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LETTER

The gut microbiome reflects ancestry despite dietary shifts across a hybrid zone

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Abstract

The microbiome is critical to an organism's phenotype, and its composition is shaped by, and a driver of, eco-evolutionary interactions. We investigated how host ancestry, habitat and diet shape gut microbial composition in a mammalian hybrid zone between *Neotoma lepida* and *N. bryanti* that occurs across an ecotone between distinct vegetation communities. We found that habitat is the primary determinant of diet, while host genotype is the primary determinant of the gut microbiome—a finding further supported by intermediate microbiome composition in first-generation hybrids. Despite these distinct primary drivers, microbial richness was correlated with diet richness, and individuals that maintained higher dietary richness had greater gut microbial community stability. Both relationships were stronger in the relative dietary generalist of the two parental species. Our findings show that host ancestry interacts with dietary habits to shape the microbiome, ultimately resulting in the phenotypic plasticity that host–microbial interactions allow.

KEYWORDS
16S, hybrid zone, microbiome, *Neotoma*, small mammal, trnL, woodrat

INTRODUCTION

The gut microbiome contains a diverse community of microorganisms central to host health (Arnolds & Lozupone, 2016; Sender et al., 2016). For mammalian herbivores, the gut microbiome is critical to nutrient acquisition by performing metabolic functions otherwise unavailable to the host (Dearing & Kohl, 2017) and variation in microbiome composition may impact fitness (Suzuki, 2017). Additionally, the functional diversity of the gut microbiome provides a source of phenotypic plasticity that is important for host survival and that can drive host evolution (Kolodny & Schulenburg, 2020; Moeller & Sanders, 2020). Despite general recognition of the importance of the gut microbiome in the ecology and evolution of hosts, little is known of how genetic and environmental factors interact to influence gut microbiome communities, especially in wild animal populations.

Individual-level traits such as diet, seasonal change in diet, host sex and disease state are known to influence microbiome composition (Amato et al., 2015; Gilbert et al., 2016; Kartzinel et al., 2019). However, host genotype is an important driver of microbial composition that may supersede environmental effects (Knowles et al., 2019; Spor et al., 2011). For example, host phylogeny and microbiome composition often mirror one another (Brucker & Bordenstein, 2013), suggesting that animals and their gut microbiomes remain associated over macroevolutionary timescales (Weinstein et al., 2021). However, other studies report prevailing environmental effects in shaping microbial composition (Grieneisen et al., 2019; Grond et al., 2020). Elucidating

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the relative influences of the environment and host genotype in shaping gut microbiome variation is central to understanding the role of microbial plasticity in individual fitness and dietary adaptation.

Hybrid zones that occur across ecotones—sharp environmental transitions between two ecological communities—provide an ideal arena in which to study the relative influences of environment and host genotype on microbial community composition. Such hybrid zones offer natural laboratories in which to investigate how mismatches between habitat, diet, host genomes and gut microbial composition may influence individual fitness and rates of hybridisation. For example, adaptation to divergent habitats can reinforce reproductive isolation by way of selection against migrants (Nosil et al., 2005; Via, 1999; Via et al., 2000). It is possible that gut microbial mismatches with novel habitats and diets may underlie selection against migrants, yet, to our knowledge, few in situ studies have examined microbiome variation in natural hybrid zones (Grieneisen et al., 2019; Lin et al., 2020; Wang et al., 2015).

Over a four-year period, we studied a woodrat hybrid zone that occurs at a sharp habitat transition in southern California between the southern Sierra Nevada and western Mojave Desert (Figure 1; Shurtliff et al., 2014). Here, *Neotoma bryanti* (Bryant's woodrat) occurs primarily within the rocky, and relatively mesic, more vegetated, hill habitat (hereafter referred to as "hill") and *Neotoma lepida* (desert woodrat) occurs primarily within the relatively xeric or arid, Mojave Desert scrub (hereafter referred to as "flats"; Figure 1). Here, the two species hybridise and generate a spectrum of F1 and backcross hybrid genotypes that are distributed within approximately ½ km^2 (Jahner et al., 2021; Patton et al., 2007; Shurtliff et al., 2014). The parental species maintain distinct, habitat-specific and toxic, plant-based diets across the ecotone during both wet and dry seasons (Matocq et al., 2020; Nielsen & Matocq, 2021). *Neotoma bryanti* on the hill maintain a more diverse diet with *Frangula californica* (California coffeeberry) comprising 25% or more of the "hill diet"; while *N. lepida* in the flats consume a less diverse diet with *Prunus fasciculata* (desert almond) comprising over 50% of the "flats diet" (Matocq et al., 2020; Nielsen & Matocq, 2021). Individuals of pure parental ancestry and parental backcross hybrids are largely spatially segregated (*N. lepida*-like genomes in the flats; *N. bryanti*-like genomes on the hill), and F1 hybrids are distributed throughout the ½ km^2 site. However, some *N. lepida*-like and *N. bryanti*-like individuals occupy the alternative habitat (hereafter 'mismatched'). The distribution of genotypes across the genomic spectrum and across habitat types provides an opportunity to quantify the effects of host genotype and host environment on the gut microbiome.

We characterised diet and gut microbiome composition of *N. bryanti, N. lepida* and hybrids to ask: (1) how do diet and the gut microbiome vary spatially and temporally across the genotypic spectrum between *N. lepida* and *N. bryanti*? (2) what are the relative influences of environment and host genotype on diet and gut microbiome composition? (3) are certain microbial lineages associated with genotypic classes or diet types, and what might that suggest from a metabolic perspective? To address these questions, we used high-throughput sequencing of field-collected faecal samples to characterise covariation between diet and microbiome across the genomic spectrum of woodrats at this site. Our study identifies the primary drivers of variation in diet and the gut microbiome and provides insight into the functional significance of differing gut microbial communities.

METHODS

Trapping and faecal sample collection

We collected faecal samples from live-trapped woodrats from 2016 to 2019 in Kelso Valley, Kern Co., California, as part of long-term sampling (Text S1). Here, hybridisation occurs between *N. bryanti* and *N. lepida* across a Sierra Nevada—Mojave Desert ecotone within an area approximately ½ km^2 in size (35°25′45 N, 118°15′2 W; Figure 1). We grouped samples into two seasonal categories: March–June, spring; and July–October, summer/fall. Animal handling was approved by the University of Nevada Reno Institutional Animal Care and Use Committee, the California Department of Fish and Wildlife, and were consistent with the guidelines developed by the American Society of Mammalogists (Sikes et al., 2016).

