian Central mountain range; a forest fragment located in the village El Aguila in Manizales, Caldas (5.10655 N, 75.50636 W) at approximately 2000 m.a.s.l (meters above sea level), and a conserved forest located in the Bremen-La Popa forest reserve in Filandia, Quindío (4.672131 N, 75.64066 W, Figure 1) between 1850 and 2050 m.a.s.l.

Butterflies sample collection

In total, the crew collected 14 individuals; 10 *H. cydno* and four *H. clysonymus* across the two sites. All specimens were captured using either Van Sommerer traps or through active entomological netting. Collection took place in May and June 2019. Van Sommerer traps were baited with a mixture of fermented fruit and a mixture of fish and nitrates.

They excised the abdomen for every individual under sterile conditions and preserved them in RNAlater (ThermoFisher) in Eppendorf tubes in a conventional freezer. In addition to the collection of individuals abdomens, they collected the local microbiome of the bait used in traps as the control samples, by stirring the bait and placing a tube with RNA later open next to the bait for at least 15 minutes. For specimens collected via netting, we followed the same protocol to collect control microbes by placing a tube with RNA later open in the environment for at least 15 minutes.

DNA Extraction

For the DNA extraction of the gut bacteria, I vertically cut each abdomen, leaving half for voucher specimens. Likewise, with control samples, I processed half of the material preserved in RNA later of each sample. I performed the extraction using the DNeasy PowerSoil Extraction Kit for soil bacteria following the manufacturer's protocol

(QIAGEN). Once extracted, I quantified the concentration and purity of the DNA with a Nanodrop 2000 and 1% agarose gels using SYBR Safe (ThermoFisher) for visualization.

16s amplification and sequencing

Amplification of the 16S gene was performed in triplicate for each sample following the protocol of Meyer et al. (2019). We used 0.25 µM of each of the V4 Earth Microbiome primers 515F (GTGYCAGCMGCCGCGGTAA) and 806R (GGACTACNVGGGTWTCTAAT, Apprill et.al, 2015), 2 µM of DNA, 3% of dimethyl sulfoxide and Phusion High-fidelity Master Mix for a 25 µM for PCR reaction. The triplicate samples were cleaned with MinElute PCR purification kit (QIAGEN) and DNA concentration was quantified with a Nanodrop 2000. DNA extractions were made, they included blanks without abdomen samples and DNA extractions with the butterfly abdomen samples. Two extraction blanks, one PCR blank and the butterfly gut samples were sequenced with Earth Microbiome barcodes following the same protocol. A final amplicon pool of 18 samples with 240 ng of each sample library was submitted to the Interdisciplinary Center for Biotechnology Research (ICBR) at the University of Florida for sequencing with Illumina MiSeq.

Data Cleaning and Taxonomic Assignment

Once raw sequences were obtained, we removed primers and adapters using cutadapt v. 1.8.1 (Martin, 2011) and used the DADA2 v. 1.14.1 pipeline (Callahan et al. 2016). We cleaned and removed chimeras (PCR artifacts not associated with the 16S region), and estimated the error rate and allocation of ASVs (Amplicon Sequence Variant) using DADA2 default parameters and the following filter settings: *filterAndTrim(fnFs, filtFs, fnRs, filtRs, truncLen=c(150,150),maxN=0, maxEE=c(2,2), truncQ=2,*

rm.phix=TRUE,compress=TRUE, multithread=TRUE). Once this process was completed, we made the taxonomic assignment through the SILVA rRNA database (Quast et al., 2013) up to the genus level, and then removed sequences with low prevalence or that belonged to chloroplasts or mitochondria.

Microbiome Bacterial Composition and Diversity Estimation

With the filtered data we generated rarefaction curves to observe the minimum sequences depth. We estimated the relative abundance of phyla and genera across controls, species (*H. cydno* and *H. clysonymus*) and sampling sites (Bremen, El Aguila). We visualized the composition of microbiome at the phylum and genus level using the 11 and 15 most abundant groups respectively ranked by relative abundance (figure 3 and 4). We then performed a Kruskal-Wallis test (hereafter, K-Wt) to check for significant differences in the relative abundance of specific genera of the bait controls and the butterfly samples. Statistically significant K-Wt were followed by post hoc pairwise Dunn tests. To compare microbiome across sites, we used Mann Whitney- U test (hereafter, M-Wht), grouping both *Heliconius* species for each sampling site (Bremen, El Aguila) to check for significance.

We used the R packages Phyloseq v. 1.30.0 (McMurdie & Holmes, 2013) and Microbiome v.1.10.0 (Leo et al., 2017) to estimate the following alpha diversity metrics: Inverse Simpson (i.e., diversity estimator considering the number of species), Shannon (i.e., estimator of diversity considering richness) and Fisher (i.e., estimator of diversity considering different sample sizes). For each of these metrics we performed Mann-Whitney U (M-Wht) to test for differences in diversity between sites, and Kruskal-Wallis (K-Wt) to test for differences between species, in case of significance in the latter (p values < 0.05), post hoc pairwise Dunn test would be performed.

We ordinated the microbial communities of our butterfly and control samples to explore the diversity patterns and beta diversity within and across species and sampling sites. We used a Principal Coordinate Analysis (PCoA) using the Bray-Curtis distance matrix with the Vegan R package v. 2.5.6 (Oksanen et al., 2019). Finally, we performed three Permanova analyses to test the differences between sampling sites, *Heliconius* species and controls vs *Heliconius* species.

Results

Gut-Microbiome bacteria composition

Overall, I obtained 1'715,744 good-quality reads with an average of 95,319 reads per sample. The most depauperate sample was of a control which had 9 reads, and the largest sample resulted in 155,774 reads (Supplementary Table 1). I found a total of 2,385 ASVs, the sample with least ASVs was a control with 4 ASV, and the sample with more ASV was also a control with 545 ASVs and a *H. cydno* sample with 455 ASVs (Table 1). Rarefaction curves showed that most of the species' richness was detected, as demonstrated by the asymptotic behavior of the curves (Figure 2).

I found 10 main bacteria phyla in the microbiomes of the two butterfly species (*H. cydno*, *H. clysonymus*) and controls (Figure 3). Four of these bacterial phyla (Proteobacteria, Firmicutes, Bacteroidetes and Tenericutes) were the most abundant in both the *Heliconius* and control samples. We examined the identity of the 15 most abundant bacterial genera in the *Heliconius* samples and controls from these four phyla (Figure 4); 11 genera (73%) corresponded to phylum Proteobacteria (*Orbus, Commensalibacter, Hafnia, Obesumbacterium, Wolbachia, Citrobacter, Pseudomonas, Klebsiella, MD3.55,*

Endozoicomonas, Thalassotalea and *Algicola*) while the remaining four (27%) were from Tenericutes (*Spiroplasma*), Bacteroidetes (*Dysgonomonas*) and Firmicutes (*Enterococcus* and *Lactococcus*).

