Title: Comparative Analysis of Tick Microbiomes in Remnant and Reconstructed Prairie Ecosystems

Mary York[1], Jaylon Vaugh, Lyndon Coghill[1], Samniqueka J. Halsey[2*]

[1] Bioinformatics and Analytics Core, University of Missouri, 1201 Rollins St., Columbia, MO, 65211, USA
[2]Applied Computational Ecology Lab, College of Natural Resources, University of Missouri, 1111 Rollins St. Columbia MO, 65211, USA
*Correspondence: samniquekahalsey@missouri.edu

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Abstract

Tick-borne pathogens pose an increasing public health threat, yet little is known about how ecological restoration influences the microbiomes of tick vectors. In this study, we evaluated how prairie restoration affects the bacterial communities of questing ticks collected from remnant and reconstructed prairie ecosystems in Missouri. Using 16S rRNA gene amplicon sequencing, we characterized the microbiomes of two tick species Amblyomma americanum and Dermacentor variabilis collected between 2020 and 2023. We compared microbial alpha and beta diversity across prairie types and tick species and used differential abundance analysis to identify key bacterial taxa, including symbionts and pathogens, associated with each habitat. Our results revealed that tick species identity had a stronger influence on microbiome composition than prairie type. D. variabilis exhibited greater microbial diversity, with higher abundance of Francisella, while A. americanum harbored more Rickettsia, including pathogenic strains. Although alpha diversity did not differ significantly by prairie type, community composition and pathogen profiles did, with ticks from reconstructed prairies carrying a higher proportion of pathogenic Rickettsia. These findings suggest that restoration status can influence the environmental context for tick microbiomes and pathogen dynamics. Our work highlights the importance of integrating microbial ecology into restoration assessments and provides foundational insights into how habitat restoration may affect disease risk through shifts in vector-associated microbial communities.

Introduction

Endosymbionts are microorganisms living within ticks that significantly influence their ability to transmit pathogens, a concept known as Vector Competence Modulation[1]. These symbionts can either enhance or impair the tick's immune system, affecting its efficiency as a disease vector. Symbiont-pathogen interactions also play a critical role by influencing the tick's microbiome stability and pathogen survival through mechanisms like antimicrobial production or resource competition[2,3]. Understanding these interactions under different environmental conditions may offer potential strategies for reducing tick-borne disease transmission by altering tick vector

competence.

Habitat restoration is a key strategy for conserving biodiversity and maintaining ecosystem function in landscapes that have experienced habitat loss and fragmentation. While prairie restoration efforts often focus on plant and animal communities, the effects of restoration on host-associated microbiome, particularly those of arthropod vectors like ticks remain poorly understood.

In this study, we compared the bacterial microbiomes of ticks collected from remnant and reconstructed prairie ecosystems to evaluate how restoration status shapes microbial community structure. Specifically, our objectives were to (1) compare alpha and beta diversity of tick microbiomes across prairie types, (2) assess whether microbial community composition differs by prairie type and tick species identity, and (3) identify bacterial taxa, including potential symbionts and pathogens, that are differentially abundant across environmental contexts. By integrating community diversity metrics, multivariate analyses, and differential abundance testing, this study provides new insight into how ecological restoration may influence host-associated microbial communities, with implications for understanding microbiome assembly processes and ecosystem health in restored landscapes.

Methods

Study Site:

Questing Tick Collection.

Tick flagging occurred monthly from May to August using one square-meter white corduroy cloth attached to a wooden dowel along one edge that was slowly pulled over herbaceous vegetation along each side of the five 100m sampling transects (Total: 1000 square meters per plot). Every 15-m of flagging, the clothes were checked for ticks, removed using masking tape, and placed in plastic vials filled with 70% ethanol. All ticks were be counted, sorted, and identified by referencing standard taxonomic keys[4] before DNA extraction and pathogen screening.

DNA extraction.

Purified DNA was extracted using commercially available RNA/DNA extraction kits (Quick-DNA/RNA™ Pathogen MiniPrep, Zymo Research). Immediately after extraction, the genomic DNA was by Qubit flourometer using the quant-iT HS dsDNA reagent kit (Invitrogen). Ticks were pooled by site, plot, species, life stage, year and month of collection, will no more than 6 samples pooled, resulting in 373 pools sent for 16S sequencing.

16S Sequencing Library Preparation.

