The gut microbial diversity of a Chagas disease vector varies across region, habitat type, and blood meal source

**Abstract.** Anthropogenic land use change can impact vector-borne disease transmission by influencing [or changing?] the spatial distribution and community composition of vectors and their hosts, but the underlying mechanisms of the changes are not well understood. One potential driver of increased vector-borne disease risk is the diversity and species composition of the vector gut microbiome. By focusing on how land use change impacts the gut microbiomes of disease vectors, we can better understand how the landscape [change?] influences vector-borne disease transmission . HERE WOULD BE A GOOD OPPORTUNITY TO MAKE THAT BROADER CONNECTION TO BIGGER ECOLOGICAL CONCEPTS.

Chagas disease is caused by the parasite *Trypanosoma cruzi* and vectored by hematophagous triatomines that can harbor the parasite in their guts. In this study, we sequenced the gut bacterial diversity of *T. cruzi* infected and uninfected triatomines (N = 283) in three different land use types across three regions in central Panama. Our data indicate that the gut bacteria [genus?] diversity and composition in triatomines are primarily shaped by biogeography and, to a lesser degree, palm habitat type and vertebrate blood meal identity and richness. The gut bacterial composition and structure from samples collected in Santa Fe, Veraguas, which is [describe where to contrast with the others being near the Panama canal] were significantly different from samples collected in two regions near the Panama Canal. Consistent with other arthropod microbiome studies, the gut microbial community composition is most closely associated with geographic region compared to other factors. After controlling for geographic region, we observed differences in bacterial [genus?] richness, composition, and structure of the communities both within samples (alpha diversity) and between samples (beta diversity). These results highlight the importance of examining the gut microbial communities across biological scales and in relation to more fine-scale ecological characteristics. Furthermore, this study evaluates relationships between deforestation, landscape ecology, and gut bacterial communities, which may help to inform vector control and Chagas disease management programs.

**Introduction.** Arthropodgut symbionts are important for key physiological processes such as reproduction, development, and pathogen resistance (Doremus and Hunter, 2020; Ohbayashi et al., 2020; Wilson, 2020). These microbial communities are primarily shaped by intrinsic (host-associated) and extrinsic (environmental) factors. Intrinsic factors influencing vector gut microbiota include phylogeny, ontogeny, and parasite infection. Several studies have shown that insect host species identity is strongly associated with gut bacterial diversity and composition, and even between closely related species, there are still differences in microbial diversity (Adair et al., 2020; Brown et al., 2020; Colman et al., 2012; Huang et al., 2019; Rodríguez-Ruano et al., 2018; Yun et al., 2014). Some studies have found that triatomines significantly lose bacterial diversity throughout development, and adults are primarily dominated by a single taxon (Brown et al., 2020; Murillo et al., 2022; Rodríguez-Ruano et al., 2018). *Ixodes pacificus* ticks also lose bacterial richness and evenness with development (Swei and Kwan, 2017). These findings may result from differential uptake and retention of environmental microbes throughout development. Again – so what? Think of each paragraph as a story that must have a beginning, middle and an end- and the end has to sort of wrap it up and support whatever point you set out to make in the beginning. Being vague is your biggest enemy. Unpack everything, support with examples- if you say something had a ‘big effect’- illustrate it with a couple of real numbers from the study.

In addition to intrinsic host factors, extrinsic (environmental) factors that shape the gut microbial communities of arthropods include insect-environment interactions, social interactions, climate, diet, and land use. Differences in blood meal sources have been found to influence the gut microbial communities of arthropod disease vectors. Experimental evidence has shown that altering an insect’s diet may change the gut's metabolic functions and community structure (Colman et al., 2012). Yun (2014) found that the gut bacterial diversity of omnivorous insects was significantly higher than in stenophagous (carnivorous and herbivorous) insects. Further, a comprehensive meta-analysis found significant differences in bacterial diversity and composition across 21 different orders of wild-caught Insecta that were largely shaped by not only taxonomy but diet as well (Yun et al., 2014).

Many studies have demonstrated a close association between biogeography and gut microbial composition and structure. In most insects, the gut microbiota is typically acquired during feeding or from the environment. For example, many insects, such as cockroaches, termites, and triatomines, acquire their initial gut microbiome by feeding on adult feces, known as coprophagy, after hatching (De Fuentes-Vicente and Gutiérrez-Cabrera, 2022). Social insects can acquire and transfer symbionts by grooming or trophallaxis (transfer of food or nutrients) (Schmidt and Engel, 2021), while other non-social insects transfer symbionts by contaminating the egg or oviposition site so that hatchlings feed on the microbes.

While most studies of gut microbial communities of arthropod vectors of infectious disease examine factors that influence insect gut microbial communities and have been conducted in controlled laboratories and experimental systems, there is limited research on how various environmental conditions affect vector gut microbial communities in the wild.

Because vector-borne diseases are impacted by anthropogenic disturbance (Dharmarajan et al., 2021; Erazo et al., 2017; Gottdenker, 2013; Gottdenker et al., 2014; Santos et al., 2021), these gut microbial communities may also interact with these pathogens, it is also essential to understand in situ patterns of how gut microbial diversity and composition vary as a function of land use. Environmental disturbances, such as deforestation, urbanization, or pollution, may cause dysbiosis of the gut microbiota and can lead to disruptions in the vectors’ physiological processes, including pathogen resistance. By further understanding how anthropogenic environmental disturbances can shape and shift these gut microbial communities, we can better predict the impacts on vector-borne disease transmission.