Commensalibacter was the most abundant bacterial genus in all butterfly and control samples (Figure 4) and did not significantly vary in relative abundance between *Heliconius* and control samples (K-Wt, chi-squared = 3.4751, df = 2, p-value = 0.176). We found high abundances of the bacterial genera *Wolbachia* and *Spiroplasma* in two butterfly samples. One *H. cydno* from El Aguila showed a high presence of *Wolbachia*, 74% of that individual's total reads (Figure 4, sample ESK0777). A single *H. clysonymus* from Bremen showed *Spiroplasma* in high relative abundance, 44% of the individual's total microbiota reads, (Figure 4, sample DNA0688). *Thalassotalea* and *Endozoicomonas* were two bacterial genera abundant in the control samples but nearly absent in the butterfly samples, K-Wt between controls and butterfly samples showed significant differences in relative abundance of controls and *Heliconius* species (*Thalassotalea* K-Wt: chi-square = 9.2231, df = 2, p-value = 0.009936, *Endozoicomonas* K-Wt: chi-squared = 10.328, df = 2, p-value = 0.005719, see Table 2).

Genus *Enteroccocus* was more abundant in *Heliconius* samples than controls (Figure 4, K-Wt between controls and butterfly samples: chi-squared = 7.8649, df = 2, p-value = 0.0196, Table 2), when I performed post hoc pairwise Dunn test, these differences were due to a higher relative abundance in *H. cydno* than in the control samples (Table 2). Genus *Orbus* was also more abundant in *Heliconius* samples than in controls (K-Wt: chi-squared =

6.6244, df = 2, p-value = 0.03644), however post hoc Dunn tests did not reveal significant pairwise differences between controls and each *Heliconius* species' samples.

Alpha diversity differences between species and sample sites

I found no statistically significant differences in the Shannon nor InvSimpson diversity indices between butterfly species and controls using Kruskal-Wallis test (Shannon: chi-squared = 2.6281, df = 2, p-value = 0.2687, InvSimpson: chi-squared = 3.2754, df = 2, p-value = 0.1944). There was no difference in ASV richness of control and *Heliconius* samples, however I observed more ASV richness in the microbiome of *H. cydno* compared to *H. clysonymus* (K-Wt, chi-squared = 6.5123, df = 2, p-value = 0.03854; Figure 5), as well as a higher diversity in the microbiome of *H. cydno* compared to *H. clysonymus* (Fisher: K-Wt, chi-squared = 6.5123, df = 2, p-value = 0.03854, Figure 5).

When I grouped microbiome composition by site, I found no significant differences in observed or estimated diversity across sites (InvSimpson: M-Wht, W = 28, p-value = 0.7104; Shannon: M-Wht, W = 27, p-value = 0.8048; Observed microbiome alpha diversity: M-Wht, W = 28, p-value = 0.7104, see Supplementary Figure 1).

Beta diversity and PCoA Analysis

Principal Coordinate Analysis (PCoA) for species (Figure 6) demonstrates differentiation in bacterial composition of butterfly species compared with controls as shown by the ellipse of the control group separated from those of the *Heliconius* groups. Permanova analysis showed a significant difference in diversity between controls and butterfly samples (F.model = 1.6278, R2 = 0.1783, Pr = 0.004), suggesting that the microbiome diversity found in both *Heliconius* species abdomen is independent of the microbial community of

the environment. On the other hand, PCoA of sites did not show differentiation in bacterial composition by site (Figure 7); Permanova was not significantly different between the two sampling sites (Bremen, El Aguila) (F.Model = 1,07, R2 = 0.082, Pr = 0,37). Permanova also did not show significant differences between the two *Heliconius* species (F.Model = 0,72, R2 = 0,056, Pr = 0,86) which means both *Heliconius* species share a similar bacterial community composition.

Regarding the comparison with other studies (Table 3), the most common bacterial genera in comparison across our and the previous studies were: *Commensalibacter, Orbus, Enterococcus, Lactococcus, Pseudomonas* and *Spiroplasma*. However, I was available to recover unique genera in this study like, *Wolbachia* and *Citrobacter* (Table 3). In addition is important to notice that some ASV's in Van Schooten et al., (2018) were not identified to genera, only family, like Enterobacteriaceae and Acetobacteriaceae.

Discussion

The results showed that gut-microbiome bacteria of the two *Heliconius* species are generally similar in composition (Figure 3). In addition, they showed low microbial richness compared to controls as demonstrated by the presence of a small number of dominant ASVs (see Figure 2, 4 and 5). These results are consistent with other studies where the dominant in abundance phyla were Proteobacteria and Firmicutes (Figures 3, 4, van Schooten et al., 2018). My results suggests that gut-microbiome bacteria of the two *Heliconius* species shared similar bacterial genera, with *Commensalibacter* (family: Acetobacteraceae, phylum: Proteobacteria) being the most abundant genus in the microbial community of both species. The latter has been documented in other insects with sugar-heavy diets like fruit flies (e.g., *Drosophila)* and honeybees (e.g., *Apis mellifera*); and has been suggested to be involved in

gut immune homeostasis (Chandler et al., 2011; Siozios et al., 2019). In addition, other top genera are similar to other previously reported for this butterfly genus, including: *Orbus*, *Pseudomonas*, *Lactococcus*, *Spiroplasma* and *Enterococcus* as the most common (Table 3; Hammer 2014, 2020; van Schooten et al., 2018). For example, *Orbus* (Phylum: Proteobacteria) was also found *H. erato i*n Panama (Hammer et al., 2020) and non-*Heliconius* species (*Sasakia charonda*) (Kim et al., 2013). *Enterococcus* (Phylum: Firmicutes), has also been reported in the gut community of *Heliconius erato* (Huff et al., 2020). In contrast, *Enterobacter* was found in low abundance in our samples while it was found in high abundance in *Heliconius erato* from Panama and Ecuador (Hammer 2014, 2020).

Wolbachia (Phylum: Proteobacteria), a genus that is known to have associations ranging from mutualism to parasitism, was found in only one individual of *Heliconius cydno* albeit in extremely high abundance. These bacteria can induce feminization and sperm-egg incompatibility and play an important role in population control of butterflies and other insects like *Drosophila* (Chandler et al., 2011). Currently *Wolbachia* is used for sterilization and therefore control of the mosquito *Aedes aegypti*, a primary vector of dengue, zika, yellow fever and chikungunya. *Wolbachia*-infected males that mate with non-infected females produce undeveloped zygotes causing a reduction in their population (Crawford et al., 2020). In addition, we found the presence of the intracellular bacteria *Spiroplasma* (Phylum: Tenericutes) in *H. clysonymus*. It was reported by Van Schooten et al. (2018) for other *Heliconius* species like *Heliconius doris* and *Heliconius ismenius*. *Spiroplasma* has been found in other butterflies (Jiggins et al., 2000) and has been shown to influence the survival rate of some *Drosophila* species (Xie et al., 2010). How these

symbionts affect *Heliconius* butterflies throughout their life cycle remains to be investigated.

The alpha and beta diversity estimates for both butterfly species were quite similar, however we identified that *H. cydno* showed more variation in the estimates than *H. clysonymus* (Figures 5, 6). Richness measurement is affected by sample size, which makes it difficult to find which species has a higher richness if we compare groups with different sizes (Kim et al., 2017). However, Fisher index estimation suggests that *H. cydno* is more diverse than *H. clysonymus* despite differences in sample size. While I did not find distinct spatial or species-level patterns in the gut bacterial communities for both butterfly species studied (Figure 6 & 7), I was able to classify the common bacterial genera in both species and sites (Figure 4). I can conclude that our sampling sites separated by approximately 50 km and despite the different conservation states (i.e.; fragmented vs non fragmented) were not enough to show differences in the abdominal microbiome bacteria for these two butterfly species. It will be necessary to sample different sites across multiple geographical regions (e.g.; Andean vs Amazon), if possible, from different geographical distances, since different regions can show changes in the microbiome as demonstrated by the study of Luna (2021).