Bacterial 16S ribosomal DNA amplicons were constructed by amplification of the V4 hypervariable region of the 16s rRNA with primers flanked by Illumina standard adapter sequences containing unique dual indexes.[5,6]. The final amplicon pool was evaluated using the Advanced Analytical Fragment Analyzer automated electrophoresis system, quantified, and diluted according to Illumina's standard protocol for sequencing on the MiSeq.

Amplicon sequencing analyses and taxonomic identification. Amplicon sequencing analyses and taxonomic identification will use a custom Nextflow v 21.10.6-based workflow.[7] This workflow is based on QIIME2 v2021+.[8] Extraction of representative sequences by taxon was accomplished using a custom "feature-classifier" built for taxa of interest under a naive-bayes model[9].

In QIIME 2[1], the classification steps involve assigning taxonomy to amplicon sequence variants (ASVs) using reference databases (*e.g.*, we used SILVA 138[4]). This process uses algorithms like Naive Bayes classifiers to match sequences to known organisms, enabling the identification of the microbial composition of samples down to the species level.

Rarefaction

Rarefaction in QIIME 2[1] is a process used to standardize the number of sequences per sample by randomly subsampling, ensuring that comparisons between samples are not biased by differing sequencing depths. This method allows for the equitable comparison of microbial diversity and composition across samples by normalizing the effort of sequencing, thereby facilitating accurate ecological and diversity analyses within a dataset. Before rarefaction, the feature table was first filtered to only contain species AA and DV and to only contain samples that had a DNA concentration > 2 ng/ul (determined from Qubit data). Rarefaction depth was 15,400.

Phylogeny

Phylogeny analysis in QIIME 2[1] involves constructing a phylogenetic tree that represents the evolutionary relationships among the microbial species identified in the samples. This tree is built using sequences of ASVs or OTUs, and it enables various downstream analyses such as phylogenetic diversity calculation, which can give insights into the ecological and evolutionary dynamics of microbial communities. Understanding these relationships helps in assessing microbial diversity and the evolutionary context of the community structure within the environmental, clinical, or experimental samples analyzed.

Species-Specific Rickettsia Identification

We subjected three to five single infected samples from each *Rickettsia* OTU ID to conventional PCR to figure out *Rickettsia* species. We utilized primers CS-78 (5'-GCAAGTATCGGTGAGGATGTAAT-3'; IDT, Iowa, USA) and CS-323 (5'-GCTTCCTTAAAATTCAATAAATCAGGAT-3'; IDT, Iowa, USA) to target the *gltA* gene for the *Rickettsia* genus under the following cycles: 95°C for 5 minutes, 40 cycles of 15 seconds at 95°C, 30 seconds at 48°C, 30 seconds at 72°C, and a 7-minute hold at 72°C (Labruna et al. 2004). The PCR product was then loaded into 1.8% low melt agarose and ran at 100V for one hour. We then excised positive bands, purified the product (Wizard SV Gel and PCR Clean-up System; Promega, Hidden, Germany), and sent the newly created samples for Sanger sequencing (Genomics Technology Core; University of Missouri—Columbia, Missouri, USA). We utilized NCBI Nucleotide BLAST to determine the species of returned sequences.

Statistical Analysis.

Before calculating diversity metrics, the feature table was first filtered to contain only species AA and DV and to contain samples that had a DNA concentration > 2 ng/ul (determined from Qubit data). Sampling depth was 15,400 for such analyses. We then calculated alpha and beta diversity to assess how communities differ across environmental contexts. We then used principal component analysis (PCA) to identify clustering (i.e., microViz package in R) [10,11]. PCA plots are a great way to see any clustering or other patterns of microbiota (dis)similarity in samples. Ordinations like PCA show the largest patterns of variation in your data. Data was aggregated at the taxonomic rank of genus and counts were transformed using the clr (centered log ratio) transformation. To identify whether there are significant differences between the microbiome community composition by prairie type and tick species, we used PERMANOVA (i.e., ADONIS via Vegan package in R), with a Bray-Curtis distance matrix.

