Methods

Title: Quantifying how evolutionary history and diet shape the mammalian gut microbiome

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Animal capture, husbandry and sample collection

To examine factors influencing mammalian gut microbiome structure and stability, we sampled wild woodrats from 25 populations, representing seven species at 18 sites across the southwestern United States (Figure 2). We captured animals using live traps (H.B. Sherman Traps, Inc) baited with oats and peanut butter. Woodrats were identified to species using morphology, except *N. lepida* and *N. bryanti* which were distinguished using microsattelites as per Shurtliff et al. (2014). To assay wild diet and gut microbiome, we collected feces at the time of capture (Kohl et al. 2015) and stored samples on dry ice while in the field. Following capture, we transported animals to the University of Utah School of Biology Animal Facility. At this facility, woodrats were housed in individual cages (48x27x20cm), and fed commercial high fiber rabbit chow (Harlan Teklad, formula 2031) ad libitum. After three to four weeks in captivity, we collected a second captive fecal sample, storing all feces at -80°C until DNA extraction.

Poster Figure 2: A. Phylogeny of seven sampled *Neotoma* species (Matocq et al. 2007, Patton et al. 2007). B. Locations of 25 sampled populations, colored by species, and coded according to species and sampling site (i.e. L17 are *N. lepida* from site 17).

Molecular analyses and bioinformatics

We isolated DNA from woodrat feces using QIAamp PowerFecal DNA kits (Qiagen), following manufacturer protocols. To evaluate sources of novel microbes and contamination, we also extracted DNA from kit reagents ("blanks", n=23), the captive diet ("chow", n=3) and swabs of empty animals cages with bedding and enrichment items ("facility", n=3). We quantified DNA concentration and quality using a NanoDrop spectrophotometer. The extracted DNA was processed at the DNA Service Facility at the University of Illinois-Chicago. To sequence woodrat microbiomes, we amplified the V4 hypervariable region of the 16S rRNA gene using the 515F and 806R primers (Caporaso et al. 2012). To sequence diets, part of the chloroplast *trn*L (UAA) intron was amplified using the *g* and *h* primers (Taberlet et al. 2007, Kartzinel et al. 2015). Amplicons were sequenced using the Illumina MiniSeq platform (2 x 151 base paired-end reads), with between sequencing run variation examined using a mock bacterial community (ZymoBIOMICS Microbial Community Standard #D6300).

Illumina sequencing reads were processed with DADA2 v1.14.1, using Cutadapt to remove primers and phyloseq v1.30.0 for additional filtering (Martin 2011, McMurdie & Holmes 2013, Callahan et al. 2016a, Callahan et al. 2016b). For bacteria, we identified 16S rRNA amplicon sequence variants (ASVs) using the Silva v138 reference database (Quast et al. 2012) and constructed a tree using FastTree v 2.1.11 (Price et al. 2010). Woodrat (n=300) and additional control samples generated 7 million reads. After removing non-bacterial sequences (e.g., archaea, chloroplasts, mitochondria), the dataset contained 6.3 million reads and 12,517 ASVs. We examined kit and PCR blanks for potential contaminants, and, after examining ASV distribution and prevalence in woodrat samples, removed ASVs present in less than 2% of

animals. The resulting dataset contained 2,754 ASVs and an average of 16,666 ± 12,830 and 20,259 ± 6,432 reads per wild (n=164) and captive (n=136) woodrat, respectively. To reduce the impact of differential sequencing depths (Weiss et al. 2017), samples were subsampled to an even depth of 5100 reads, removing two wild samples. After examining taxonomy and clustering patterns, five additional wild samples with poor DNA quality were excluded from subsequent analyses. All analyses used R version 3.6.3 (R Core Team 2020), with two alpha diversity (Observed, Shannon) and four beta diversity (Bray-Curtis (BC), Jaccard (J), Weighted Unifrac (WU), and Unifrac (U)) metrics calculated in phyloseq, (McMurdie & Holmes 2013). Permutation tests use 9999 permutations, unless otherwise stated.

For plant sequences, initial processing generated a total of 6.3 million reads representing 610 ASVs from 159 wild woodrats. To accommodate the full range of *trn*L amplicon lengths (Taberlet et al. 2007) and plant geographic distributions, we assigned taxonomy using a custom python script. We removed ASVs likely derived from bait (genera *Avena* (oats), *Glycine* (soy), or *Arachis* (peanut) and then, for each animal, we removed any ASVs representing less than 1% of the diet (Ando et al. 2018), creating a dataset of 104 ASVs assigned to 54 plant families and 88 genera. All reads were identified to family, and 84% were identified to genus. Prior to downstream analyses, we compared sequence-derived diets to previous diet studies and observed plant communities. Although sequencing results generally matched expectations, *trn*L primers appeared to poorly amplify cactus. We confirmed this observation using captive feeding trials and then analyzed carbon stable isotopes in woodrat feces to calculate the percentage of cactus in diets (contact authors for more details). We adjusted dietary cactus proportions using stable isotope data, and then subsampled diet

samples to an even depth, removing two animals with fewer than 3600 reads. We grouped plant taxa at the family level and then calculated observed and Shannon diet diversity in phyloseq. As these alpha diversity metrics were significantly correlated (Pearson correlation, $r = 0.83$, $df=145$, $t=17.7$, $p < 0.001$), we used observed richness in subsequent analyses.