Furthermore, anthropogenic environmental disturbances can result in shifts in the gut microbial community, due to many circumstances, including modified food sources, altered abiotic conditions, and changing parasite dynamics. Li et al. (2022) demonstrated that hard ticks (*Haemaphysalis longicornis*) acquire gut symbionts from their habitats, and thus disturbances to these habitats would likely result in shifted bacterial profiles. Onyango et al. (2020) found that an increase in mosquito rearing temperatures resulted in the enrichment of specific taxa, while Hussain et al. (2017) found a reduction in symbiont richness with increasing rearing temperatures. And finally, parasite infections can impact the gut bacterial communities of arthropods (Díaz et al., 2016; Mann et al., 2020; Murillo et al., 2022; Orantes et al., 2018), and changing parasite dynamics in the field may subsequently shift vector gut microbial communities. More studies are necessary to untangle the interactions between anthropogenic disturbance and disease vector gut microbial communities. A more comprehensive understanding of these interactions may provide a basis for vector control and public health policies.

The current study aimed to evaluate the gut bacterial communities of the primary Chagas disease vector in Panama, *Rhodnius pallescens.* Chagas disease, a neglected tropical disease, is responsible for millions of human infections and is a leading cause of heart disease in Latin America (Lidani et al., 2019). Chagas disease is caused by the protozoan parasite *Trypanosoma cruzi* and is transmitted via triatomine bugs that feed exclusively on vertebrate blood. While hundreds of triatomine species that are known to harbor *T. cruzi* can be found in a wide range of habitats, *R. pallescens* live in the crowns of royal palm trees (*Attalea butyracea*). Studies have shown that deforestation is associated with increased vector abundance and vector infection with *T. cruzi* in Panama (Gottdenker et al., 2011, 2012); however, potential impacts of trypanosome infection-related changes to gut microbial communities on triatomine bugs are unknown. In this study, we used a 16S rRNA metabarcoding approach to explore the gut bacterial communities of wild-caught *R. pallescens* across a deforestation gradient in central Panama and analyzed the bacterial alpha (within sample) and beta (between sample) composition and structure. The main objective of this study was to investigate associations between landscape-scale environmental variability, including habitat type and geographic region variations, on vector gut microbial composition and diversity.

**Materials and Methods.**

**Sampling efforts and insect collection.** *R. pallescens* (n = 288) were opportunistically collected from 41 *Attalea butyracea* palm crowns across central Panama using Noireau traps (Noireau et al., 2002) during the summer of 2017. The samples consisted of adults (n =54), nymphal stage 5 (n = 27), nymphal stage 4 (n = 14), nymphal stage 3 (n = 60), nymphal stage 2 (n = 60), and nymphal stage 1 (n = 74). These samples were collected from 3 regions, La Chorrera (8°52′49″N 79°47′0″W) and Capira (8°45′0″N 79°52′12″W), which are located in Panama Oeste province, and Santa Fe district (8°30′N 81°04′W), located in Veraguas province (Fig. 1). Among these, samples were collected in habitat types categorized as peridomestic (Capira (n = 15), La Chorrera (n = 57), Veraguas (n= 37)), pasture (Capira (n = 54), La Chorrera (n = 59), Veraguas (n= 51)), and forest remnants (Veraguas (n = 28)). Peridomestic palms were classified as palms found in yards or areas within 100 meters from a lodging, pasture palms were classified as cleared areas for cattle grazing, and forest remnants were classified as mid to late secondary forest patches surrounded by land previously deforested for agriculture or cattle.

*Figure . Map of sampling effort*

**Sample preparation and DNA extraction**

After capture, each triatomine was placed in 70% ethanol and kept at 4˚C. The body surface of each individual was rinsed in sterile water, and then the mid and hind guts were dissected using sterile techniques. Between each dissection, forceps and scalpels were rinsed in 70% ethanol and then sterilized using a glass beads sterilizer. Each triatomine gut sample was macerated and digested overnight in digest ATL buffer with Proteinase K and extracted using phenol-chloroform-isoamyl alcohol. Extractions were reconstituted in TLE buffer (10 mM Tris, pH 8; 0.1 mM EDTA), and impurities were removed with Sera-Mag SpeedBeads™ (Thermo Fisher Scientific, Waltham, MA, USA; (Faircloth and Glenn, 2012) with a final reconstitution of 30 μL TLE. In addition, 14 negative samples were included during extractions, *16S* rRNA amplification, and library sequencing for a total of 307 samples.

**DNA amplification, *16S,* and *12S* rRNA sequencing**

We amplified bacterial *16S* rRNA DNA using the S-D-Bact-0341-b-S-17 (5′-CCT ACG GGN GGC WGC AG-3′) forward and S-D-Bact-0785-a-A-21 (5′-GAC TAC HVG GGT ATC TAA TCC-3′) reverse primer pair following the protocol from Kieran et al. (2019). Blood meal DNA was amplified in the samples using *12S* rRNA gene-specific primers for vertebrates following the protocol from Kieran et al. (2017). All libraries were sent to the Georgia Genomics and Bioinformatics Core (http://dna.uga.edu) for sequencing on an Illumina MiSeq using a v3 PE300 kit (Illumina, San Diego, CA, USA).