Establishing genotypic classes

We extracted DNA from woodrat ear tissue using the Qiagen DNeasy blood and tissue kit as previously described in Nielsen and Matocq (2021). For faecal samples collected from active woodrat nests (n = 64), we identified genotypes using amplification of microsatellites previously described and used for *N. lepida* and *N. bryanti* at our study site (Nielsen & Matocq, 2021; Shurtliff et al., 2014). For woodrats from which ear biopsies were collected (n = 178), we generated a Single-Nucleotide Polymorphism dataset (SNPs) using a double-digest restriction site associated sequencing (ddRADseq) protocol previously used in this hybrid zone (Jahner et al., 2021; Parchman et al., 2012; Peterson et al., 2012). We used `Stacks` v. 2.53 (Catchen et al., 2013) to align reads to the *N. lepida* genome (Greenhalgh et al., 2022), and identify SNPs and call genotypes for each locus. Ancestry and population structure were inferred using `FastSTRUCTURE` (Raj et al., 2014; see Text S2).

Rather than treat genetic variation as a continuous variable, we categorised individuals into genotypic classes based on the proportion of their genome assigned

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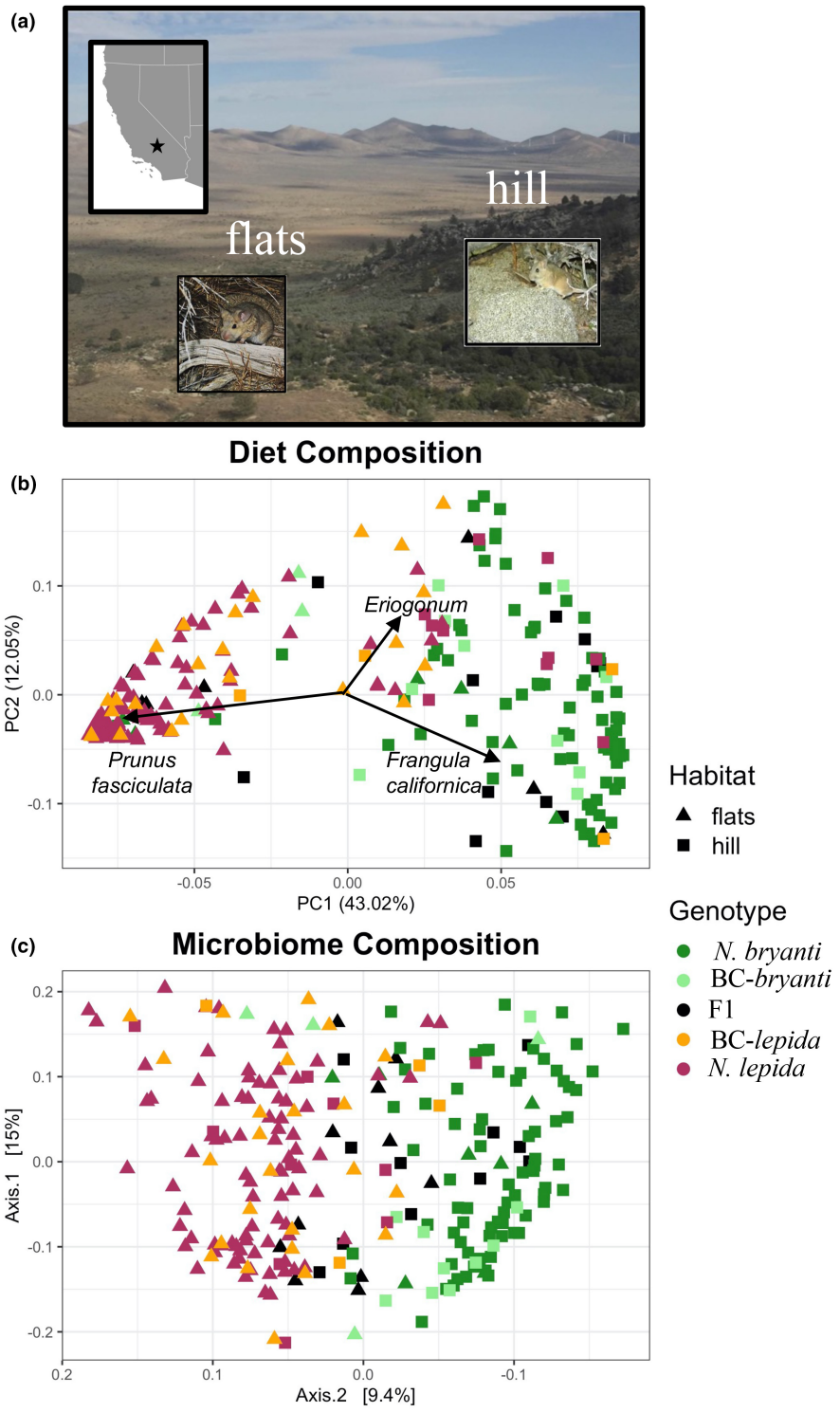

FIGURE 1 The study site in southern California, where the mesic hill habitat transitions to the xeric flats. Photo taken from north looking southeast. Inset photos of *N. lepida* in flats, and *N. bryanti* on hill (a). Diet composition was largely distinct among genotypic classes and across habitats (b), with loadings from principal components analysis (PCA). Microbiome composition also differed among genotypic classes and across both habitats (c).

to *N. bryanti* ($q_{bryanti}$) using $K = 2$, consistent with previous work at this site: >0.90 = *N. bryanti*, 0.90–0.60 = BC-*bryanti*, 0.60–0.40 = F1, 0.40–0.10 = BC-*N. lepida*, and <0.10 = *N. lepida* (Nielsen & Matocq, 2021). We did not classify advanced hybrids as the previous analysis found no evidence of F2 or advanced generation hybrids (Jahner et al., 2021). Four individuals were removed from the STACKS analysis due to low coverage and were assigned genotypes using microsatellites as described above.

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Amplicon sequencing for characterising diet and microbiome

We submitted trap-collected faecal samples to Jonah Ventures LLC for DNA extraction, PCR amplification and sequencing. DNA was sequenced for both *trnL*, a common plant metabarcoding gene to identify diet composition, and 16S rRNA, a common microbial barcoding gene to identify microbiome composition (Valentini et al., 2009), on the Illumina MiSeq platform using the v2 500-cycle kit. Methods of DNA extraction, amplification and sequencing for diet followed Nielsen and Matocq (2021). For *trn*L data, we removed plants that fell below a 5% relative read abundance threshold, and we filtered out reads originating from the small amount of peanut butter used as bait (genus *Arachis*). After filtering and taxonomic identification, we retained 4,015,325 reads across 46 plant taxa. We restricted our analyses to the five most common plants identified previously as dietary components: *Prunus fasciculata*, *Frangula californica*, *Eriogonum*, *Pinus* and *Phacelia*. The remaining plant reads were grouped together as an 'other' category (Table S6 for full plant list).

