Sexual dimorphism and host genetics shape the gut microbiome of northern elephant seal pups (*Mirounga angustirostris*)

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Abstract

Due to a population bottleneck, northern elephant seals (*Mirounga angustirostris*) have very low genetic diversity, making them ideal model organisms for assessing the impact of genetic and non-genetic factors on the gut microbiome. In our study, we were especially interested in the role of sex given the northern elephant seal's extreme sexual dimorphism. We investigated 54 northern elephant seal pups that were rescued from along the California coastline and brought to The Marine Mammal Center, a rehabilitation facility. Using a metabarcoding approach, we characterized microbial communities shortly after admission to the facility and found that both sex and geographic origin explained microbial variation. We detected significant differences in microbial class and order composition between sexes. We further analyzed paired samples from 24 seals at two time points, shortly after admission to the rehabilitation facility and a month post-acclimation in the facility. Between these two time points, microbial diversity increased, likely due to changes in diet. While there was an overall convergence of microbiome composition in a shared environment over time, remaining differences in microbial composition were explained by sex and host genetics.

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Running Head: Intrinsic factors shaping seal gut microbiome

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Abstract

Due to a population bottleneck, northern elephant seals (*Mirounga angustirostris*) have very low genetic diversity, making them ideal model organisms for assessing the impact of genetic and non-genetic factors on the gut microbiome. In our study, we were especially interested in the role of sex given the northern elephant seal's extreme sexual dimorphism. We investigated 54 northern elephant seal pups that were rescued from along the California coastline and brought to The Marine Mammal Center, a rehabilitation facility. Using a metabarcoding approach, we characterized microbial communities shortly after admission to the facility and found that both sex and geographic origin explained microbial variation. We detected significant differences in microbial class and order composition between sexes. We further analyzed paired samples from 24 seals at two time points, shortly after admission to the rehabilitation facility and a month post-acclimation in the facility. Between these two time points, microbial diversity increased, likely due to changes in diet. While there was an overall convergence of microbiome composition in a shared environment over time, remaining differences in microbial composition were explained by sex and host genetics.

Keywords: Gut microbiome, sexual dimorphism, seals, metabarcoding, shared environment

Introduction

The mammalian gut microbiome is composed of more than a trillion diverse microorganisms (Thursby & Juge 2017) that aid in metabolic functioning, pathogen defense, and immune signaling and regulation (Belkaid & Hand 2014; Jandhyala *et al*. 2015; Kinross *et.al* 2011; Shreiner *et. al* 2015). Studies in humans have shown that microbial composition and diversity vary with age, sex, genetics, diet, environment, stress, and other factors (Hasan & Yang 2019; Kurilshikov *et al.* 2017; Tasnim *et al.* 2017). In model organisms and wild populations, interactions between host genetics and environmental factors can strongly influence an individual's microbiome composition (DeCandia *et al.* 2021, de Jonge *et. al* 2022; Rojas *et al*. 2020; Turnbaugh *et al.* 2009; Zhu *et al*. 2021). Model organisms, such as traditionally inbred mouse lines, have been used to control for genetic variation in order to understand the impact of environmental factors on differences in the gut microbiome (Spor *et. al* 2011). Further, biological sex appears to have a weak effect on shaping the vertebrate gut microbiome (Bennett *et al*. 2016; Bolnick *et al*. 2014; Maurice *et al*. 2015; Park & Im 2020), even in wild species with pronounced sexual dimorphism (e.g., gorillas [Pafčo *et al*. 2019] and baboons [Tung *et al.* 2015]). However, differences in microbiome composition between sexes remain understudied, particularly in wildlife.

Due to their history of a population bottleneck, northern elephant seals (*Mirounga angustirostris*; NES) have very low genetic variation and high levels of inbreeding (Hoelzel *et al.* 2002; Weber *et al.* 2000), which should result in lower levels of genetically controlled phenotypic variation compared to outbred populations (Fowler & Whitlock 1999). Therefore, NES are one of the few natural systems in which non-genetic intrinsic and extrinsic factors, such as sex, can be assessed on a background of minimal genetic variability. The extreme

sexual dimorphism of NES renders them particularly suitable for considering the role of sex in shaping microbial communities (Beltran *et. al* 2022). At birth, female and male NES are equivalent in size but extreme sexual size dimorphism develops around 4-5 years of age during puberty, due to exponential growth rates in males (Le Boeuf *et. al* 1994). As adults, males weigh up to ten times more than females (Deutsch *et al.* 1994; Stewart 1997) and have sex-specific behavioral, dietary, ecological, and physiological traits (Kienle *et al.* 2022; Le Boeuf *et. al* 2000; Reiter *et. al* 1981; Stewart 1997). These dramatic sex-specific differences are likely the result of divergent sex-specific social and energetic needs for reproductive success, as predicted by evolutionary theory (Slatkin 1984; Williams & Carroll 2009).