**Bioinformatic processing.** Amplicon indices were demultiplexedusing Mr. Demuxy 1.20 (https://pypi.org/project/Mr\_Demuxy/), and the resulting files were imported into QIIME2 v. 2022.2 (Bolyen et al., 2019). Using the QIIME2 plugin *q2-cutadapt* (Martin, 2011), primers were trimmed, paired, and reads were merged. The quality of the sequences were verified, the sequences were filtered, and any chimera sequences were removed using the QIIME2 plugin *q2-demux,* followed by denoising with DADA2 via the *q2-dada2* plugin (Callahan et al., 2016) to a final set of amplicon sequence variants (ASVs). All ASVs were aligned using mafft via the *q2-alignment* plugin (Katoh, 2002) and then used to construct a phylogeny with fasttree2 via the *q2-phylogeny* plugin (Price et al., 2010). Taxonomy was assigned the *q2-feature-classifier* plugin (Bokulich et al., 2018) against the Naive Bayes classifier Geengenes 13\_8 99% OTUs (McDonald et al., 2012) that was trained on this sample dataset. We filtered out non-bacterial sequences (i.e., chloroplast and mitochondria) and Wolbachia sequences. QIIME v. 1.9 (Caporaso et al., 2011) was used to assign operational taxonomic units (OTUs) for vertebrate species identification with a custom reference database. We eliminated vertebrate species hits receiving ≤10% of total read hits for the sample. Blood meals assigned to house mice (*Mus musculus*) were not included in any blood meal analyses as house mice were used in the triatomine Nouri traps, and humans were eliminated due to likely contamination during processing. Relative abundance plots were created using the R package *phyloseq* (McMurdie and Holmes, 2013) to calculate the bacterial community composition across sampling regions, habitat type, and blood meal type and visualized using *ggplot2* (Wickham, 2009) in R v. 4.2.0 (R Core Team, 2022).

***Blood meal analyses.*** Across the triatomine samples, up to 5 distinct blood meal, vertebrate genera were identified from *12S* rRNA gene sequencing ([Supplementary File 1](https://outlookuga-my.sharepoint.com/:x:/g/personal/ka14691_uga_edu/EUS_qibeMlpPut-2CPF74hsBG89KJPSV61t_YpxxAqEKIg?e=Yw7Tya)). However, in the alpha and beta analyses, the dominant blood meal only includes a single vertebrate animal per triatomine sample with the greatest number of reads. Many genera were only identified a few times across the dataset. Therefore, for the alpha and beta analyses, only the top three vertebrates were used for the dominant blood meal: spiny pocket mice (*Heteromys*,n = 63), gray and black four-eyed opossums (*Philander*,n = 70), and Large American opossums (*Didelphis*,n = 35).

**Statistical Analyses**

***Alpha Diversity.*** Statistical analyses were completed using QIIME2 and R. For alpha and beta diversity analyses, samples were rarefied to 2133 sequences. Four alpha diversity metrics (ASV observed richness, Shannon diversity (Shannon, 1948), Faith’s phylogenetic diversity (Faith, 1992), and Pielou’s evenness (Pielou, 1966)) were calculated using *phyloseq* and QIIME2. Each test was compared among groups using Pairwise Wilcoxon Rank Sum Tests with Benjamini-Hochberg adjustments (for groups of two) or a Kruskal-Wallis analysis of variance (for more than two groups) followed by Pairwise Wilcoxon Rank Sum Tests with Benjamini-Hochberg adjustments (Benjamini and Hochberg, 1995) to test for pairwise significance. Boxplots were visualized using *ggplot2*. To evaluate the associations between sampling region, habitat type, blood meal type, and blood meal species richness on changes in bacterial alpha diversity, linear mixed models (LMMs) were implemented using the *lme4* (Bates et al., 2015)and *lmerTest* (Kuznetsova et al., 2017) packages in R. Log-transformed alpha diversity metrics were used as the response variables (ASV richness, Shannon diversity, Faith’s phylogenetic diversity, and Pielou’s Evenness) and model predictors included sampling region, habitat type, dominant blood meal taxa, blood meal richness, and *R. pallescens* developmental stage, and palm identity was treated as a random effect. The Akaike information criteria (AIC) was used to select the best model. The same LMMs were then repeated, first split by the three sampling regions (Capira, La Chorrera, and Veraguas) and then again divided by habitat type (pasture and peridomestic).

***Beta Diversity.*** Beta diversity calculations were used to measure differences in community structure and composition across sampling region, habitat type, and bloodmeal species richness. Differences in beta diversity between samples were estimated using a permutational multivariate analysis of variance (PERMANOVA) on unweighted and weighted UniFrac distance matrices with 999 permutations using the QIIME2 *q2-diversity* plugin and with Benjamini-Hochberg adjustments and were supported with beta-dispersion. Unweighted UniFrac (community composition) measures the presence/absence of ASVs that incorporates phylogenetic distances while weighted UniFrac (community structure) considers the phylogenetic distances between observed ASVs in addition to relative abundance of ASVs. Principal coordinate analysis ordinations (PCoA) were calculated based on unweighted and weighted UniFrac distance matrices with 999 permutations using the *q2-diversity* plugin in QIIME2 and visualized using *ggplot2*.

**Results.**

A total of 288 samples of *R. pallescens* were sequenced for a total of 3,124,000 raw sequencing reads. After filtering and denoising the samples with DADA2, the remaining read total was 1,444,104. The samples were then rarefied to a sampling depth of 2,133 based on rarefaction curves, which resulted in 603,639 reads, 283 samples, and 3543 unique ASVs. In total, 452 vertebrate blood meals across eight orders and 19 genera of vertebrate blood meals were identified across all sampled *R. pallescens* (Table S1).