Statistical analyses

Starting with the simplest model for community structure, we tested how well wild microbial communities fit the neutral model of prokaryote community assembly following methods described in Burns et al. (2016). We first fit the neutral model to ASVs from all wild hosts, calculating fit and classifying ASVs as either neutral (within 95% CI of model) or selected (above or below predicted neutral frequency). We then fit the model to ASV frequency and abundance data from each site with at least 7 animals (n=15), and then used linear models to test whether model fit (R^2) declined with increasing number of species at a site.

If bacteria are dispersal limited, hosts sampled from increasingly distant locations should harbor increasingly disparate microbiomes. We first tested whether microbiome composition varied by site, using the adonis() function in vegan to conduct PERMANOVAS, including species as a factor and calculating microbiome dissimilarities using the four distance metrics. We then used a partial mantel test in vegan to examine whether dissimilarity increased with geographic distance, aggregating microbiome data for each population and controlling for potential genetic effects by including a phylogenetic distance matrix. We created the phylogenetic matrix from the *Neotoma* consensus Bayesian phylogeny using the cophenetic function in ape v5.3 (Matocq et al. 2007, Paradis & Schliep 2018) and the geographic distance

matrix from site coordinates using geosphere v1.5-10 (Hijmans 2019). We first analyzed the relationship between geography and dissimilarity (BC, J) using all ASVs, and then repeated the analysis with ASVs divided into neutral and selected taxa, calculating community dissimilarity using the Jaccard index, as subsetting potentially alters relative abundance calculations.

To examine how host evolutionary history influences microbiome composition, we first tested whether bacterial communities differ between host species, using a PERMANOVA and including site as a factor. Aggregating microbiome data for each host population (n=25), we then tested for congruence between host phylogeny and microbial community dendrograms. As generalized Robinson-Foulds tree comparison methods require bifurcating trees, we randomly subsampled host populations to include no more than two populations per species, and then calculated average host and microbiome tree congruence based on 100,000 host and microbiome trees. Microbiome trees were constructed using microbiome dissimilarities (BC, J, WU, U), clustered with UPGMA (function: hclust(), method = 'average'). We calculated the distance between the microbiome dendrogram and host phylogeny using mutual clustering information with the TreeDistance function in the TreeDist package (Smith 2020). To assess significance, we calculated p-values by comparing the average host-microbiome tree distance to the distribution of distances from 100,000 randomly generated tree pairs with the same number of tips, using TreeTools to generate random trees (TreeTools).

To examine how diet influenced wild microbiome diversity and composition we tested whether microbiome diversity (observed or Shannon) correlated with diet diversity, including host species and DNA concentration as fixed effects and sequencing run and population random factors. We then examined whether populations with more diverse diets exhibited

higher microbiome dispersion using a beta regression model with species as random factor, using the betadisper function in vegan to calculating average microbiome dispersion for the four standard distance metrics. We ran all generalized mixed effects models in glmmTMB v1.0.1.9 (Brooks et al. 2017), examined residuals to assess fit, used AIC to compare candidate models, and tested for factor significance by comparing models with and without the factor of interest using Wald chi-squared tests.

We predicted that plant secondary compounds in woodrat diets would select for microbes capable of degrading these toxins. We used DESeq2 v1.26.0 to identify taxa more abundant in animals feeding on cactus, creosote, and conifer based diets (compared to all other animals). We conducted differential abundance on un-rarefied (but filtered as described above) reads using the "local" fit type and default functions settings, and the Benjamini–Hochberg method to control false discovery rate. Expanding analyses to include all diet types, we next examined whether similar diets were associated with similar bacterial communities by comparing population-level microbiome and diet dendrograms.

As we expected geography, phylogeny and diet to jointly influence microbiome structure, we used multiple regression on distance matrices (MRM) to examined the unique and combined effects of these factors. We ran MRM models with 1000 permutations in ecodist v2.0.5 (Goslee & Urban 2007), using microbiome dissimilarity matrices (BC, J, WU, U) for all wild rats (n=147) as the response, and constructed geographic, phylogenetic and diet (BC, for individual rat diets) distance matrices as factors. With the model outputs, we then used variance partitioning methods adapted from Legendre (2008) to calculated the unique and shared contributions of each factor.