Microbiome composition was characterised using the 515f and 806R 16S rRNA primers (Caporaso et al., 2011). 16S sequences were processed using the standard `MOTHUR` SOP pipeline (Kozich et al., 2013, assessed 25 October 2020). Sequences from mitochondria, chloroplast, Archaea and Eukaryota were excluded using the *remove.lineage* command of `MOTHUR`. 16S sequences were clustered into operational taxonomic units (OTUs) based on 97% similarity and taxonomy was assigned using the SILVA reference database (Quast et al., 2012, accessed Oct. 25, 2020). This initial dataset, including singletons, contained 3,552,497 reads across 242 unique samples and was used to calculate microbial richness (see below). Use of trap-collected faeces for studying the gut microbiome has been validated (Kohl et al., 2015); but to confirm that environmental contamination did not contribute to overall patterns in microbial composition of faecal samples, we conducted a validation study (see Text S1). A negative lab control was included, and we removed any OTU for which 5% or more of its reads was contained within the blank.

For the resulting diet and microbiome datasets, we used a hierarchical Bayesian approach implemented with `CNVRG` to estimate the proportion of each feature (i.e., plant or microbe) within an individual sample (Harrison et al., 2020; details in Text S3). The resulting proportional dataset included 3655 microbial OTUs across 242 unique individual woodrats. We calculated Bray-Curtis distances from the resulting diet and microbiome proportional data. Finally, we also used `CNVRG` multinomial estimates to infer differentially abundant microbial taxa (Text S3). Some individual woodrats were sampled multiple times; for these, we randomly selected only one sample per individual (woodrat ID) for analyses, unless otherwise indicated (see below and Text S4).

Variation in diet and microbiome diversity

We estimated Shannon's diversity index of diet and microbiome across genotypes, habitats and spring and summer seasons using the `phyloseq` package in `R` (McMurdie & Holmes, 2013). To assess differences in alpha diversity in diet and microbiome across genotypes, habitats and spring and summer, we performed ANOVA using the *aov* function in the `stats` library. We calculated richness for diet and microbiome as the total number of plants and microbial (after removing singletons) OTUs observed in a sample, respectively. We tested whether diet and microbial richness and distance were correlated within woodrat genotypes as well as within individuals sampled multiple times (Text S4).

Quantifying host and environmental effects on diet and microbiome

We estimated seasonal diet and microbiome turnover as the average Bray-Curtis distance for each genotypic class within each habitat between spring versus summer/fall sampling. We conducted a principal components analysis (PCA) using square-root transformed read counts from diet data with the *prcomp* function of the `stats` package. We visualised microbiome and diet composition using principal coordinates analysis (PCoA) and PCA, respectively, and with `ggplot2` (Wickham, 2016). We assessed whether F1 hybrid microbiome composition was intermediate by comparing pairwise Bray-Curtis distances among conspecific (e.g., *N. bryanti* or *N. lepida* comparisons), heterospecific (i.e. *N. bryanti* – *N. lepida* comparisons), or either parental species versus F1 hybrids (see Text S8). We plotted microbiome PCoA 2 against diet PC1 to illustrate the effect of genotype on the relationship between diet and microbiome composition in mismatched parental and backcross hybrids, and F1 hybrids (`scatterpie` package, Yu, 2021).

To guide dimensionality reduction, we calculated Spearman rank correlation coefficients (Spearman's rho) between host and environmental variables (i.e. genotype, habitat, season and year) and the first four axes of both diet (PCA) and microbiome (PCoA; Text S5). We estimated the amount of variance explained in diet and microbiome composition by individual host and environmental variables using partial distance-based redundancy analysis (dbRDA) using the *dbrda* function in `vegan` (Oksanen, 2020; see Text S6). We used variance partitioning (*varpart* function in `vegan`) to further assess the combined and individual contributions of habitat, genotype and either diet PC1 or microbiome PCoA2 on overall variation in diet and microbiome composition. By using partial RDAs, this approach estimates the proportion of variance explained by each predictor variable combined in the full model, as well as independently by partialling out the influence of the other variables. This analysis

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allows investigation of the relative influence of multiple variables, while accounting for correlation among them.

To determine if microbial community composition was significantly associated with the most common diet plants, we implemented a constrained analysis of principal coordinates (CAP) with the *ordinate* function in `phyloseq` using the model: *OTU ~ Prunus fasciculata + Frangula californica + Eriogonum + Condition (Year + Age + Sex)*. We used an ANOVA with 999 permutations to test for model significance. We conducted analyses in R (R Core Team, 2020).

RESULTS

Genotypic variation among sampled individuals

After quality filtering, our genomic dataset contained 154,022 SNPs. Of 242 unique individuals sampled between 2016 and 2019, we identified 83 *N. bryanti*, 14 BC-*bryanti*, 22 F1 hybrids, 28 BC-*lepida*, and 95 *N. lepida*. Sample sizes within genotype, habitat and seasonal groups are reported in Figure 2 (panels a and d).

Diet varies across habitats and among genotypes

We collected a total of 334 faecal samples from 242 individuals from 2016 to 2019. Overall, diet and microbiome were distinguishable among genotypic classes and across habitat types (Figure 1b,c). The three most abundant plants in spring and summer diets were: *P. fasciculata* (desert almond) predominantly in the flats diet, *Frangula californica* (Californica coffeeberry) in the hill diet and *Eriogonum* (buckwheat) was consumed in both habitats (Figures 1b and 2). *Phacelia*, a spring forb, was consumed in both habitats when available (Figure 2a). Diet diversity differed among genotypes (df = 4, $F = 6.5$, $p < 0.001$) and across habitats (df = 1, $F = 4.7$, $p = 0.03$; Figure 2b). Seasonal turnover of *N. lepida* diet in the flats was significantly lower than that of *N. bryanti* on the hill ($p < 0.05$; Figure 2c).