The field crew captured *Heliconius* butterflies using aerial netting methods and in baited Van Someren traps, a sampling method not typically associated with *Heliconius.* Our documentation of both bacterial diversity of these capture environments (i.e., ambient bacteria in the air, bacteria in baits) allowed us to examine associations between environmental and internal bacterial community structure. There was very low concordance

between environmental and butterfly bacterial microbiomes. This finding support that the microbial communities within the gut of this butterflies are selective and thus distinct from the microbial makeup of their surroundings (Figure 4, Supplementary Material Figure 2). For example, two of the main bacterial genera found were mostly from controls, *Thalassotalea* and *Endozoicomonas* (Figure 4). Genus *Thalassotalea* (Phylum: Proteobacteria) is usually found from marine environments and contributes to nutrient cycling (Kim et al., 2020), while *Endozoicomonas* (Phylum: Proteobacteria) can be found in corals and other marine invertebrates. This genus is used as a coral health indicator of since it is found in high amounts in healthy corals and in low abundance in diseased corals (Tandon et al., 2020). Both bacterial genera probably came from the bait which contained fermented fish and shrimps.

In general, microbiomes of different *Heliconius* species located in some countries from Central and South America shared similar top genera, specially belonging to phyla Proteobacteria and Firmicutes. However other less abundant genera were variable from different studies since *Heliconius* species and samples from the same species had different ASVs or OTUs abundance (Table 3, Hammer et al., 2014, 2020; Van Shooten et al., 2018).

Conclusions

My results illustrate the diversity within and variation across bacterial microbiomes of two *Heliconius* butterfly species. *H. cydno* was more diverse than *H. clysonymus* despite differences in sample size, but the full structure and composition of the communities harbored by the two butterfly species were quite similar. Further, using multivariate ordination methods, I was able to demonstrate that sampling sites with different conservation conditions did not affect the diversity nor composition of the gut microbial

communities tested in this small assay. At a broad scale, my results were consistent with other gut microbiomes studies for the genus *Heliconius*, I found a similar composition than the previously reported. Although the abundances might differ slightly across species and geographical locations. Finally, I was able to conclude that the gut-microbiome of these two species of butterflies is completely different from their environments, because their composition and diversity estimates are significantly different for the control samples.

Moving forward researchers should continue to explore the diversity of gut microbiomes of insects, ideally using a greater number of samples for each species and ensure the replicates for all sexes. With increased efforts across taxonomy and space, we will better understand patterns of microbial diversity, including intraspecific variation and geographic patterns, such as along altitudinal gradients with different types of vegetation. Future research should also be dedicated to studying the larval and adult states of individuals, as bacterial composition may change due to ontogeny and/or changes in diet across life stages.

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Tables

Table 1. Richness, Simpson Index and Evenness of all the samples and controls.

SampleID	Site	Species	Richness (#of ASVs)	Evenness
BBCntl22519	BB	Control	161	0.72
BBJCntrl-261518	BB	Control	260	0.85
BBJCntrl-281519	BB	Control	4	0.99
EABCont322019	EAB	Control	545	0.93
DNA0528	BB	*Heliconius clysonymus*	36	0.39
DNA0688	BB	*Heliconius clysonymus*	53	0.51
ESK0781	EAB	*Heliconius clysonymus*	53	0.60
DNA8797	EAB	*Heliconius clysonymus*	54	0.53
ESK0571	BB	*Heliconius cydno*	118	0.46
ESK0659	BB	*Heliconius cydno*	199	0.39
ESK0666	BB	*Heliconius cydno*	347	0.52
ESK0667	BB	*Heliconius cydno*	235	0.57
ESK0702	BB	*Heliconius cydno*	119	0.43
ESK0777	EAB	*Heliconius cydno*	103	0.21
ESK0785	EAB	*Heliconius cydno*	455	0.24
ESK0796	EAB	*Heliconius cydno*	86	0.53
ESK0918	EAB	*Heliconius cydno*	115	0.53
DNA0937	EAB	*Heliconius cydno*	114	0.40

Table 2. Statistical test comparing the four genera which contributed strongly to differences between butterfly and control samples. * significance $p < 0.05$

	Kruskal-Wallis test			**Dunn test**	
Genus	**Chi-squared**	**Df**	**P-value**	**Pairwise comparison**	**P-value**
Thalassotalea	9.2231	2	0.009936*	Control-*H.Cydno*	0.022*
				Control-*H.Clysonymus*	0.018*
				H.Cydno-H.Clysonymus	1
Endozoicomonas	10.328	2	0.005719*	Control-*H.Cydno*	0.0371*
				Control-*H.Clysonymus*	0.0056*
				H.Cydno-H.Clysonymus	0.67
Enterococcus	7.8649	2	0.0196*	Control-*H.Cydno*	0.016*
				Control-*H.Clysonymus*	0.164
				H.Cydno-H.Clysonymus	1
Orbus	6.6244	2	0.03644*	Control-*H.Cydno*	0.057
				Control-*H.Clysonymus*	0.073
				H.Cydno-H.Clysonymus	1

Table 3. Comparison between the principal bacterial genera of the abdominal microbiome of *Heliconius* butterflies from different studies in Panamá, Brazil and Ecuador. Red highlighted genera were found in most of the studies.

Bacterial genera	Our study (Colombia)	(Hammer et al., 2014) Panama	(Hammer et al., 2020) Panama and Ecuador	(van Shooten., 2018) Brazil
Commensalibacter	x	x	x	(unidentified genus)
Orbus	x	x	x	
Enterococcus	x	x	x	x
Lactococcus	x	x	x	x
Pseudomonas	x		x	x
Spiroplasma	x		x	
Enterobacter		x	x	(unidentified genus)
Acinetobacter		x	x	x
Asaia		x	x	
Apibacter			x	
Klebsiella	x		x	
Hafnia.Obesumbacterium	x			
Wolbachia	x			
Citrobacter	x			
Bacillus				x
Fructobacillus				x
Leuconostoc				x
Algicola	x			
Dysgonomonas	x			

Figure Legends

Figure 1. Butterfly sampling locations in the *eje* cafetero region of the central Colombian Andes. Forest fragments in the departments of Quindio and Caldas included Bremen and El Aguila respectively.

Figure 2. Rarefaction curve of the ASVs sequenced from the microbiomes of 14 individuals of *Heliconius cydno*, *Heliconius clysonymus* and control reads, showing a major richness from *H. cydno* compared to *H. clysonymus* (M-Wht, p-value=0,0019).

Figure 3. **Relative abundance of the main phyla of microorganisms found in the microbiomes of *H.cydno* and *H.clysonymus.*** Proteobacteria, Firmicutes and Bacteroidetes were the most abundant phyla in all samples, Tenericutes was abundant in one sample of *H. clysonymus* corresponding to the genus *Spiroplasma*.