Next, we performed differential abundance analysis of the microbiome count data using ANCOM-BC2 (Analysis of Compositions of Microbiomes with Bias Correction 2) to identify the effect of Prairie Type and tick species on Francisella spp and Rickettsia spp [12]. ANCOM-BC2[8] create a bias-corrected log abundance table for testing, which accounts for taxon-specific and sample-specific biases. It uses test statistic W and calculates significance via a two-sided Z-test using the test statistic W. The analysis was carried out at the genus taxonomic level and for samples with a DNA concentration (> 2ng/uL, n=203). The Holm method was used to adjust p-values. A sensitivity analysis was performed to assess the impact of different pseudo-counts on zero counts for each taxon. Any taxa present in less than 10% of samples were not considered in the analysis. No samples were discarded based on library sizes. A small positive constant, chosen as the 5th percentile of standard error values for each fixed effect, was added to the denominator of the test statistic for each taxon to avoid significance due to extremely small standard errors, especially for rare taxa. Structural zeros were not detected, and a taxon was not classified as a structural zero using its asymptotic lower bound. The level of significance was set at 0.05.

We conducted a chi-square analysis to observe pathogenic and endosymbiotic *Rickettsia* prevalence differences by site and species.

Results

From 2020-2023, we collected a total of 3,026 ticks from two prairie ecosystems: Tucker Prairie (remnant, n = 1,097) and Prairie Fork Conservation Area (reconstructed, n = 1,929). Ticks belonged to two species: the lone star tick (Amblyomma americanum, n = 1,966; 65.0%) and the American dog tick (Dermacentor variabilis, n = 1,060; 35.0%). Ticks were nearly evenly distributed between life stages, with 1,561 nymphs (51.6%) and 1,465 adults (48.4%, Figure 1). Among adult ticks, 833 (56.9%) were female and 632 (43.1%) were male.

A total of 433 tick pools were submitted for 16S rRNA gene amplicon sequencing. DNA extractions yielding <2 ng/µL, as quantified by Qubit fluorometry, were excluded from downstream analysis, resulting in 200 samples retained for sequencing. After demultiplexing and quality filtering, a total of 24,614,229 paired-end reads were generated across these samples, with per-sample read counts ranging from 2 to 1,300,937 (mean: 123,071; median: 59,588; SD:

180,149). All retained samples passed quality filtering, and sequencing depth was sufficient to characterize the bacterial communities associated with each tick. A total of 12,666 unique bacterial operational taxonomic units (OTUs) were identified, spanning 38 phyla, 99 classes, and 1,080 genera. Several OTUs were classified within tick pathogen–associated genera of interest, including *Francisella spp.* (73.5% of samples), *Rickettsia spp.* (54.0%), *Ehrlichia spp.* (5.5%), *Borrelia spp.* (1.5%), *Anaplasma spp.* (4.5%), and *Bartonella spp.* (1.5%).

Alpha diversity

There were no significant differences in alpha diversity between sites for any metric (Observed: $\chi^2 = 0.007$, $p = 0.93$; Chao1: $\chi^2 = 0.007$, $p = 0.93$; Shannon: $\chi^2 = 0.007$, $p = 0.93$; Inverse Simpson: $\chi^2 = 0.074$, $p = 0.79$, Figure 2). In contrast, Shannon and Inverse Simpson indices differed significantly by tick species, with higher diversity observed in *Dermacentor variabilis* compared to *Amblyomma americanum* (Shannon: $\chi^2 = 7.92$, $p = 0.0049$; Inverse Simpson: $\chi^2 = 14.15$, $p < 0.001$). No significant differences by species were observed for richness-based metrics (Observed: $\chi^2 = 0.50$, $p = 0.48$; Chao1: $\chi^2 = 0.50$, $p = 0.48$).

Beta Diversity

There were significant differences in the microbiome composition between both remnant and reconstructed prairie ($F_{(1, 139)} = 2.206$, $P = 0.026$) and *A. americanum* and *D. variabilis* (($F_{(1, 139)} = 35.709$, $P = 0.001$).

We found clustering in the bacterial microbiome between *A. americanum* and *D. variabilis* at the genus level (Figure 3), which indicates that different tick species have distinct microbiomes. Community composition differed significantly by tick species ($R^2 = 0.040$, $F_{(1, 139)} = 8.23$, $p = 0.001$) and to a lesser extent by prairie site ($R^2 = 0.010$, $F_{(1, 199)} = 2.06$, $p = 0.024$), based on PERMANOVA of CLR-transformed genus-level bacterial communities. Tick life stage was not a significant predictor of microbial composition ($R^2 = 0.005$, $F_{(1, 199)} = 1.02$, $p = 0.598$). A combined model including tick species, site, and their interaction explained 5.7% of the total variation ($F_{(1, 199)} = 3.94$, $p = 0.001$), suggesting that microbiome structure is influenced by both host identity and environmental context. A PERMDISP test for homogeneity of group dispersions showed no significant difference in dispersion between tick species ($F = 0.68$, $p = 0.41$), supporting the interpretation that observed PERMANOVA differences reflect true shifts in community structure rather than within-group variability