***Relative Bacterial Abundance.*** Across all samples, *Sphingomonas* was the dominant genera at 74.5% mean relative abundance. After *Sphingomonas,* the most abundant genera were *Erwinia* (6.1% mean relative abundance), *Stenotrophomonas* (5.4% relative abundance), and *Dietzia* (3.6% relative abundance). Across the three sampling regions, *Sphingomonas* was the dominant genera (mean relative abundance at Capira: 70.6%, La Chorrera: 74.4%, and Veraguas: 58.8%) (Fig. 2). At Veraguas, *Sphingomonas* displayed a lower abundance than at the other two regions, with *Erwinia* and *Dietzia* having greater mean relative abundance than at the other two sampling regions (13.3% and 7.6%, respectively). Across habitat types, *Sphingomonas* was the dominant genera (mean relative abundance at pasture palms: 69.8%, peridomestic palms: 68.1%, and forest remnant palms: 55.6%) (Fig. 3). Similar to Veraguas regions, in bugs captured from forest remnant palms, *Sphingomonas* displayed a lower relative abundance, with *Erwinia* displaying 20% mean relative abundance, which was greater than pasture and peridomestic palms. Accounting for dominant blood meal genera, individuals that fed on *Didelphis* displayed lower *Sphingomonas* (46.0%) and *Erwinia* (21.4%) than the *Heteromys* and *Philander*-fed individuals. Notably, the *Didelphis*-fed samples also had a greater mean abundance of *Dietzia* than those fed on the other vertebrates (Fig. 4).

**Gut microbial composition and structure across sampling regions.**

***Alpha Diversity (within sample diversity).*** At Veraguas, alpha diversity was greatest across ASV richness, Shannon diversity, and Faith’s phylogenetic diversity (Fig. S1) and was significantly different compared to samples from both La Chorrera (Richness: *p* < 0.001; Shannon diversity: *p* < 0.001; Faith’s phylogenetic diversity: *p* < 0.001; Pielou’s evenness: *p* = 0.92) and Capira (Richness: *p* = 0.0025; Shannon diversity index: *p* < 0.001; Faith’s phylogenetic diversity: *p* = 0.0059; Pielou’s evenness: *p* = 0.92) (Table S2). Between the two Panama Canal sites, Capira had greater ASV richness and Shannon diversity (Richness: *p* = 0.017; Shannon diversity: *p* = 0.044) (Fig. S1) compared to La Chorrera; however, there was no difference in Faith’s phylogenetic diversity or Pielou’s evenness (*p* = 0.501 and *p* = 0.917, respectively) (Fig. S1; Table S2).

To examine associations between sampling region and gut bacterial diversity more closely, the dataset was split by habitat type. When only examining pasture palms, a slightly different pattern was found. Vector gut microbial diversity in Veraguas was significantly greater than La Chorrera in ASV richness (*p* = 0.0019) and Shannon diversity (*p* < 0.001) and only significantly greater than Capira in Shannon diversity (*p* = 0.019) (Fig. 5; Table 1). Additionally, Capira had greater richness (*p* = 0.017) and Shannon diversity (*p* = 0.019) when compared to La Chorrera palms (Fig. 5; Table 1). Across all peridomestic palms, samples collected at Veraguas were also significantly greater than samples collected at Capira and La Chorrera in richness (*p* < 0.001; *p* < 0.001, respectively), Shannon diversity (*p* = 0.0015; *p* < 0.001), and Faith’s phylogenetic diversity (*p* < 0.001; *p* < 0.001). However, at peridomestic palms, there was no difference in alpha diversity between Capira and La Chorrera samples (Fig. 6, Table 2). Forest remnant palms were only sampled in Veraguas and were therefore not compared to any other sampling regions.

***Mixed linear models for alpha diversity.*** Across all alpha diversity metrics, the best fit models (lowest AIC value) were either the null model (no fixed effects), had sampling region as the sole fixed effect (Richness, Shannon diversity), habitat type as the sole fixed effect (Pielou’s evenness) or were best explained by both sampling region and habitat type (Faith’s phylogenetic diversity). Sampling region was strongly only correlated with two of the alpha diversity metrics (Richness: R2 = 0.13, *p* = 0.002; Shannon: R2 = 0.19, *p* < 0.001), while habitat type was not correlated any diversity metric (Table S3).

At pasture palms, the best fit models included sampling region as the sole fixed effect (richness and Shannon diversity), vertebrate blood meal richness as the sole fixed effect (Pielou’s evenness), or best explained by sampling region, developmental stage, and vertebrate blood meal richness (Faith’s phylogenetic diversity). Vertebrate blood meal richness and developmental stage were both strongly correlated with Faith’s phylogenetic diversity (R2 = 0.045, *p* = 0.009; R2 = 0.086, *p* < 0.001, respectively), but sampling region was not strongly correlated with diversity (Table 3). At peridomestic palms, the best fit models included either sampling region as the sole fixed effect (richness and Shannon diversity), dominant blood meal as the sole fixed effect (Faith’s phylogenetic diversity), or both sampling region and vertebrate blood meal richness (Pielou’s evenness). Sampling region is strongly correlated with ASV Richness (R2 = 0.21, *p* = 0.005) and Shannon diversity (R2 = 0.22, *p* < 0.001), dominant blood meal was strongly correlated and had a large effect on Faith’s phylogenetic diversity (R2 = 0.40, *p* < 0.001), and vertebrate blood meal richness was marginally correlated with Pielou’s evenness (R2 = 0.08, *p* = 0.041) (Table 4).

***Beta Diversity (between sample diversity).*** We performed a permutational multivariate analysis of variance (PERMANOVA) and a permutational multivariate dispersion (beta-dispersion) on weighted UniFrac (community structure) and unweighted UniFrac (community composition) distances that were calculated in QIIME2. Sampling region was a significant predictor of triatomine gut bacterial composition (UniFrac PERMANOVA: pseudo-F statistic = 13.033199, *p* = 0.001, beta-dispersion on 999 permutations: *p* = 0.261) (Fig. S2a; Table S4), whereas abundance-weighted composition (wUniFrac) showed more overlap and Veraguas with significantly greater variance (wUniFrac PERMANOVA: pseudo-F statistic = 25.136, *p* = 0.001, beta-dispersion on 999 permutations: *p* = 0.001) (Fig. S2b, Table S5).