Figure 4. Bubble plot of butterfly and control samples showing n=15 top genera, ranked by relative abundance for the two variables (Site, BB= Barbas Bremen, EAB= El Aguila) and species (*H. cydno*, *H. clysonymus* and controls). Asterisks show significant differences between controls and samples of 3 genera. Black asterisk indicates a higher abundance in *Heliconius* samples than in controls for the genus *Enterococcus* (Kruskal-wallis test, chi-squared = 7.8649, df = 2, p-value = 0.0196). Orange asterisks indicate a higher abundance in controls than *Heliconius* samples for the genera *Endozoicomonas* (Kruskal Wallis test, chi-squared = 10.328, df = 2, p-value = 0.005719) and *Thalassotalea* (Kruskal Wallis test, chi-squared = 9.2231, df = 2, p-value = 0.009936). (See table 2 for pairwise comparisons)

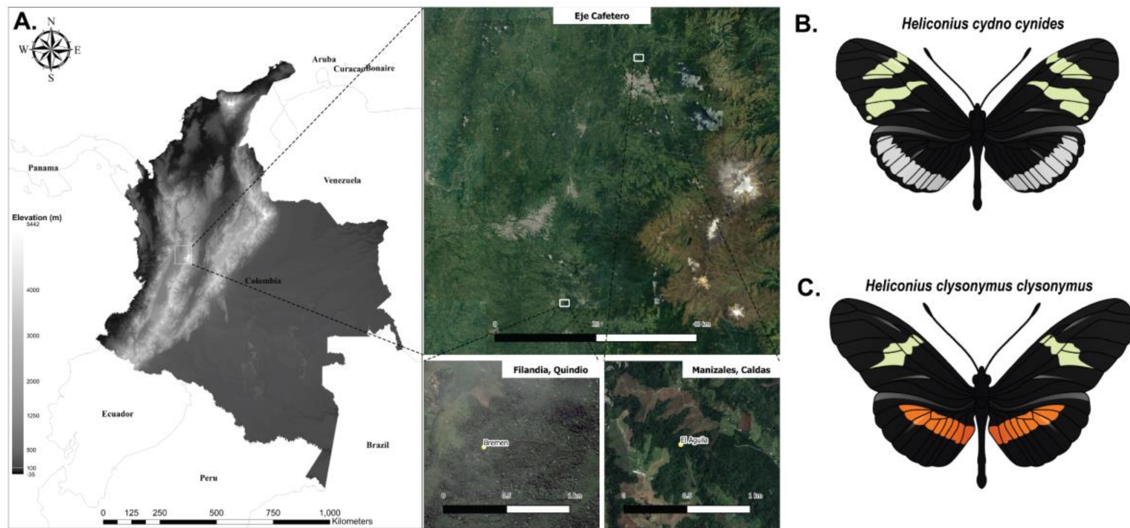
Figure 5.

Alpha diversity index (InvSimpson, Shannon and Observed) of two species of *Heliconius* butterflies (*H. cydno* and *H. clysonymus*) and controls. A) Inverse Simpson index. B) Shannon index where Mann-Whitney U test showed significant differences between controls and *H. cydno* (p-value= 0,03), *H.cydno* was less diverse than the controls. C) Observed data where Mann-Whitney U test showed significant differences between both *Heliconius* species (p-value = 0.002) *H. cydno* had a higher richness than *H. clysonymus*.

Figure 6. Principal coordinate analysis (PCoA) of the 14 samples of *H. cydno*, *H. clysonymus* and Controls. There is no patterns related to both *Heliconius* species (F.Model = 0,72, R2 = 0,056, Pr = 0,86), however controls were separated from the others butterfly samples, this shows that microbiome diversity found in both *Heliconius* species is independent of the microbial community of the controls (F.model = 1,6278, R2 = 0,1783, Pr = 0.004).

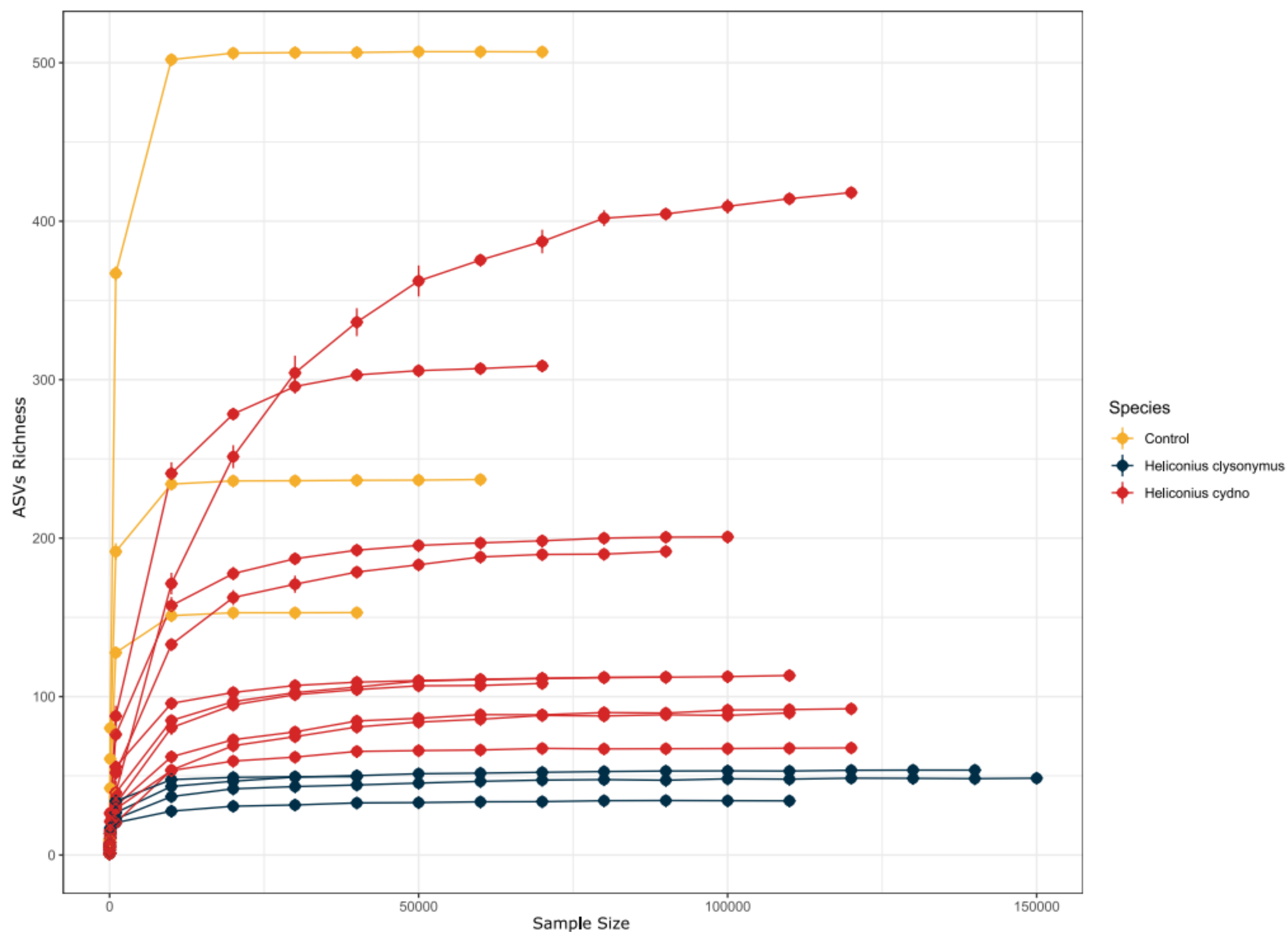
Figure 7. Principal coordinate analysis of the 14 samples of both sampling sites (BB= Barbas Bremen, EAB=El Aguila) showing no significant difference between sampling sites (F.Model = 1,07, R2 = 0.082, Pr = 0,37).

Figure 1.