Differential abundance testing using ANCOM-BC revealed significant shifts in the relative abundance of *Rickettsia* and *Francisella* between tick species with higher abundance of *Rickettsia* in *Amblyomma americanum* compared to *Dermacentor variabilis* ($W = -4.05$, $p < 0.001$, Figure 4A). Conversely, *Francisella* was significantly more abundant in *D. variabilis* than in *A. americanum* ($W = 4.18$, $p < 0.001$). Furthermore, for *Rickettsia,* there was no significant difference in abundance between prairie sites ($W = 0.56$, $p = 0.577$), whereas for *Francisella*, a marginal, non-significant trend was observed ($W = -1.67$, $p = 0.097$), likely driven by *D. variabilis (*Figure 5*)*.

Pathogenic and endosymbiotic *Rickettsia* prevalence

Of the 200 samples tested, 108 (54%) were positive for *Rickettsia*, and species-level identification was achieved for 105 of these, encompassing four distinct *Rickettsia* species: *R. amblyommatis*, *R. rickettsii*, *R. montanensis*, and *R. adanae*. Co-infection with multiple *Rickettsia* species was rare, occurring in only 5 of the 200 samples (2.5%). These included three *Dermacentor variabilis* and two *Amblyomma americanum* ticks, with three collected from the reconstructed prairie and two from the remnant site. No consistent pattern of co-occurrence was observed among the species-level combinations.

A strong contrast was observed between tick species. Pathogenic *Rickettsia* species were detected in 43 of 52 (*Amblyomma americanum*) samples (82.7%) but only in 8 of 53 (*Dermacentor variabilis*) samples (15.1%). Conversely, endosymbiont *Rickettsia* were more common in *D. variabilis* (44 of 53; 83.0%) than in *A. americanum* (12 of 52; 23.1%).

The distribution of *Rickettsia* types differed markedly between tick species. Pathogenic *Rickettsia* species were detected in 46 of 61 (*Dermacentor variabilis*) samples (75.4%) compared to only 5 of 44 (*Amblyomma americanum*) samples (11.4%). In contrast, endosymbiont *Rickettsia* were much more common in *A. americanum* ticks (41 of 44; 93.2%) than in *D. variabilis* (15 of 61; 24.6%).

A Chi-square test of independence revealed no significant association between prairie site and pathogen type ($\chi^2 = 1.30$, $p = 0.254$). In contrast, pathogen type was significantly associated with tick species identity ($\chi^2 = 45.45$, $p < 0.001$), indicating that the distribution of pathogenic versus endosymbiont Rickettsia differed strongly between tick species.

When comparing sites, we recorded that 78% of *Rickettsia* found within the restorative prairies are pathogenic, contrasting with the 63% of pathogenic *Rickettsia* recorded in remnant prairies (p=0.01708). This finding highlights how restorative practices facilitate the lifecycle of pathogenic *Rickettsia,* similar to our documentation of *R. parkeri.* We recorded a more drastic difference between species of ticks, with 93% of *Rickettsia* found in *A. americanum* ticks being pathogenic versus the 27% found in *D. variabilis* ($p < 2.2e-16$). This suggests that *A. americanum* ticks may play an essential role in the lifecycle of local pathogenic *Rickettsia*.