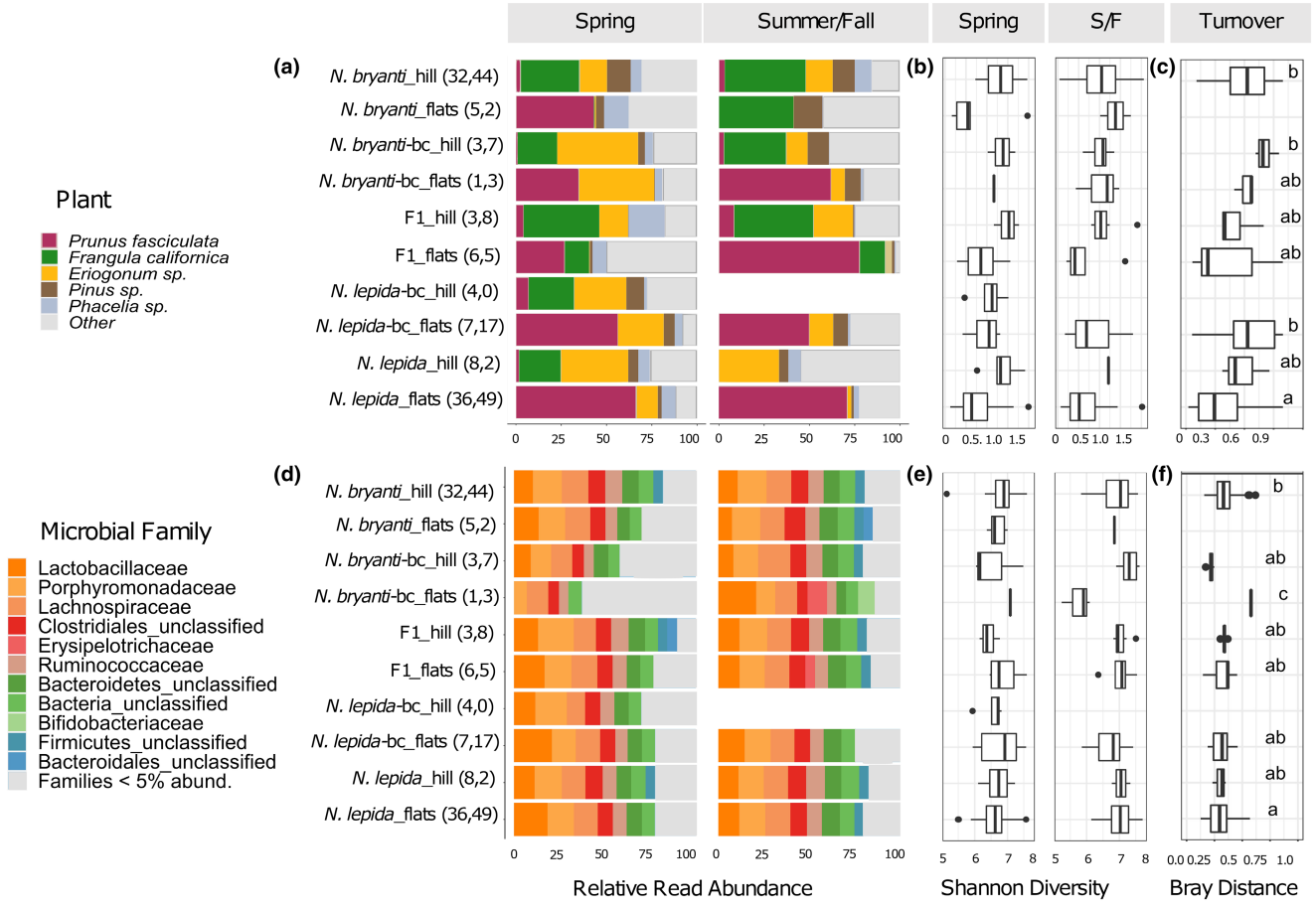

FIGURE 2 Composition, diversity, and turnover varied among genotype, habitat, and season for diet (panels a–c) and microbiome (panels d–f). Numbers in parentheses on the y axis are sample sizes of each category of woodrat samples for spring and summer/fall, respectively. Turnover for each group was calculated as the average Bray-Curtis distance between spring and summer/fall samples from each category. Letters indicate significant differences (Tukey; $p < 0.05$).

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Microbiome varies among genotypes and across habitats

Microbiome composition varied among genotypic classes and habitat types, with F1 hybrids exhibiting a microbial community composition intermediate to pure parental individuals (Figure 1c and Figure S5). Firmicutes and Bacteroidetes were the most abundant microbial phyla across samples. Common microbial families included Lactobacillaceae, Porphyromonadaceae, Lachnospiraceae, Erysipelotrichaceae, Ruminococcaceae and Bifidobacteriaceae (Figure 2d). Shannon diversity of the microbial community did not differ among genotypic classes, but did differ across seasons (df = 1, $F = 11.3$, $p < 0.001$; Figure 2e). Seasonal turnover of the gut microbiome community was greater in *N. bryanti* than in *N. lepida* ($p < 0.05$; Figure 2f).

Across the entire dataset microbiome richness was positively correlated with dietary richness ($p < 0.001$, conditional $R^2 = 0.12$; Figure 3a). When evaluated individually, only *N. bryanti* on the hill exhibited a positive relationship between diet and microbiome richness ($R = 0.27$; $p = 0.02$, Figure 3b). There was a significant linear relationship between diet and microbiome distance across the entire dataset (Mantel, $r = 0.13$, $p = 0.001$; Figure S3), but no linear relationship within individual genotypes. Among individuals sampled multiple times, *N. bryanti* on the hill exhibited a negative relationship between diet and microbiome distance (Spearman; $r = -0.49$, $p = 0.03$; Figure 3c), and while not significant, *N. lepida* in the flats exhibited a positive trend. Diet richness was negatively correlated with microbiome distance in *N. bryanti* individuals sampled multiple times (Spearman; $r = -0.51$, $p = 0.03$; Figure 3d).