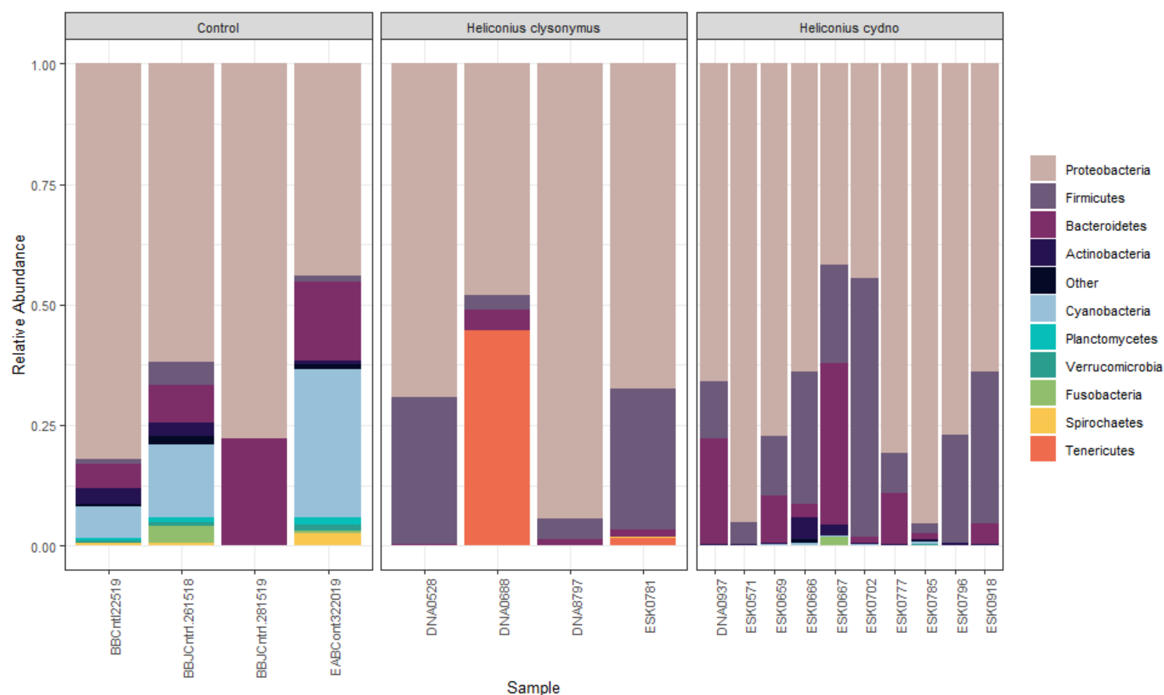
Butterfly sampling locations in the *eje* cafetero region of the central Colombian Andes and *Heliconius* species. A. Forest fragments in the departments of Quindío and Caldas included Bremen and El Aguila respectively. B. *Heliconius cydno cydnides* and C. *Heliconius clysonymus clysonymus*.

Figure 2.



Rarefaction curve of the ASVs sequenced from the microbiomes of 14 individuals of *Heliconius cydno*, *Heliconius clysonymus* and control reads, showing a major richness from *H. cydno* compared to *H. clysonymus* (M-Wht, p-value=0,0019).

Figure 3.

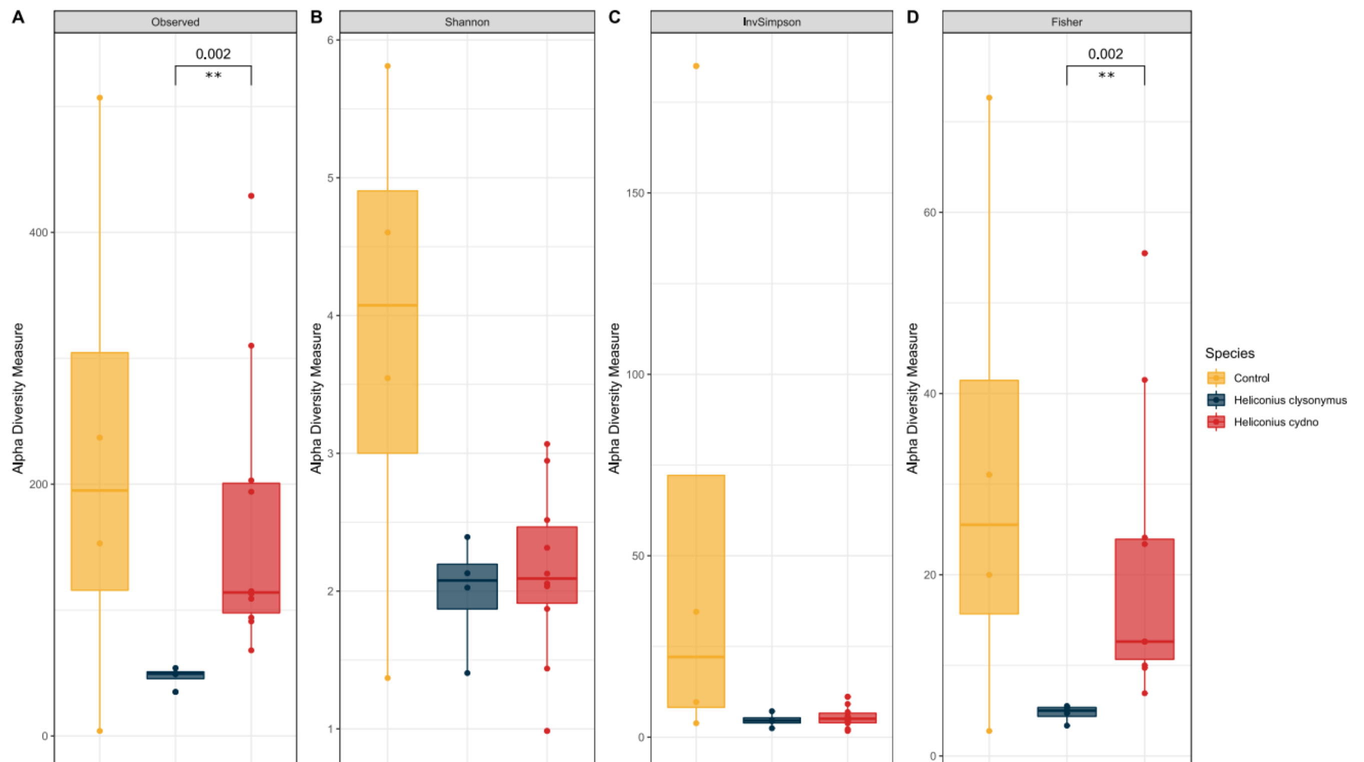


Relative abundance of the main phyla of microorganisms found in the microbiomes of *H.cydno* and *H.clysonymus*. Proteobacteria, Firmicutes and Bacteroidetes were the most abundant phyla in all samples, Tenericutes was abundant in one sample of *H. clysonymus* corresponding to the genus *Spiroplasma*.

Figure 4.