Discussion

Acknowledgements

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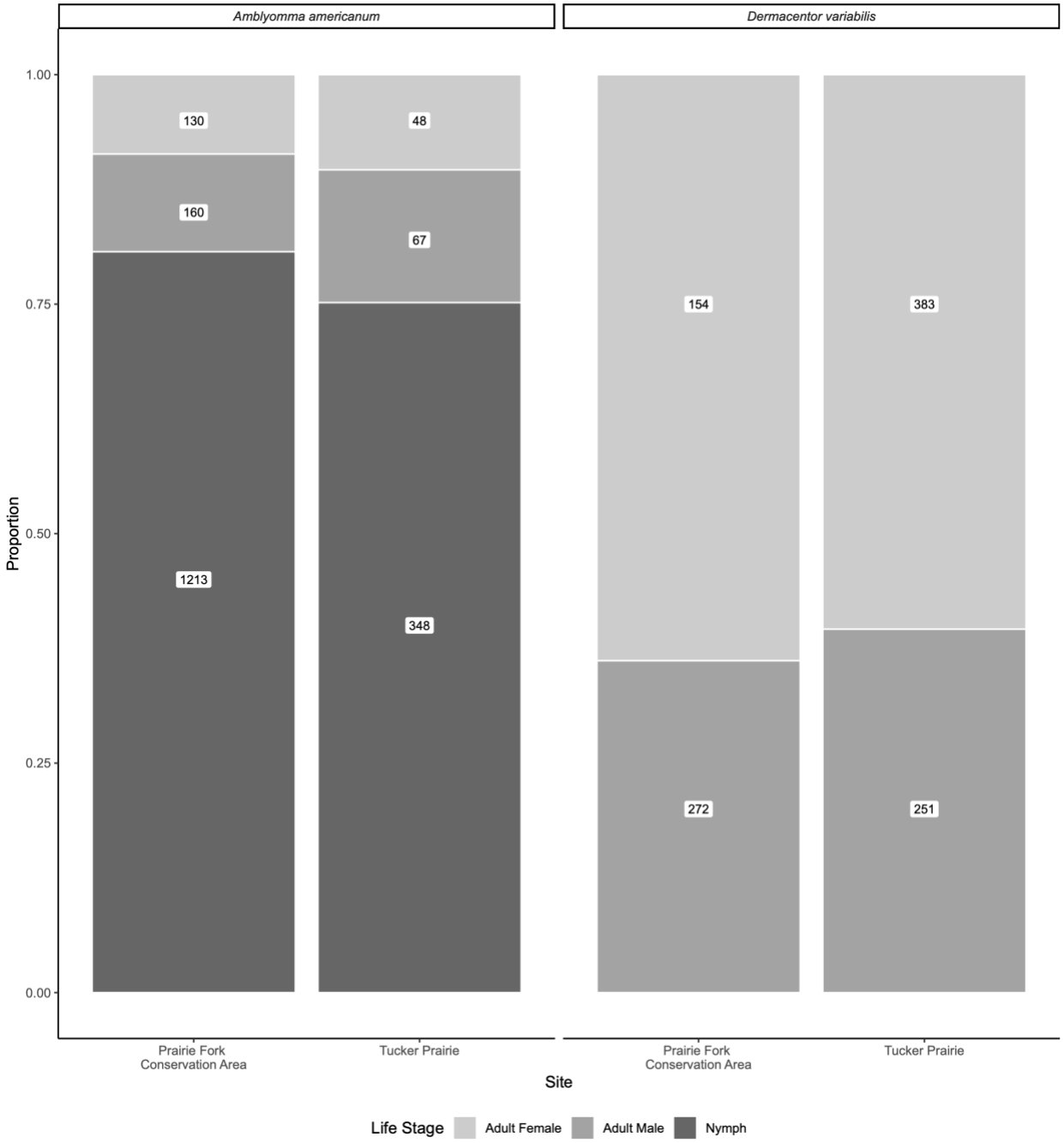

Figure 1. Proportional composition of tick life stages and sexes by species and prairie type from 2020 to 2023.Bars represent the relative proportions of Amblyomma americanum (Lonestar tick) and Dermacentor variabilis (American dog tick) collected between May and August, 2020–2023, from two prairie sites: Tucker Prairie (remnant) and Prairie Fork Conservation Area (reconstructed in Callaway County, Missouri. Numbers within each bar indicate the total number of ticks in each life stage and sex category.