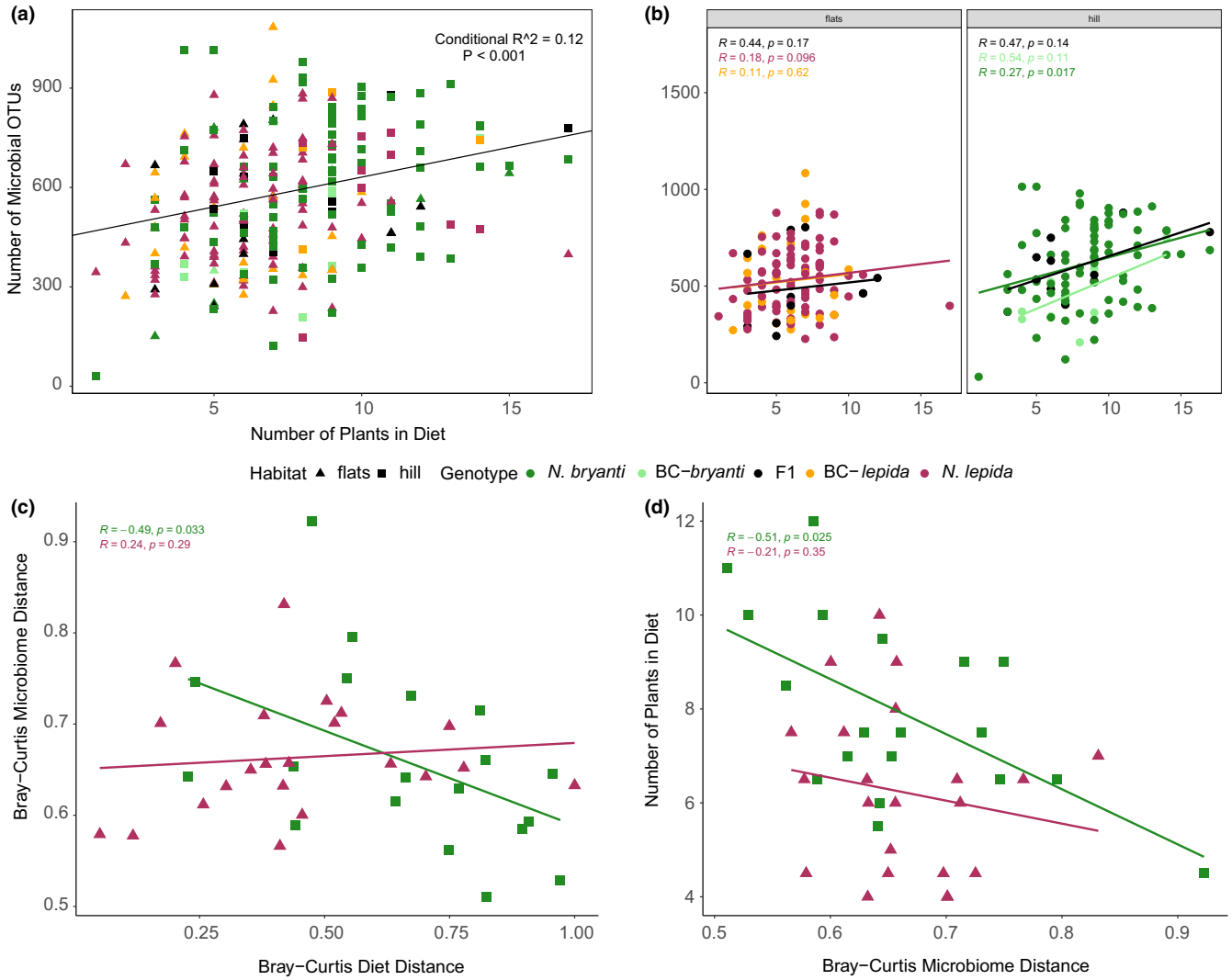

FIGURE 3 Correlation between diet and microbiome diversity. Overall, diet and microbial richness were significantly correlated (a). The regression line in panel (a) is from the *glmmTMB* model using diet richness, season, habitat, and genotype as fixed effects. The relationship between diet and microbiome richness varied when evaluated individually among genotypic classes and habitats (b). Diet and microbiome distance were negatively correlated in *N. bryanti* sampled multiple times (c). Diet richness was negatively correlated with microbiome distance in *N. bryanti* individuals sampled multiple times (d).

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Drivers of diet and microbiome composition

Habitat and genotype were correlated with diet PC1 which explained 43% of variation; whereas habitat and genotype were correlated with axis 2 of the microbiome PCoA which explained 9.4% of variation (Tables S2 and S3). Based on these results, we used diet PC1 and microbiome PCoA2 as variables in further analyses (see below).

Partial distance-based redundancy analysis (dbRDA) revealed that habitat explained the greatest amount of variation in diet, while host genotype explained the greatest amount of variation in the gut microbiome (Table S4). See supporting information for more detail.

Variance partitioning provided further insight into the relative contributions of genotype, habitat, diet and microbiome on variation in diet and microbiome across this ecotone (Table 1). For this analysis and based on the results summarised above (Tables S2 and S3), we simplified diet and microbiome to values along PC1 and PCoA2, respectively. When evaluated individually, habitat (*PVE* = 68.6%) explained more variation in diet than genotype (*PVE* = 49.7%) or microbiome (PCoA2; *PVE* = 54.5%). Considering the partial contributions of these variables (i.e., after removing the influence of other variables), habitat (adj. r^2 = 15.5%, Table 1) explained the most variation in diet while microbiome (PCoA2) only explained 1.4% and genotype did not explain any further variation. In contrast, microbiome appears to be primarily constrained by ancestry. Genotype (*PVE* = 70.1%) explained more variation in the microbiome than habitat (*PVE* = 54.5%) and diet (PC 1; *PVE* = 54.5%). When considering the partial contributions, genotype (adj. r^2 = 15%) still explained the most variation in microbiome while diet PC1 only explained 1.3%, and habitat did not explain any further variation. Variance partitioning estimates explained variance of predictor variables, both combined and independently; however, as genotype and habitat are highly correlated, we confirmed these results by analysing only hybrid and mismatched individuals (Table S5). Also, to verify that these results were not biased due to dimension reduction, we confirmed the order of variable importance by performing variance partitioning using all diet and microbiome dimensions.

Microbiome community composition was significantly associated with the most common diet plants, with *F. californica* and *Eriogonum* exhibiting strong associations with microbiome composition of *N. bryanti* on the hill, and *P. fasciculata* significantly associated with *N. lepida* microbiome composition in the flats (Figure 4). BC-*lepida* individuals exhibited a *P. fasciculata*-associated microbiome like their parental counterparts in the flats. F1 hybrids exhibited a range of diet by microbiome associations, but an overall intermediate microbiome composition in comparison to purebred individuals (Figures 4, 5, Figure S5).