After controlling for habitat type, similar patterns were present, such that our samples still clustered by sampling region at pasture palms (UniFrac: pseudo-F statistic = 9.195085, *p* = 0.001 with beta-dispersion on 999 permutations: p = 0.127) (Fig. 7a; Table 5) while abundance-weighted composition showed more overlap and Veraguas with significantly greater variance than the other regions (wUniFrac: pseudo-F statistic = 13.223361, *p* = 0.001, beta-dispersion on 999 permutations: *p* = 0.019) (Figs. 7b; Table 6) and at peridomestic palms (UniFrac: pseudo-F statistic = 6.491517, *p* = 0.001, beta-dispersion on 999 permutations: *p* = 0.051; wUniFrac: pseudo-F statistic = 12.048014, *p* = 0.001, beta-dispersion on 999 permutations: p = 0.028) (Figs 8a-b; Tables 7-8). Furthermore, at pasture and peridomestic palms, Veraguas samples had significantly dissimilar bacterial communities than both Capira samples (UniFrac: pseudo-F statistic = 11.664, *p* = 0.001 and wUniFrac: pseudo-F statistic = 12.633, p = 0.001 for pasture palms, Tables 5-6; UniFrac: pseudo-F statistic = 5.648, *p* = 0.001 and wUniFrac: pseudo-F statistic = 7.911, *p* = 0.001 for peridomestic palms, Tables 7-8) and La Chorrera samples (UniFrac: pseudo-F statistic = 8.093, *p* = 0.001 and wUniFrac: pseudo-F statistic = 20.516, *p* = 0.001 for pasture palms, Tables 5-6; UniFrac: pseudo-F statistic = 10.339, *p* = 0.001 and wUniFrac: pseudo-F statistic = 20.712, *p* = 0.001 for peridomestic palms, Tables 7-8). At pasture palms, Capira and La Chorrera samples were significantly dissimilar (UniFrac: pseudo-F statistic = 7.822, *p* = 0.001 and wUniFrac: pseudo-F statistic = 4.956, *p* = 0.001) (Fig. 7a-b), however at peridomestic palms, Capira and La Chorrera samples are only dissimilar as measured by unweighted UniFrac (pseudo-F statistic = 5.981, *p* = 0.001), but not by weighted UniFrac (pseudo-F statistic = 1.152, *p* = 0.329) (Fig. 8a-b).

**Gut microbial composition and structure across palm habitat type.**

***Alpha Diversity***. Across habitat types, samples from forest remnant palms were consistently different from samples at both pasture (Richness: *p* = 0.0204; Shannon diversity: *p* < 0.001; Faith’s phylogenetic diversity: *p* = 0.00270; Pielou’s evenness *p* < 0.001) and peridomestic palms (Richness: *p* < 0.001; Shannon diversity: *p* < 0.001; Faith’s phylogenetic diversity: *p* = 0.00631, Pielou’s Evenness: *p* = 0.001). Pasture and peridomestic samples, however, were only significantly different across ASV richness (Richness: *p* = 0.045; Shannon diversity: *p* = 0.880; Faith’s phylogenetic diversity: *p* = 0.377; Pielou’s evenness: *p* = 0.534) (Fig. S3, Table S6).

To further examine the relationships between bacterial communities and habitat type, the dataset was split by sampling region. At Capira, pasture and peridomestic samples were statistically similar as measured by Shannon diversity, Faith’s phylogenetic diversity, and Pielou’s evenness, however pasture palms displayed greater ASV richness compared to peridomestic palms (*p* = 0.0074) (Fig. 9, Table 9). At La Chorrera, pasture and peridomestic palms were not significantly different across any alpha diversity metrics (Fig. 10, Table 10). Similarly, at Veraguas, pasture palms were also not significantly different across any alpha diversity metric than peridomestic palms (Fig. 11, Table 11). However, forest remnant palms were significantly greater than pasture palms when measured by Shannon diversity (*p* = 0.0087) and Pielou’s evenness (*p* = 0.00043), but not Faith’s phylogenetic diversity or ASV richness, and forest remnant palms were significantly greater than peridomestic palms as measured by Shannon diversity (*p* = 0.0159) and Pielou’s evenness (*p* = 0.00533), but not Faith’s phylogenetic diversity or ASV richness (Fig. 11, Table 11).

***Mixed linear models for alpha diversity across habitat types.*** After controlling for sampling region, habitat was the best fit model after the null model for three alpha diversity metrics at Capira but did not display any significant effects (Richness: R2 = 0.0849, *p* = 0. 0.2158; Shannon diversity: R2 = 0.030, *p* = 0.3583; Pielou’s evenness: R2 = 0.0275, *p* = 0.2722). Habitat type and vertebrate blood meal richness were both the best fit model that explained Faith’s phylogenetic diversity; however, only vertebrate blood meal richness significantly correlated with diversity (R2: 0.10, *p* = 0.004) (Table 12). At Chorrera, the best fit models across all alpha diversity metrics were either the null model or had habitat as the sole fixed effect, however, habitat was not significantly associated with any alpha diversity metrics (Table 13). Lastly, at Veraguas samples, the best fit models across all alpha diversity metrics included dominant blood meal as the sole fixed effect and were strongly correlated with Shannon diversity (R2 = 0.0915, *p* = 0.0037) and evenness (R2 = 0.011, *p* = 0.011) (Table 14).