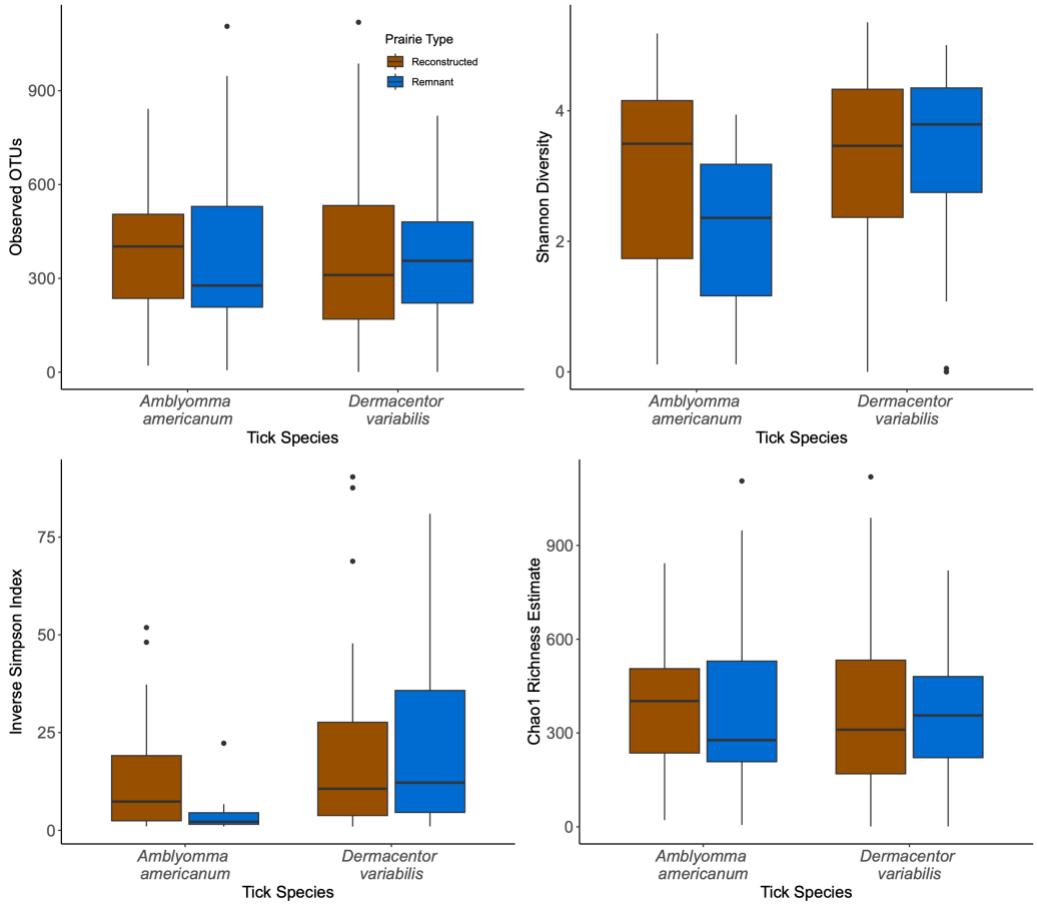

Figure 2. Alpha diversity of tick-associated bacterial communities by species and prairie type. Boxplots show four alpha diversity metrics—Observed OTUs, Chao1 richness estimate, Shannon diversity, and Inverse Simpson index stratified by tick species and prairie site (Reconstructed = Prairie Fork, Remnant = Tucker Prairie)..