Mismatched individuals exhibit more variable diets and microbiomes

Individuals occupying the "mismatched" habitat (i.e., *N. bryanti* in flats, *N. lepida* on hill) exhibited reduced preference for the plant most consumed in that habitat. For instance, the relatively rare *N. bryanti* in the flats consumed some *P. fasciculata* (frequency of occurrence in diet (FOO) = 42.8%, relative read abundance in diet (RRA) = 36.8%), but much less in comparison to *N. lepida* in the flats (FOO = 94%, RRA = 70.7%). Likewise, the rare *N. lepida* on the hill consumed some *F. californica* (FOO = 60%, RRA = 17.1%), but less than *N. bryanti* on the hill (FOO = 88.7%, RRA = 40.3%). *Neotoma bryanti*

TABLE 1 Variance partitioning of the individual and combined influences of habitat and genotype on variation in diet PCA axis 1 (explained 43.0% of total variation; Figure 1 panel b) and microbiome PCoA axis 2 (explained 9.4% of total variation; Figure 1 panel c)

Model	Adjusted r^2 (%)	Proportion of variance explained (%)
Diet ~ Habitat + Genotype + Microbiome PCoA 2	46.0—	72.2*
Diet ~ Habitat	15.5*	68.6*
Diet ~ Genotype	0.2	49.7*
Diet ~ Microbiome PCoA 2	0.4*	54.5*
Residuals	28.0	
16S ~ Habitat + Genotype + Diet PC 1	47.0—	74.5*
16S ~ Habitat	0.2	54.5*
16S ~ Genotype	15.0*	70.1*
16S ~ Diet PC 1	1.3*	54.5*
Residuals	25.5—	

*model significance p 0.001—cannot be tested

Note: Respective axes of variation for diet and microbiome were also included in models for each other. Habitat, genotype, and microbiome explained 72.2% of diet variation (PC1), while habitat, genotype, and diet explained 74.5% of microbiome variation (PCoA 2). Adjusted r^2 values represent the amount of variation each variable explained after removing the effects of the other variables.

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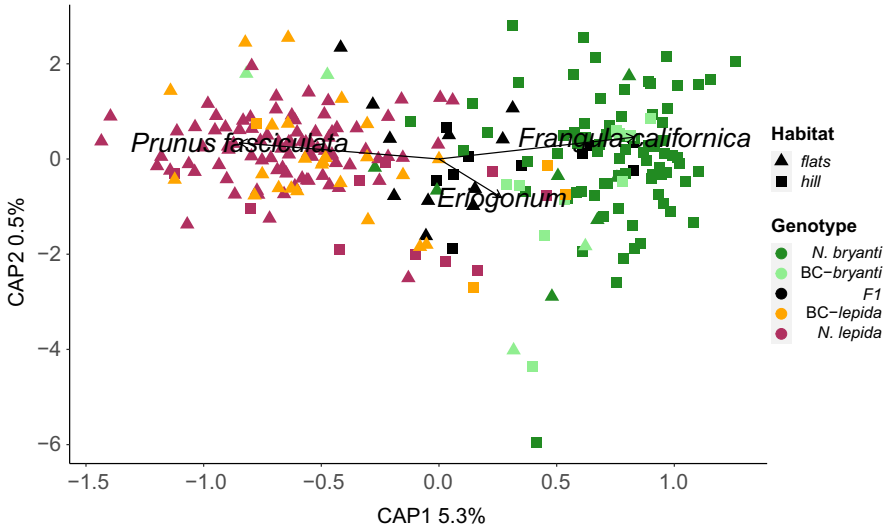

FIGURE 4 Constrained analysis of principal coordinates (CAP). Each point represents an individual's microbiome community composition using Bray–Curtis dissimilarity. We tested for significant associations between diet plants and microbiome composition, and removed the effects of year, age and sex with the following formula: OTU ~ *Prunus fasciculata* + *Frangula californica* + *Eriogonum* + *Condition (year + sex + age)*. The association between microbiome and diet plants included was significant (ANOVA with 999 permutations; df = 3, $F = 4.95$, $p = 0.001$, adj. $r^2 = 4.6$).