***Beta Diversity.*** Using a PCoA for visualization, both beta diversity metrics demonstrated that the samples clustered by habitat type (UniFrac: pseudo-F statistic: 4.385148, *p* = 0.001, beta-dispersion on 999 permutations: p = 0.464; wUniFrac: pseudo-F statistic= 6.847971, *p* = 0.001, beta-dispersion on 999 permutations: p = 0. 0.097) and the pairwise PERMANOVA comparisons between forest and pasture samples had the largest pseudo-F statistic across each metric (UniFrac: pseudo-F statistic: 6.765, *p* = 0.001; wUniFrac: pseudo-F statistic: 11.630, *p* = 0.001) (Fig. S4, Tables S7-S8).

We then split the samples by sampling region to explore the data further. At Capira, pasture and peridomestic palms had significantly different variances using unweighted UniFrac distances (pseudo-F statistic: 5.146824, *p* = 0.001, beta-dispersion on 999 permutations: *p* = 0.049, Fig. 12a, Table 15) but showed no difference in clustering or variance using weighted UniFrac (pseudo-F statistic: 3.046044, *p* = 0.061, beta-dispersion on 999 permutations: *p* = 0.631) (Fig. 12b, Table 16), while La Chorrera samples some differences in composition by habitat using unweighted UniFrac distances (pseudo-F statistic: 2.754805, *p* = 0.004, beta-dispersion on 999 permutations: *p* = 0.152) (Fig. 13a, Table 17) but no differences using weighted UniFrac (pseudo-F statistic: 1.909, p = 0.058, beta-dispersion on 999 permutations: p = 0.129) (Fig. 13b, Table 18). At Veraguas, the forest and peridomestic samples clustered together, indicating little difference between the two habitat types using unweighted UniFrac (pseudo-F statistic: 1.601603, *p* = 0.078, beta-dispersion on 999 permutations: p = 0.427) and pasture palms displaying different compositions (pseudo-F statistic: 3.638, p = 0.001 for pasture vs. forest palms; pseudo-F statistic: 1.652, *p* = 0.004 for pasture vs. peridomestic palms). And finally, abundance-weighted composition at Veraguas displayed some clustering across habitats (pseudo-F statistic: 3.98348, *p* = 0.001, beta-dispersion on 999 permutations: *p* = 0.222)

**Associations between gut microbial diversity and dominant blood meal identity**.

After specifically examining dominant blood meal identities, *Didelphis­*- and *Heteromys*-fed individuals were only collected at Veraguas. Compared to *Heteromys*-fed individuals, *Didelphis*-fed individuals were significantly greater when measured by Shannon diversity (*p* < 0.001), Faith’s phylogenetic diversity (*p* = 0.007), and Pielou’s evenness (*p* < 0.001). Across habitat types, *Didelphis*-fed individuals collected from pasture palms displayed the greatest ASV richness (Pasture vs Forest: *p* = 0.001; Pasture vs Peridomestic: *p* = 0.001) but were not significantly different in Pielou’s evenness (p = 0. 0.552) or Shannon diversity (*p* = 0.3702) from forest remnant samples. Peridomestic palms were significantly lower in Pielou’s evenness and Shannon diversity than forest (*p* = 0.006, *p* = 0.0225; respectively) and pasture palms (*p* = 0.012, *p* = 0.0029; respectively) There was no difference in phylogenetic diversity between the samples (Fig. S5). *Heteromys*-fed individuals collected from forest remnant palms were significantly greater than pasture palms measured by Shannon diversity (*p* = 0.03) and Pielou’s evenness (*p* = 0.018) but were not difference across any metric from peridomestic palms. Additionally, *Heteromys*-fed individuals collected from pasture palms were not significantly different from individuals collected in peridomestic across any alpha diversity metric (Fig. S6). *Philander* blood meals were only identified at La Chorrera and Capira across each habitat type. *Philander*-fed individuals collected at Capira were not significantly different from individuals collected at La Chorrera across any alpha diversity metric, and individuals collected in pasture palms were not significantly than individuals collected at peridomestic palms across any metric (Figs. S7-S8).

**Discussion**

Host-associated microbes perform a myriad of beneficial functions within their host. Across arthropod disease vectors, microbes are involved in increasing tolerance to environmental perturbations, priming the immune system, pathogen resistance, and aiding in digestion and nutrition. Triatomines have co-evolved with specific gut-associated bacteria to primarily supplement their nutrient-poor diet. Currently research suggestions that ontogeny, species identity, and the environment are the primary factors that shape the gut bacterial communities of triatomines (Brown et al., 2020). This study was designed to closely examine the influence of environmental factors on the gut communities of triatomines, after controlling for variations across ontogeny. Studies have shown strong associations between collection site and gut microbial diversity of disease vectors in mosquitoes (Akorli et al., 2016; Bascuñán et al., 2018; Coon et al., 2016; Osei-Poku et al., 2012; Zouache et al., 2011), ticks (Kwan et al., 2017; Li et al., 2022; René-Martellet et al., 2017), and triatomines (Brown et al., 2020; Kieran et al., 2019); however, few studies have closely examined differences in the microhabitat of these collection sites. As *R. pallescens* primarily live in palm tree crowns, it is important to understand whether their gut bacterial community is influenced by microhabitat characteristics, particularly as land conversion and deforestation continues in areas of high vector prevalence. In this paper, we demonstrate that sampling region, palm type, and blood meal source drive differences in bacterial communities of triatomines in central Panama.