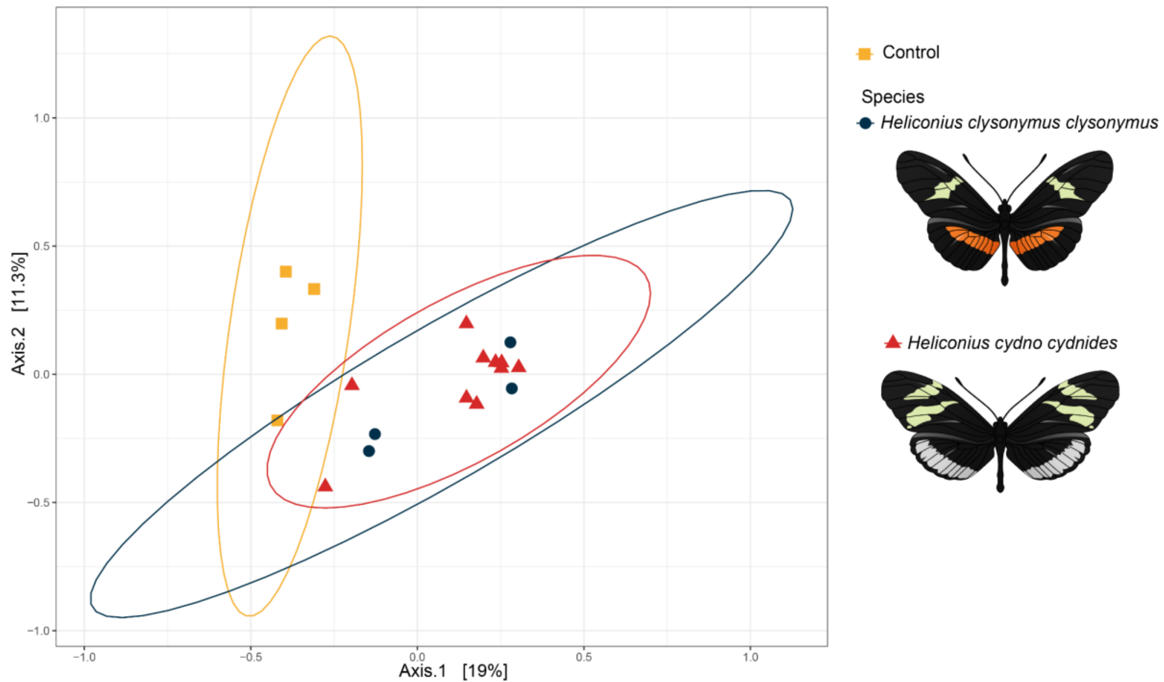
Bubble plot of butterfly and control samples showing n=15 top genera, ranked by relative abundance for the two variables (Site, BB= Barbas Bremen, EAB= El Aguila) and species (*H. cydno*, *H. clysonymus* and controls). Asterisks show significant differences between controls and samples of 3 genera. Black asterisk indicates a higher abundance in *Heliconius* samples than in controls for the genus *Enterococcus* (Kruskal-wallis test, chi-squared = 7.8649, df = 2, p-value = 0.0196). Orange asterisks indicate a higher abundance in controls than *Heliconius* samples for the genera *Endozoicomonas* (Kruskal Wallis test, chi-squared = 10.328, df = 2, p-value = 0.005719) and *Thalassotalea* (Kruskal Wallis test, chi-squared = 9.2231, df = 2, p-value = 0.009936). (See table 2 for pairwise comparisons)

Figure 5.



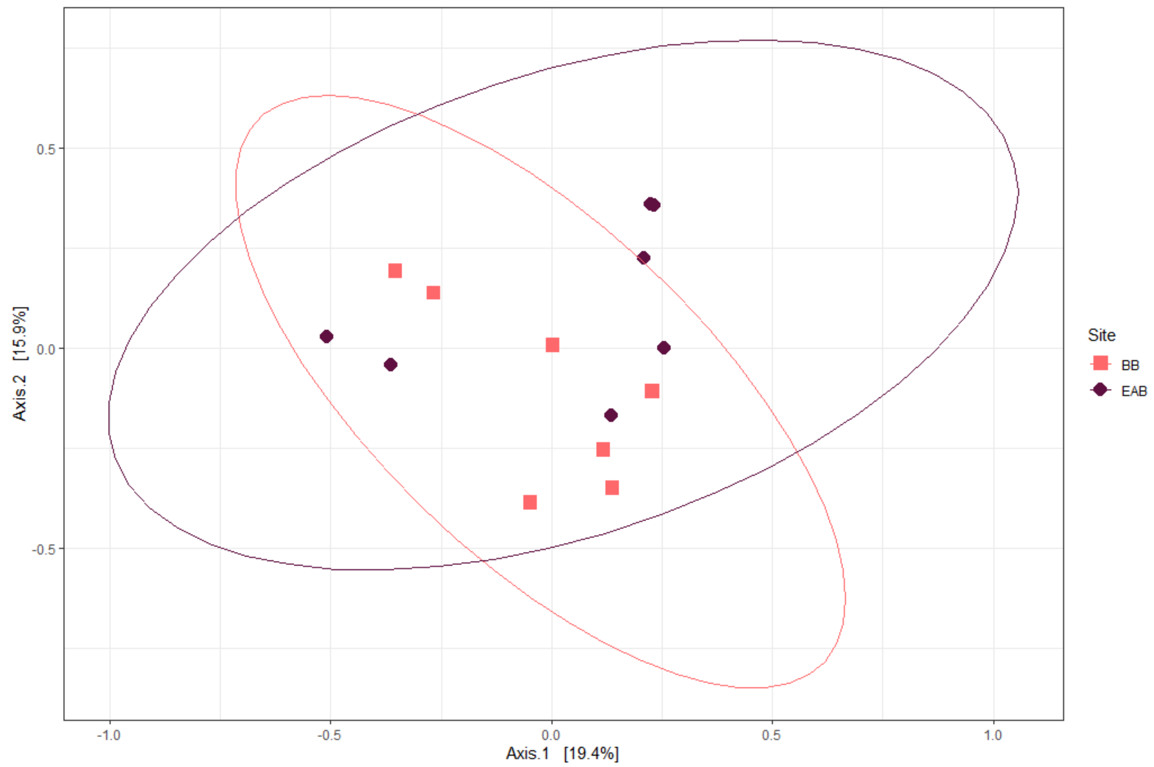
Alpha diversity index (Observed, Shannon, InvSimpson and Fisher) of two species of *Heliconius* butterflies (*H.cydno* and *H.clysonymus*) and controls. A) Observed richness data where Mann-Whitney U test showed significant differences between both *Heliconius* species (p-value = 0.002) *H. cydno* had a higher richness than *H. clysonymus*. B) Shannon index. C) Inverse Simpson index. D) Fisher index where Mann-Whitney U test showed significant differences between both *Heliconius* species (p-value = 0.002) *H. cydno* was more diverse than *H. clysonymus*.

Figure 6.



Principal coordinate analysis (PCoA) of the 14 samples of *H. cydno*, *H. clysonymus* and Controls. There is no patterns related to both *Heliconius* species (F.Model = 0,72, R2 = 0,056, Pr = 0,86), however controls were separated from the others butterfly samples, this shows that microbiome diversity found in both *Heliconius* species is independent of the microbial community of the controls (F.model = 1,6278, R2 = 0,1783, Pr = 0.004).

Figure 7.



Principal coordinate analysis of the 14 samples of *Heliconius* butterflies from both sampling sites (BB= Barbas Bremen, EAB=El Aguila) Permanova did not show significant difference between sampling sites (F.Model = 1,07, R2 = 0.082, Pr = 0,37).