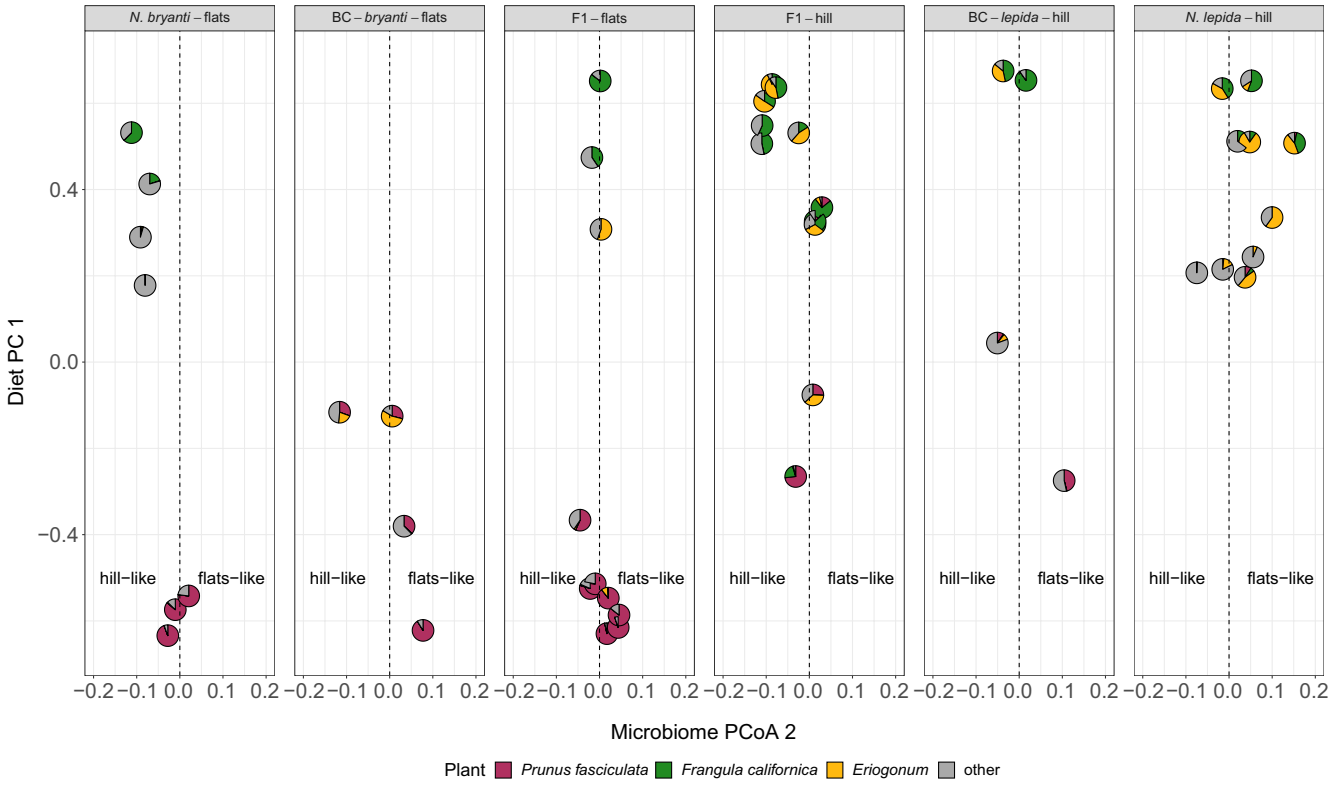

FIGURE 5 Microbiome PCoA 2 plotted against diet PC 1 for mismatched parental and backcross hybrids, and F1 hybrids in either habitat. Pie charts represent the relative abundance of *Prunus fasciculata*, *Frangula californica*, *Eriogonum* or the remaining 'other' plants identified in diets using *trn*L metabarcoding. Among parental *N. bryanti* and *N. lepida* individuals, the imprint of ancestry is evident by the maintenance of a genotype-specific microbiome, with some exceptions in individuals that consumed habitat-specific diets. The strong influence of genotype on microbiome is also evident in F1 hybrids which exhibit intermediacy (particularly in the flats).

in the flats that consumed more *P. fasciculata* exhibited a more intermediate microbiome than those that consumed a more hill-like diet (Figure 5). *Neotoma lepida* on the hill consumed more diverse diets, including increased consumption of *Eriogonum*, and exhibited variable microbiome composition (Figure 5). Backcross and

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F1 hybrids primarily consumed habitat-specific plants and exhibited more intermediate microbiome composition (Figure 5 and Figure S5).

Differential abundance of microbial taxa

More than 80% of differentially abundant taxa between *N. bryanti* and *N lepida* belonged to Bacteroidetes and Firmicutes (CNVRG analysis; Figure S4). *Lactobacillus* were more abundant in woodrats consuming a *P. fasciculata*-dominated diet, and in *N. lepida* and F1 hybrids in the flats (Figure S6). We also detected microbial lineages that are expected to modify hydrogen cyanide, the toxin associated with consumption of *Prunus*, including members of the Pseudomonadaceae (Zhu et al., 2018). The complete list of genera that differed with greater than 95% probability is provided in the supporting information (Tables S7–S10).

DISCUSSION

The distribution of *N. bryanti, N. lepida* and their hybrids across an ecotone allowed us to investigate the individual and joint effects of environment, host genotype and diet on gut microbial composition. We found that habitat-specific diets are accompanied by distinct microbial communities and that, at the individual level, microbial diversity is correlated with diet diversity. Nonetheless, we found that diet is most influenced by habitat, while microbial composition is primarily determined by host genotype. The latter of the two findings was further supported by our observation that admixed genomes were more likely to harbour microbial communities that were intermediate to those typically associated with pure parental genomes, regardless of diet (Figures 1, 4 and 5). Our findings suggest that gut microbiome composition in woodrats is primarily driven by host genotype, yet within that overall constraint, individual variation in diet is accompanied, and could be facilitated by (Kohl et al., 2014), gut microbial community diversity.

Relationship between diet and gut microbial community composition

We found a strong signature of habitat-specific diets within parental species, their respective backcross hybrids and F1 hybrids. *Frangula californica* and *P. fasciculata* were the most abundant diet plants in the hill and flats, respectively, and consumption of these plants was maintained across seasons. Given that both plants are known to contain compounds that can be toxic to mammals—anthraquinones in *F. californica* and cyanogenic glycosides in *P. fasciculata* (Matocq et al., 2020; Qin et al., 2016; Vetter, 2000)—we would expect these animals to diversify their diets when given the seasonal opportunity to shift away from these toxins (Nielsen & Matocq, 2021). Diet turnover across seasons was significantly higher for *N. bryanti* in the hill habitat than *N. lepida* in the flats, suggesting *N. bryanti* is more of a dietary generalist than *N. lepida*—a result consistent with previous studies (Nielsen & Matocq, 2021). Further, *N. lepida* maintains a high proportion of *Prunus* in their diet even in spring when a higher diversity of plants become available, consistent with findings that this species is a facultative specialist (Nielsen & Matocq, 2021; Shipley et al., 2009).

We found that diet and microbial richness were positively correlated at the fine spatial scale of this study, a pattern evident at broad spatial scales across populations and species of woodrats (Weinstein et al., 2021). The positive relationship between diet and microbial richness could be the result of multiple factors. It is possible that consuming a more nutritionally and chemically diverse diet requires or results in a more functionally diverse microbiome (Heiman & Greenway, 2016), consistent with the expectation of close ecological interactions between the gut microbiome and specific dietary components (Kartzinel et al., 2019; Knowles et al., 2019; Ren et al., 2017). Alternatively, microbial lineages detected in the faeces of woodrats may have been associated with the plants consumed and not persistent members of the gut microbiome (Kohl et al., 2014). Nonetheless, even transient plant-associated microbial lineages can contribute to metabolic functioning in the intestinal microflora (Zeibich et al., 2019), and may augment the functional capacity of host gut microbes through horizontal gene transfer (Hehemann et al., 2010; Wybouw et al., 2014). However, when broken down by genotype alone, diet and microbiome richness was only significantly positively correlated in *N. bryanti*. As this species exhibits a more generalist dietary strategy, this result supports the supposition that microbial diversity is at least partly driven by feeding strategy (Reese & Dunn, 2018).

Given the broad concordance between diet and microbiome richness and the close ecological association this relationship suggests, we anticipated that changes in diet would be correlated with changes in microbiome composition, resulting in a positive relationship between individual pairwise diet distances and their gut microbial distance. We found this relationship to be significantly positive for the entire dataset, but not when evaluated by individual genotypic class (Figure S3). We resampled some individuals across time, allowing further examination of whether changes in diet are correlated with changes in the microbiome, and found a positive trend within *N. lepida*, and a negative relationship in *N. bryanti*. The relative dietary specialist, *N. lepida*, may have a gut microbial community more tuned to a low diversity, albeit toxic, diet and when these diets shift, more microbial turnover occurs. On the other hand, the relative

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dietary generalist *N. bryanti* maintains a more diverse diet and microbiome, the latter of which may have the capacity to metabolise new dietary components without a compositional shift. This would lead to the expectation that *N. bryanti* individuals with the most diverse diets would exhibit greater stability (less turnover) in their microbial composition from one sampling point to the next, and that is indeed what we observe (Figure 3d). As seen in humans (Johnson et al., 2019), *N. bryanti* individuals with the most diverse diets appear to have the greatest stability in their microbial community composition. The potential relationships between specialists and generalists and their respective microbial communities warrants further investigation. Overall, though, despite the associations we detected between diet and microbiome, both appear to be primarily driven by different factors.