**There are differences in gut microbial composition and structure in samples collected across sampling regions.** Per the linear mixed models, sampling region is factor that most explains the alpha diversity among all individuals (Tables S3). Figure S1 demonstrates that the greatest differences in alpha gut microbial diversity were found between samples collected in Veraguas and samples collected around the Panama Canal (i.e., Capira and La Chorrera). Alpha richness and diversity were greatest at Veraguas samples, indicating that there was a greater number of ASVs present in the guts of triatomines there (Fig. S1). However, evenness is the same across all regions, meaning region is not differentially selecting for greater or fewer amounts of each taxon. Similarly, Veraguas samples displayed the greatest Faith’s phylogenetic diversity measurements, therefore indicating that not only is there an increase in amplicon diversity, but phylogenetic diversity as well.

Furthermore, after controlling for habitat type, we were able to closely examine any patterns of diversity across sampling regions within all pasture palms and all peridomestic palms. When only examining peridomestic palms, similar alpha diversity patterns emerge between sampling regions. Veraguas samples displayed greater alpha richness and diversity than both the Canal sites, however samples from Capira and La Chorrera were not significantly different (Fig. 6). Conversely, when only examining pasture palms, Veraguas samples had greater ASV richness compared to both Capira and La Chorrera, but only displayed greater Shannon diversity compared to the La Chorrera samples (Fig. 6). This indicates that there may be ecological differences driving these patterns between pasture and peridomestic palms.

Beta diversity, like alpha diversity, was found to be driven most by sampling region. In pasture palms, the composition and structure of the Veraguas samples were significantly dissimilar from both the Canal sampling regions, and the composition and structure of the gut communities at two canal sites were significantly dissimilar from each other. However, in peridomestic palms, there was no difference in beta diversity between the two Canal regions. Similar to alpha diversity, this may indicate that pasture palms and forest remnants are driving some differences in triatomine gut diversity.

Many other studies have demonstrated a close association between biogeography and gut microbial composition and structure. Mosquitoes acquire their initial gut microbiota as larval from their aquatic habitats, which results in distinct gut composition between sites (Akorli et al., 2016; Bascuñán et al., 2018; Coon et al., 2016). Various studies found shared soil and other environmental bacteria between ticks and their habitats (Li et al., 2022; René-Martellet et al., 2017). One study also demonstrated that bees reared in identical conditions had different gut bacteria composition after being place in different landscape types for 6 weeks (Jones et al., 2017). And finally, the gut microbiota of triatomines have also been found to be associated with locality, with distinct compositions of microbes that differed across sites (Brown et al., 2020; Kieran et al., 2019). However, any differences in the environmental factors between these areas were not closely examined to identify any ecological drivers of these patterns. As demonstrated thus far, there may be factors associated specifically with Veraguas, aside from forest remnant samples, that may be driving some of the differences in gut bacterial composition and structure, and some marginal differences associated with Capira that may be driving differences between those samples and samples from La Chorrera.

Compositionally, samples from Veraguas displayed a great deviation from samples collected at the two canal sites. The mean relative abundances of *Sphingomonas*, the dominant genera across all samples, was slightly displaced by *Erwinia* and *Dietzia.* *Dietzia* has been described in other triatomine species (Díaz et al., 2016; Gumiel, 2015; Montoya-Porras et al., 2018; Rodríguez-Ruano et al., 2018; Waltmann et al., 2019) and other hematophagous insects, including *Aedes albopictus* (Yadav et al., 2015) and *Glossina pallidipes* (Malele et al., 2018), which suggests *Dietzia* might be an important mutualist. However, Brown et al. (2020) did not identify *Dietzia* in their N1 samples across several species of *Triatoma*, and concluded that *Dietzia* may not be transmitted vertically. *Erwinia* has also been found across several species of triatomines (Carels et al., 2017; da Mota et al., 2012). The bacterial genus *Erwinia* contains mostly plant pathogenic species and is most commonly found only in the guts of insects that feed on plant tissue. This likely indicates that *Erwinia*, like *Dietzia*, is acquired from the environment, rather than exposure to or ingestion of maternal feces.

**There are differences in gut bacterial composition and structure between samples collected in different palm habitat types.** In addition to microbial differences between samples collected across Veraguas and the Canal regions, there were also significant differences in gut bacterial composition and structure across palm habitat types. Figure S3 shows that overall, samples collected in forest remnant palms displayed greater microbial richness and diversity than samples collected at pasture and peridomestic palms. Importantly, all of the samples from forest remnant palms were collected from the Veraguas region, so therefore it was expected for the forest samples to have greater richness and diversity. For this reason, our data was split by sampling region to better assess any associations with palm microhabitat irrespective of region. There were little to no differences between pasture and peridomestic palms at each of the three regions (Figs. 9-11), however at Veraguas, forest remnant samples had greater alpha diversity measurements than pasture and peridomestic palms. As the gut microbial communities may be largely influenced by the environment, it is important to consider the microhabitat of the palms.

As previously discussed, gut microbial communities are likely to be largely shaped by environmental variables. *R. pallescens* live primarily in the crowns of *Attalea* palms, and the amount of dead organic matter, surrounding canopy cover, and number of connected trees can influence temperature and humidity fluctuations within the palm crown. Preliminary data from one study found that temperature and humidity fluctuations stabilized as these environmental variables increased, by providing a buffer to the *Attalea* palm crown’s microclimate under changing ambient climate conditions throughout the day (Padukone, 2016). Another (unpublished) study that sampled within the same the regions and similar palms as this project, found that the microclimate conditions of the palm crowns stayed within an ideal range for triatomines (25-28° C and 79 – 98 % RH), although pasture palm crowns experienced larger microclimate fluctuations within a 24 hour period compared to palms in forest remnants and peridomestic areas. These stabilized microclimates within the palm crowns may have contributed to the marginal differences between pasture and peridomestic palms seen in this project across each sampling region.