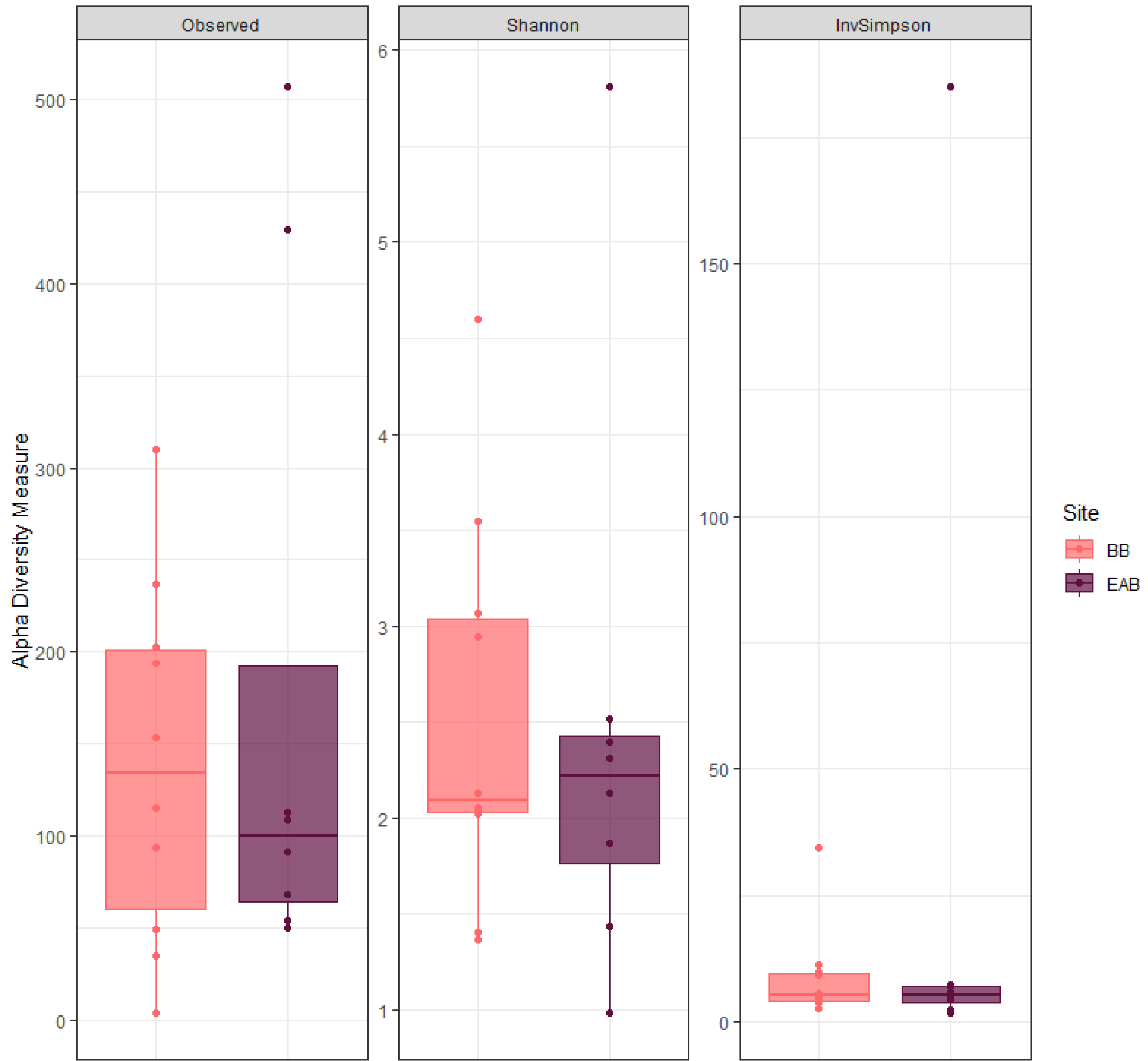
Supplementary Material

Supplementary Table 1. Total of read counts per sample.

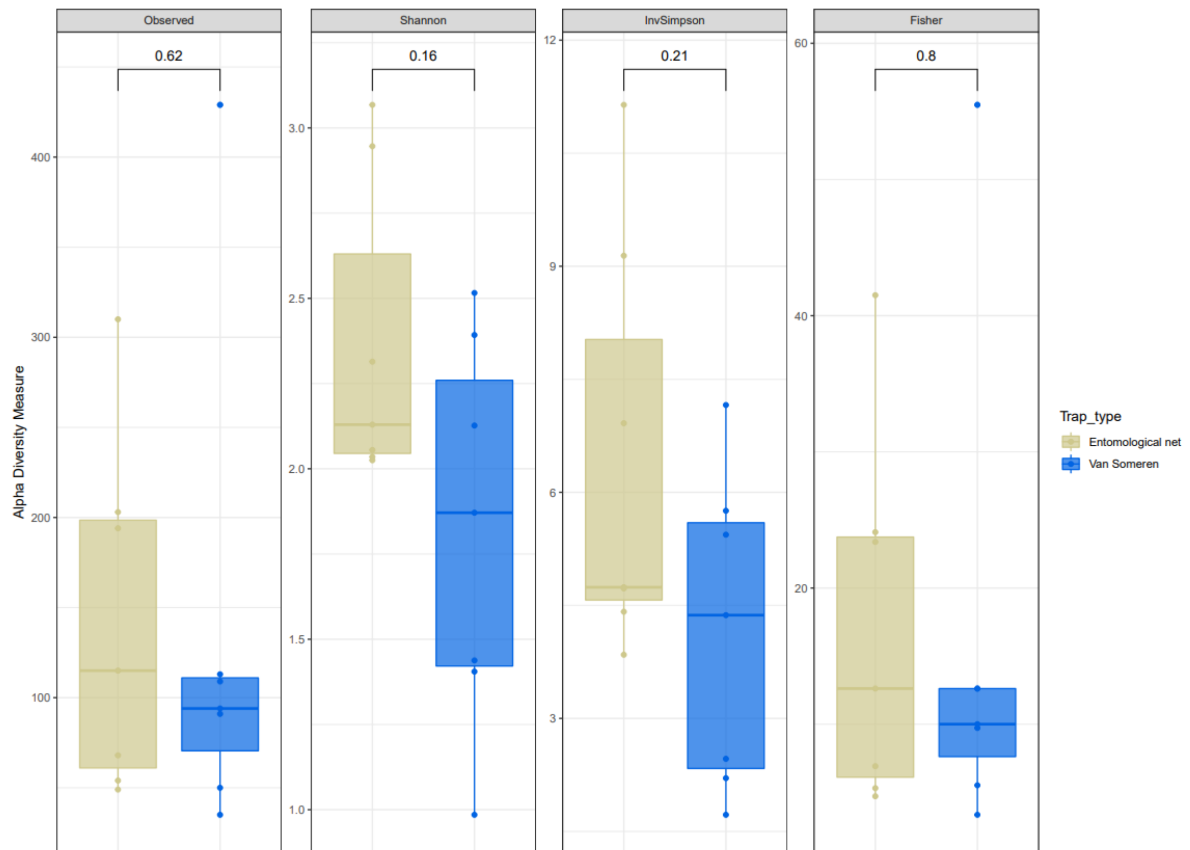
SampleID	Site	Species	Sequences per sample
BBCntl22519	BB	Control	43633
BBJCntrl-261518	BB	Control	70257
BBJCntrl-281519	BB	Control	9
EABCont322019	EAB	Control	86297
DNA0528	BB	*Heliconius clysonymus*	116385
DNA0688	BB	*Heliconius clysonymus*	155774
ESK0781	EAB	*Heliconius clysonymus*	47860
DNA8797	EAB	*Heliconius clysonymus*	140219
ESK0571	BB	*Heliconius cydno*	122837
ESK0659	BB	*Heliconius cydno*	93743
ESK0666	BB	*Heliconius cydno*	74710
ESK0667	BB	*Heliconius cydno*	110715
ESK0702	BB	*Heliconius cydno*	114668
ESK0777	EAB	*Heliconius cydno*	112826
ESK0785	EAB	*Heliconius cydno*	126914
ESK0796	EAB	*Heliconius cydno*	129417
ESK0918	EAB	*Heliconius cydno*	98509
DNA0937	EAB	*Heliconius cydno*	70971
Total			1715744