Influence of environment and host genotype on diet and gut microbial composition

Diet and microbiome composition were differentially influenced by each other, host genotype and habitat (Tables 1 & S4). In this system, diet is influenced most by habitat, then moderately by an individual's microbiome, with no additional variation explained by host genotype. Conversely, the microbiome is influenced most by genotype, then moderately by diet, with no additional variation explained by habitat.

The importance of genotype, or individual ancestry, in shaping microbiome composition is further supported by the microbial communities that characterise F1 hybrids. With half the *N. lepida* genome and half the *N. bryanti* genome, we might expect the microbiomes of F1 hybrids to be intermediate between parental types regardless of habitat diet. Indeed, F1 hybrids exhibited intermediate microbiome composition, particularly in the flats (Figure S5 and Figure 5). This pattern was evident even though most F1 individuals inhabiting the flats eat a *Prunus*-rich diet, and most on the hill eat a characteristic hill diet. Due to an ancient mitochondrial capture all *N. bryanti* at this site have an *N. lepida*-like mtDNA (Patton et al., 2007; Shurtliff et al., 2014), as such, we cannot confirm an individual's mother's genotype using mtDNA. However, hybridisation at this site is thought to primarily occur via female *N. bryanti* mating with male *N. lepida* (Shurtliff et al., 2013). Given the importance of maternally inherited microbes in mammalian microbiomes (Funkhouser & Bordenstein, 2013), it is possible that the pronounced intermediacy of microbiome composition of F1 hybrids in the flats is due to having *N. bryanti* mothers from which they inherit a more "hill-like" microbiome. The primacy of host genotype as a driver of microbiome composition has recently been shown at broad spatial scales in woodrats (Weinstein et al., 2021), but ours is the first investigation to support these findings with hybrid individuals and at a fine spatial scale. If intermediate microbial communities allow these individuals to have greater flexibility in habitat association, this could be an important mechanism determining rates of hybridisation in this system.

In our study, genotype and habitat were highly correlated due to the strong spatial segregation of parentals and their respective backcrosses. However, although rare, parental and backcross individuals that occupy the mismatched habitat provide a decoupling of the dominant habitat and genotype association at this site. For *N. bryanti*, the relative dietary generalist, some individuals ($N = 3$; Figure 5) that occupy the flats maintain a characteristic *Prunus* diet and their microbiome is intermediate between hill- and flats-like microbial communities, while other *N. bryanti* ($N = 4$) maintain a more diverse or hill-like diet and microbiome all while living in the flats. On the other hand, mismatched *N. lepida* on the hill consumed little *Prunus* and shifted their diet to a more hill-like diet, albeit dominated by *Eriogonum* rather than *Frangula*. However, these *N. lepida* genomes appear to sustain a wide range of microbial communities including those with intermediate composition, and even one example of a hill-like community. It appears that at least some *N. lepida* and *N. bryanti* can diversify their diets and sustain themselves in the alternate habitat. *Neotoma lepida* primarily rely on *Eriogonum* and other plants on the hill, while some *N. bryanti* can consume large amounts of *Prunus*, as might be expected for this facultative generalist. Many of these mismatched individuals have microbial communities that are intermediate in composition in comparison to their counterparts in their native habitat. This result suggests that the microbiome is not absolutely constrained by host genotype, and that plastic response to environmental conditions can occur.

Ecological and evolutionary implications

Our work adds to a small number of studies that investigate the relative role of host genotype and host environment in shaping the gut microbiome across mammalian hybrid zones (Grieneisen et al., 2019; Lin et al., 2020; Wang et al., 2015). There is complexity in the diet and microbiome datasets stemming from temporal variation (Tables S2–S4), as well as individual-level variation evident in high baseline Bray-Curtis distances within individuals sampled multiple times (Figure 3c,d). However, we still detected signals from habitat and genotype which allowed us to explore the relative contributions of these variables in shaping diet and microbiome composition. Diet was most influenced by habitat; and, consistent with other studies, we find that host genotype is the primary driver of microbial composition (Knowles et al., 2019; Weinstein et al., 2021). Additionally, dietary richness and composition were associated with microbial richness, composition and stability, but the nature

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of these relationships may differ between dietary specialists and generalists. Among differentially abundant microbial taxa detected, some may be functionally important in this system. *Lactobacillus* was strongly associated with *N. lepida* and F1 hybrids in the flats, as well as diets composed of greater than 50% *P. fasciculata*. Some *Lactobacillus* species degrade cyanogenic glycosides (Lei et al., 1999), which have been found in *P. fasciculata* at this site (Matocq et al., 2020). Presence of specific microbial taxa, even lineages in low abundance, may be critical to individual fitness across this hybrid zone.

Individual variation in dietary and gut microbial plasticity can influence the ability to acclimate to new habitats (Alberdi et al., 2016), which may have important ecological and evolutionary consequences (Vander Zanden et al., 2010), including the facilitation or encumbrance of gene flow. This hybrid zone is characterised by strong ecological segregation, with across-habitat dispersal occurring in only ~4% of captured individuals, despite spatial distances between these habitats being within individual dispersal capabilities (Shurtliff et al., 2014). Of the relatively few "mismatched" individuals that occur in the alternate habitat, many had a novel diet and gut microbial composition that suggests there may not be strict selection against migrants in this system. However, further genomic and demographic study is needed to identify the host metabolic and microbial traits that determine survival and reproductive success of these mismatched individuals and the potential for interspecific gene flow they create.

AUTHOR CONTRIBUTION

MDM obtained funding for this research. DPN and MDM conceived the ideas and methods; DPN-collected field samples, and all authors contributed to the analysis of data. DPN and MDM drafted the manuscript and all authors contributed to drafts and gave final approval for publication.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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DATA AVAILABILITY STATEMENT

Amplicon sequences are available on the NCBI sequence read archive under the Bioproject PRJNA887535. Data and code related to this publication can be found on Dryad DOI https://doi.org/10.5061/dryad.cfxpnvx8v.

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SUPPORTING INFORMATION

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