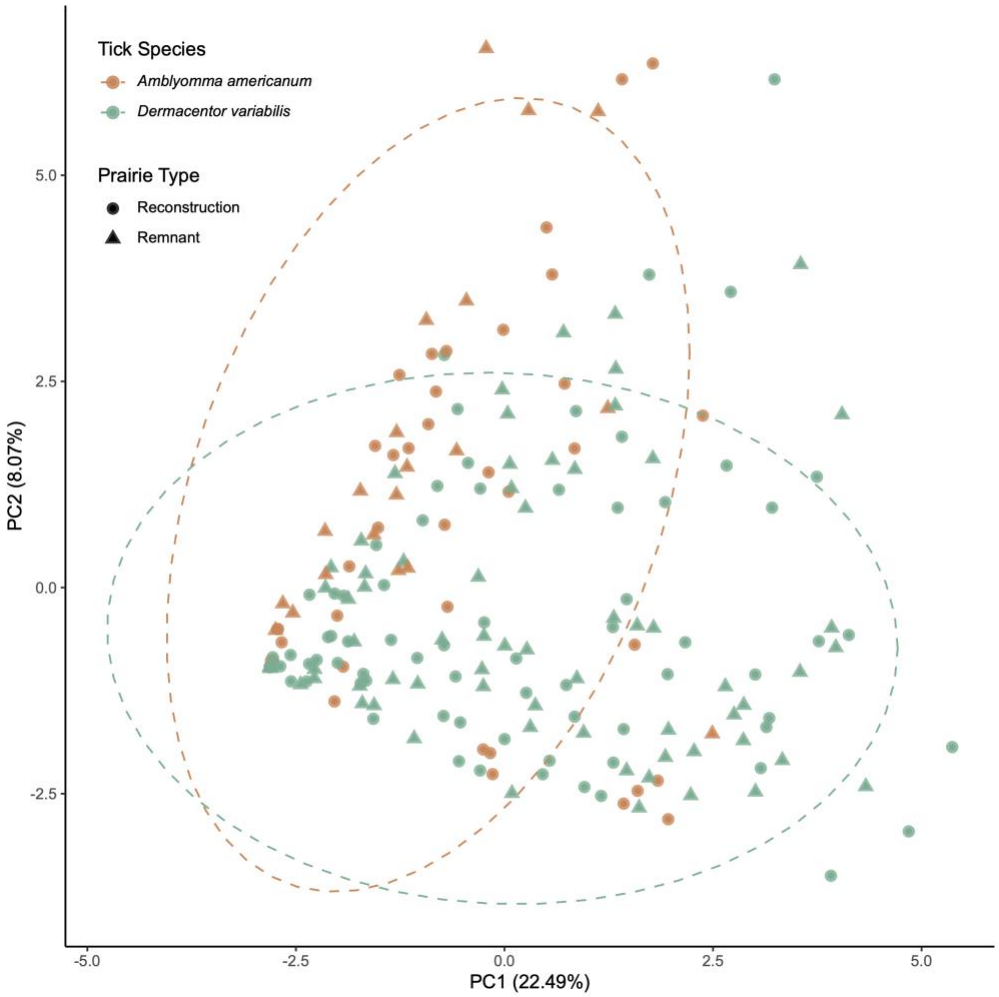

Figure 3. Principal component analysis (PCA) of genus-level CLR-transformed bacterial communities from ticks. Ordination is based on Euclidean distances calculated from centered log-ratio (CLR) transformed genus-level abundances. Each point represents a tick sample, colored by species (Amblyomma americanum [AA] and Dermacentor variabilis [DV]) and shaped by prairie site (Reconstructed = Prairie Fork, Remnant = Tucker Prairie). Dashed ellipses represent 95% confidence intervals around species centroids.

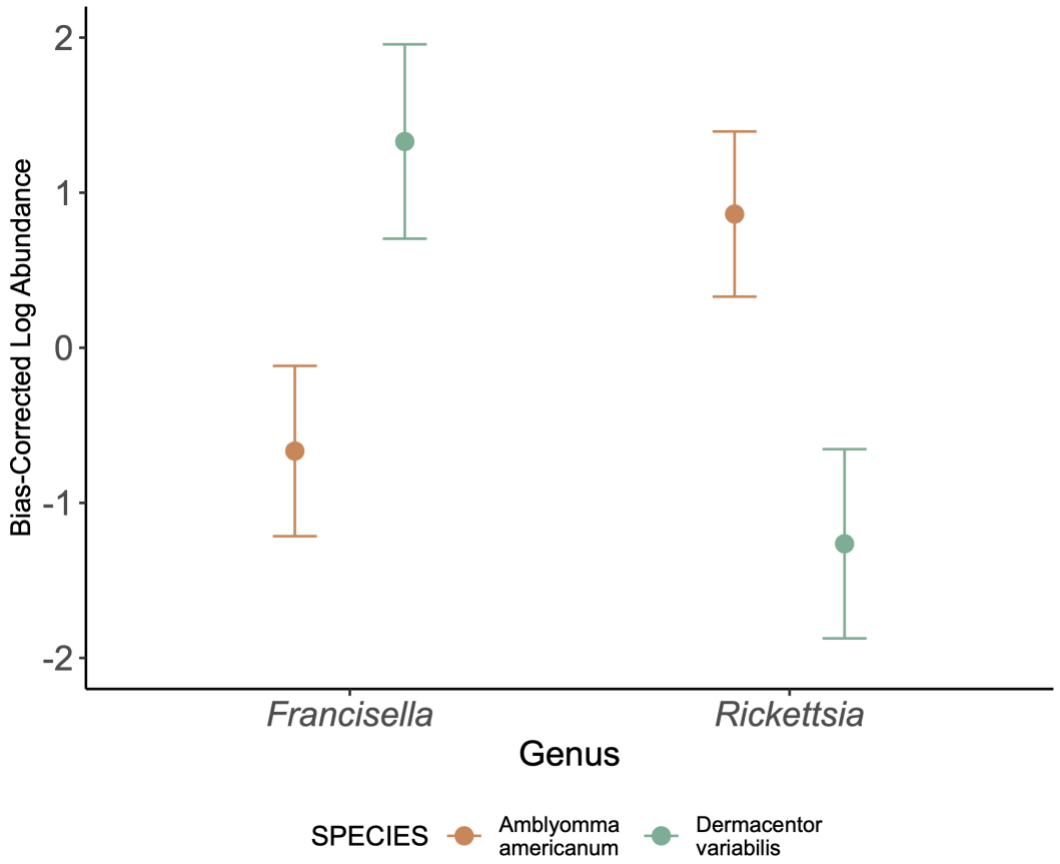

Figure 4. Mean Bray-Curtis Log log-transformed abundances (±95% confidence intervals) of differentially abundant genera between *Amblyomma americanum* and *Dermacentor variabilis*.