In addition to microclimate of the palm crowns, vertebrate blood meal may have influenced the gut microbial communities of the triatomines, particularly at Veraguas. While the null linear mixed models were the best fit for all alpha diversity metrics, dominant blood meal was the second best fit for all metric, which may indicate a slight association with bacterial diversity. The samples collected at Veraguas had fed on a larger number of blood meals and had significantly more rodent blood meals than the other two sampling regions. Capira, on the other hand, had the fewest detected blood meals, which may have influenced any differences in gut bacterial diversity between Capira and La Chorrera. There were some associations between blood meal identity and alpha bacterial composition and diversity. Individuals that fed on *Didelphis* displayed the greatest Shannon diversity and Pielou’s evenness were significantly different at some alpha metrics compared to *Heteromys*-fed individuals. Furthermore, *Didelphis*-fed individuals displayed greater relative abundance *Erwinia* and *Dietzia*, while *Heteromys*-fed individuals showed a similar, but smaller, difference in relative abundances of these bacteria compared to other triatomines (Fig. 4). As discussed previously, *Erwinia* and *Dietzia* are likely acquired from the environment.

Previous studies have found that blood meal source can have strong associations with gut bacterial diversity. One study showed that there is a strong impact of host blood meal identity on tick (*Ixodes* *pacificus*) bacterial species richness and composition (Swei and Kwan, 2017). These researchers found that lizard-fed ticks exhibited lowered bacterial diversity compared to mammal-fed ticks. In mosquitoes, a recent study showed a significantly greater in alpha diversity of the gut bacteria of laboratory-reared individuals fed on either sugar, rabbit blood, or a mixture of chicken and rabbit blood, to that of chicken-fed mosquitoes (Muturi et al., 2021). Another study found that gut bacterial diversity shifts after ingestion of a blood meal but will then return to its original structure within 6 days. If this remains true for *R. pallescens*, the composition and structure may be temporarily altered by a blood meal. It is important to note, however, while multiple blood meals were detected from each triatomine, the results from this project only incorporated a single blood meal for each individual sample (i.e., blood meal identity). And importantly, *Didelphis*-fed and *Heteromys*-fed individuals were only sampled in Veraguas. Furthermore, we relied on 12S rRNA sequencing to identify vertebrate blood meals, but triatomines are able exploit other food sources, such as plant sap, will engage in coprophagy (feces-eating), and may also feed on the hemolymph of other triatomines.

**The gut bacterial composition and structure of samples collected at Veraguas different significantly from Canal samples, which may be influenced by taxonomy and landscape features.**

The samples collected in Veraguas were significantly different across all metrics than the samples collected from the two Canal regions, while the two Canal sites were similar to each other. There may be several reasons for this phenomenon, including biogeography, blood meal diversity, and vector taxonomy. Geographically, Veraguas is about 136.82 km away from the middle of the two Canal regions, while the two Canal regions are only about 16.56 km away from each other. Researchers recently found that there was high migration of *R. pallescens* between sampling regions around the Panama Canal but very low rates of migration from Santa Fe, Veraguas to sites around the Canal (Kieran, 2020). These researchers concluded that the differences in migration are likely due to the greater distance between the Canal sites and Santa Fe, Veraguas (~ 150 km), topographical/elevation effects, and proximity and similarity in biogeographic features between the Canal sampling regions. Additionally, the similar gut bacterial patterns between triatomines collected in Capira and La Chorrera may be the result of microbial dispersal dynamics across small spatial scales that may, in part, drive community patterns between close populations of insects.

Furthermore, there is also evidence that triatomines found in Veraguas are genetically differentiated from the triatomines found around the Panama Canal. The samples found in Veraguas have a darker pigmentation and are slightly larger, but are otherwise morphologically similar to the Canal triatomines (Saldaña et al., 2018). In addition to different migration patterns between across central Panama, Kieran et al. (2020) found that triatomines in Veraguas were genetically differentiated from the triatomines collected around the Canal, while samples from various regions and sites around the Canal had little genetic differentiation. Several studies have shown that host taxonomy is typically a significant driver of gut bacterial diversity within arthropods, even within closely related species (Adair et al., 2020; Brown et al., 2020; Huang et al., 2021; Lim and Bordenstein, 2020). Although we cannot pinpoint the precise factors that drive the gut bacterial communities of triatomines collected pasture and peridomestic palms to be more similar to each other than triatomines collected in forest remnants, nor precise factors that drive Veraguas samples to differ from the other sampling regions, these results motivate further research into the influence of microhabitats and landscape features on gut communities.

In conclusion, we provide an evaluation of the gut bacteria R. pallescens, the primary vector of Chagas disease in Panama. We found that there are differences between sampling regions, with samples collected from Veraguas that were distinctly different alpha and beta diversity composition and structure. These patterns likely reflect limited migration patterns of triatomines between Veraguas and the Canal regions and greater migration patterns amongst the Canal regions and genetically isolated Veraguas triatomines. We also found differences between samples collected at Veraguas across habitat type, with forest samples that were distinctly different than the samples collected in peridomestic and pasture palms. Furthermore, samples collected in pasture and peridomestic palms had very similar gut bacteria communities. These patterns across habitat type are likely the result of greater biogeographic similarities between pasture and peridomestic palms compared to forest remnant palms. Moreover, these data provide important insights for further investigations of the gut bacterial communities of triatomines across anthropogenically disturbed landscapes, which is imperative for vector control and vector-borne disease management.

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