Supplementary Table 2. Kruskal-Wallis Test comparing each genus between species and controls. * significance of $p < 0.05$

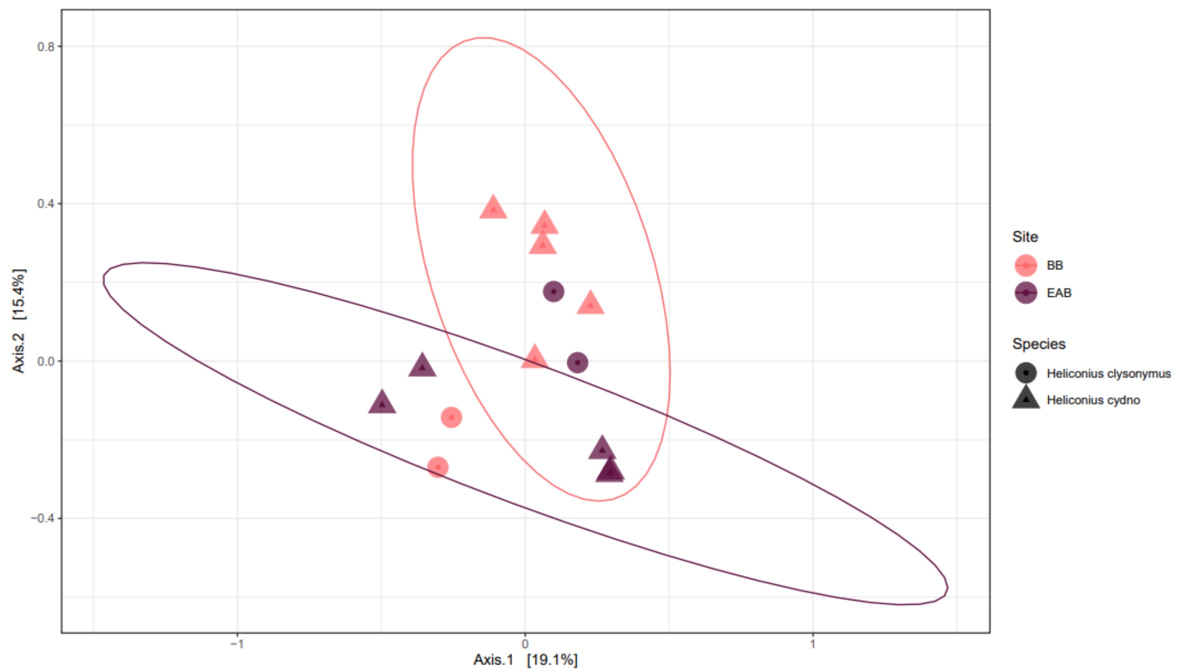
Genera	**Chi-square**	**P-value**	**Abundance differences**
Orbus	6.6244*	0.03644	Species > Controls
Commensalibacter	3.4751	0.176	
Spiroplasma	3.0565	0.2169	
Wolbachia	0.18543	0.9115	
Pseudomonas	4.7804	0.09161	
Enterococcus	7.8649*	0.0196	Species > Controls
Klebsiella	4.8492	0.08851	
Thalassotalea	9.2231*	0.009936	Controls > Species
Hafnia.Obesumbacterium	2.0984	0.3502	
Citrobacter	2.047	0.3593	
Lactococcus	1.9441	0.3783	
Dysgomonas	2.4973	0.2869	
MD3.55	2.8727	0.2378	
Endozoicomonas	10.328*	0.005719	Controls > Species
Algicola	4.805	0.09049	



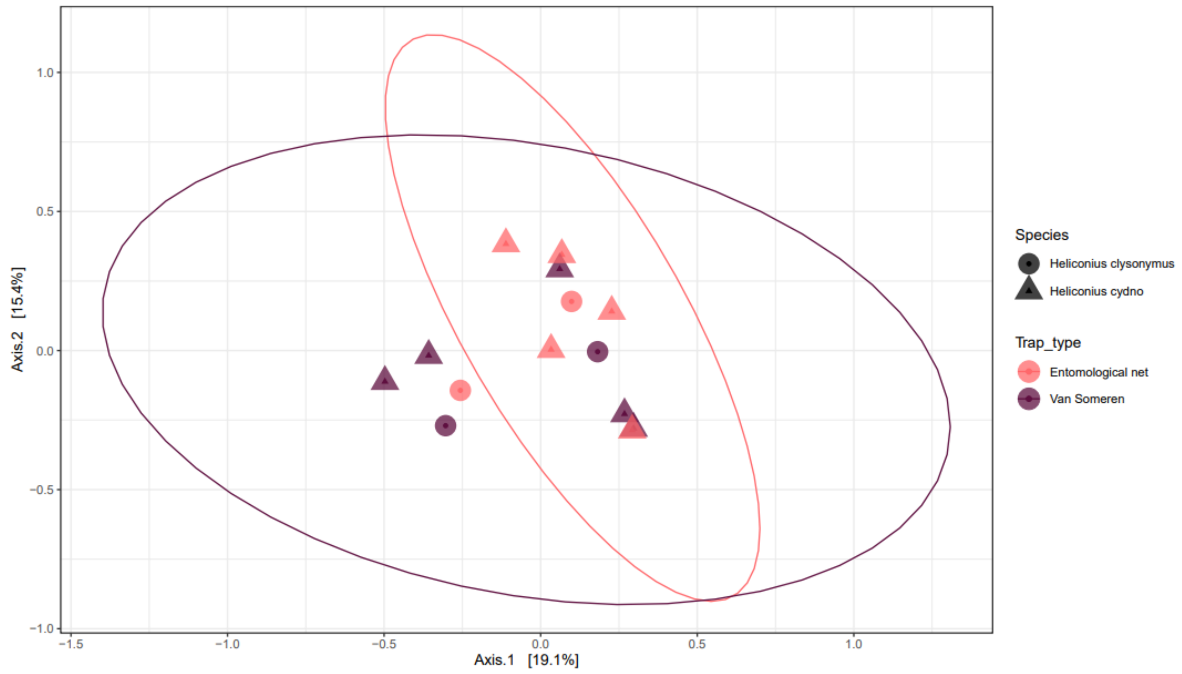
Supplementary Figure 1. Alpha diversity measures of the 14 butterfly samples and the 4 controls for each sample site, boxes show no differences between sites.



Supplementary Figure 2. Alpha diversity measures of the 14 butterfly samples and no controls for each capture method (active entomological netting and passive Van Someren-Rydon traps).



Supplementary Figure 3. Principal coordinate analysis of the 14 samples of two *Heliconius* butterflies (*H. clysonymus* and *H. cydno*) from both sampling sites (BB= Bremen, EAB=El Aguila).



Supplementary Figure 4. Principal coordinate analysis of the 14 samples of two *Heliconius* butterflies (*H. clysonymus* and *H. cydno*) caught by two methods (Entomological netting and Van Someren-Rydon traps).