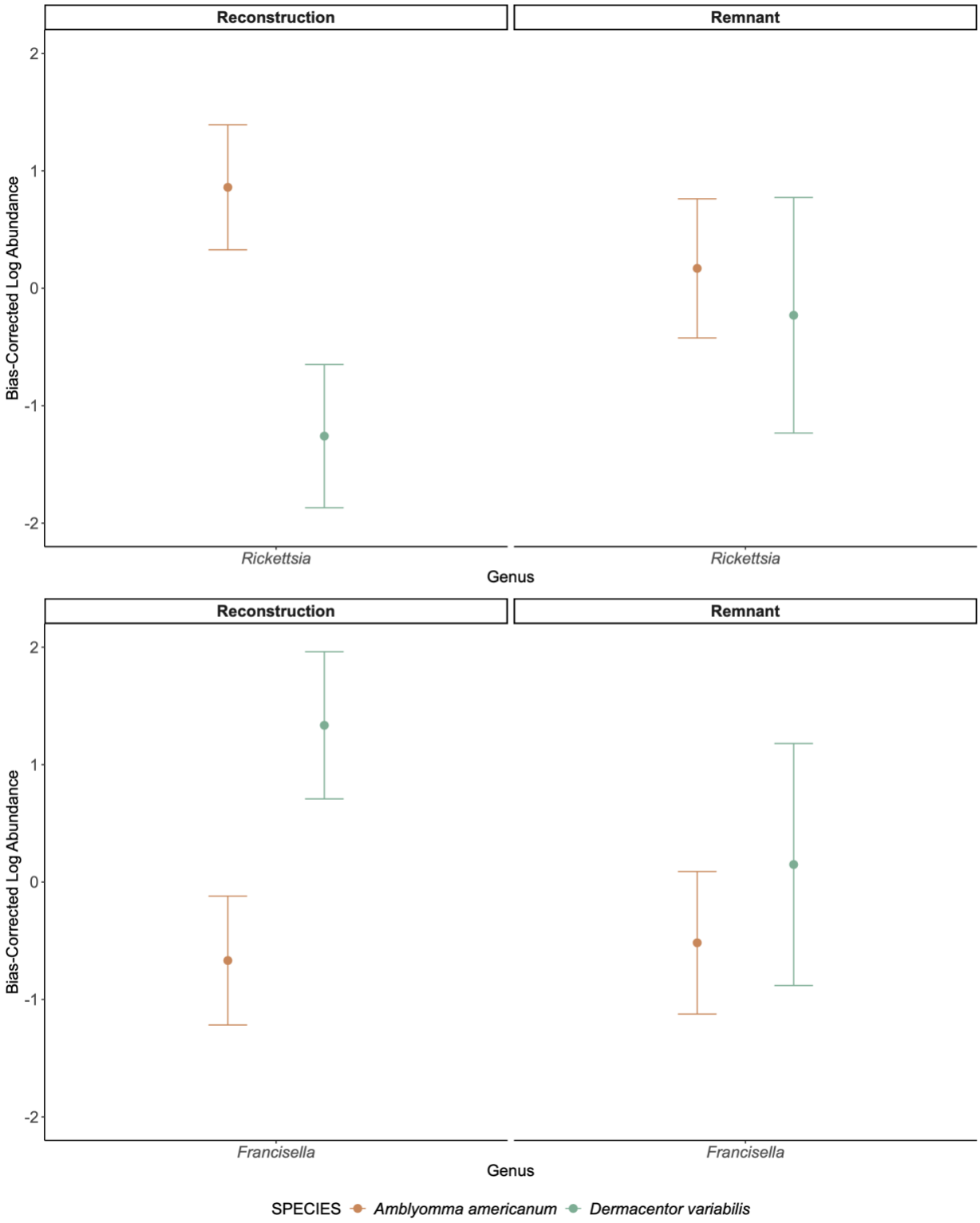

Figure 5. Mean Bray-Curtis Log log-transformed abundances (±95% confidence intervals) of differentially abundant genera (*Rickettsia spp.*) between Amblyomma americanum and Dermacentor variabilis.