Bringing rats into captivity removed natural environmental variation and created a standardize disturbance. To test whether captivity reduced the structuring effects of site and diet, we repeated MRM analyses using bacterial communities from captive hosts (n=136). As we also obtained similar when using only animals (n=123) with paired wild and captive samples, we report analyses with the full dataset on the poster. We next tested how host, diet, and microbiome factors influence microbiome responses to captivity. We first tested whether wild microbiome diversity predicted captive diversity using mixed models which included population as a random effect. We then examined whether shifts in microbiome diversity (Shannon, observed) were influenced by wild diet diversity, wild microbiome diversity, and species identity, again controlling for population. To look at how disturbance altered community composition, we first tested whether captive communities differed from those in the wild using PERMANOVAs, including population as a factor. We then tested whether homogenous captive conditions homogenized the gut microbiome, using the betadisper function to calculate dispersion in wild and captive microbiomes. Controlling for population differences, we next examined how wild diet diversity, wild microbiome diversity, and species identity predicted pairwise distances between wild and captive bacterial communities. Finally, for each host species, we calculated the average change in alpha and beta diversity and then tested for a phylogenetic signal using Moran's I in the package phylosignal (Keck et al. 2016). For beta diversity comparisons, we used the four standard metrics and adjusted for multiple comparisons (Benjamini & Hochberg 1995).

References

Ando, H., C. Fujii, M. Kawanabe, Y. Ao, T. Inoue, and A. Takenaka. 2018. Evaluation of plant contamination in metabarcoding diet analysis of a herbivore. Scientific Reports 8:15563.

Benjamini, Y. and Y. Hochberg. 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. Journal of the Royal Statistical Society Series B (Methodological) 57:289-300.

Brooks, M. E., et al. 2017. glmmTMB balances speed and flexibility among packages for zero-inflated generalized linear mixed modeling. The R journal 9:378-400.

Burns, A. R., et al. 2016. Contribution of neutral processes to the assembly of gut microbial communities in the zebrafish over host development. The ISME Journal 10:655-664.

Callahan, B., K. Sankaran, J. Fukuyama, P. McMurdie, and S. Holmes. 2016a. Bioconductor Workflow for Microbiome Data Analysis: from raw reads to community analyses [version 2; peer review: 3 approved]. F1000Research 5.

Callahan, B. J., P. J. McMurdie, M. J. Rosen, A. W. Han, A. J. A. Johnson, and S. P. Holmes. 2016b. DADA2: High-resolution sample inference from Illumina amplicon data. Nature Methods 13:581-583.

Caporaso, J. G., et al. 2012. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. The ISME Journal 6:1621-1624.

Goslee, S. C. and D. L. Urban. 2007. The ecodist Package for Dissimilarity-based Analysis of Ecological Data. 2007 22:19.

Hijmans, R. J. 2019. geosphere: Spherical Trigonometry. Ver. R package version 1.5-10. https://CRAN.R-project.org/package=geosphere.

Kartzinel, T. R., et al. 2015. DNA metabarcoding illuminates dietary niche partitioning by African large herbivores. Proceedings of the National Academy of Sciences 112:8019-8024.

Keck, F., F. Rimet, A. Bouchez, and A. Franc. 2016. phylosignal: an R package to measure, test, and explore the phylogenetic signal. Ecology and Evolution 6:2774-2780.

Kohl, K. D., K. Luong, and M. D. Dearing. 2015. Validating the use of trap-collected feces for studying the gut microbiota of a small mammal (*Neotoma lepida*). Journal of Mammalogy 96:90-93.

Legendre, P. 2008. Studying beta diversity: ecological variation partitioning by multiple regression and canonical analysis. Journal of Plant Ecology 1:3-8.

Martin, M. 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnetjournal 17:10-12.

Matocq, M. D., Q. R. Shurtliff, and C. R. Feldman. 2007. Phylogenetics of the woodrat genus *Neotoma* (Rodentia: Muridae): implications for the evolution of phenotypic variation in male external genitalia. Molecular Phylogenetics and Evolution 42:637-652.

McMurdie, P. J. and S. P. Holmes. 2013. phyloseq: An R package for reproducible interactive analysis and graphics of microbiome census data. PLoS ONE 8:e61217.

Paradis, E. and K. Schliep. 2018. ape 5.0: an environment for modern phylogenetics and evolutionary analyses in R. Bioinformatics 35:526-528.

Patton, J. L., D. G. Huckaby, and S. T. Álvarez-Castañeda. 2007. The evolutionary history and a systematic revision of woodrats of the *Neotoma lepida* group. Univ of California Press.

Price, M. N., P. S. Dehal, and A. P. Arkin. 2010. FastTree 2 – Approximately Maximum-Likelihood Trees for Large Alignments. PLoS ONE 5:e9490.

Quast, C., et al. 2012. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Research 41:D590-D596.

R Core Team. 2020. R: A Language and Environment for Statistical Computing. Ver. R version 3.6.3. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/.

SHURTLIFF, Q. R., P. J. MURPHY, AND M. D. MATOCQ. 2014. Ecological segregation in a small mammal hybrid zone: Habitat-specific mating opportunities and selection against hybrids restrict gene flow on a fine spatial scale. Evolution 68:729-742.

TABERLET, P., et al. 2007. Power and limitations of the chloroplast *trn*L (UAA) intron for plant DNA barcoding. Nucleic Acids Research 35:e14.

WEISS, S., et al. 2017. Normalization and microbial differential abundance strategies depend upon data characteristics. Microbiome 5:27.