Despite strong sexual dimorphism in NES, little is known about the molecular mechanisms underlying this dimorphism. Sex-linked genes are predominantly responsible for major sex-specific differences, mediated through the expression or repression of genes on the X- or Y-chromosomes (Deegan & Engel 2019; Sekido & Lovell-Badge 2009), although autosomal genes, epigenetics, and gene expression patterns also likely play a role. For example, pre-pubescent physiological dimorphism has been measured in yearling NES (Jelincic *et al.* 2017; Kelso *et al.* 2012), which suggests that molecular dimorphism connected to differences in hormone expression or resource allocation may begin at early ages. Given that diet, ecological niche, hormones, and physiology are known to influence the gut microbiome, it is unsurprising that a few studies have already detected sex as a significant factor in microbiome composition in NES (Stoffel *et. al* 2020) and its closely related species, the southern elephant seal (Kim *et al.* 2020). Despite previous evidence that sex had no significant impact on Pacific harbor seals of unknown ages (Pacheco-Sandoval *et. al* 2019), a recent study in newborn harbor seals suggests otherwise (Switzer *et. al* 2023).

In this study, we used a metabarcoding approach to characterize the gut microbiome of NES pups, during a period of transition from maternal dependence to independence, at multiple time points in a rehabilitation facility in California, USA. We investigated associations between microbial composition and sex while controlling for age, diet, health status, and environment. We also used genetic relatedness derived from genome-wide SNP genotypes to test for an effect of host genetic background on microbial composition. Given the extreme sexual dimorphism of NES, we hypothesized that sex would be a major factor in shaping microbiome composition. Due to its history of a population bottleneck, NES have minimal genetic variation and thus we further hypothesized that host genetics would have minimal impact on microbiome composition.

Materials and Methods

Sample and data collection

We collected data, rectal swabs, and blood samples from stranded NES pups admitted to a rehabilitation facility (The Marine Mammal Center, TMMC; Sausalito, California, USA) in March 2021 (Fig. 1A, 1B). We collected rectal swabs from each animal during admission evaluation (typically within 3 days of entering care) and opportunistically while in care. We reviewed animal history and health data including rescue date and location, reason for rescue and admission to rehabilitation, sex, medical diagnoses and treatments in rehabilitation, and outcome (released, died, or euthanized) (Table S1).

We housed pups in groups of 3-8 conspecifics in concrete pens with a closed system pool. Our water was maintained at salinity of 24-30 parts per thousand with a continuous turnover rate of approximately 30 minutes and disinfected by ozone filtration. We fed pups a slurry of herring (*Clupea* spp.) with salmon oil and water by orogastric tube three times daily. Concurrently, pups

were introduced to frozen/thawed whole herring and tube feeding was discontinued when pups were reliably eating whole fish. We gave pups vitamin B complex via intramuscular injection daily for three doses upon admission to care and an oral multi-vitamin supplement (Pinnivite, Mazuri, Richmond, IN, USA) daily while in care.

Microbial DNA extraction

We randomly sorted rectal samples across two 96-well plates to minimize batch effects between plates. We used a modified Qiagen DNeasy PowerSoil Kit protocol. Briefly, we first transferred swab tips to their predetermined location in a 96-well PowerBead Plate. Across both plates, we reserved seven wells for negative controls and five wells for positive controls (empty well and a mock microbial community extraction standard, respectively) to assess potential sources of contamination and ensure successful amplification of bacteria. We used ZymoBIOMICS Microbial Community Standard D6300 as our positive control when extracting DNA. We added 750µL of PowerBead Solution to each sample and control, and then placed the plate on a Qiagen TissueLyser II for 12 minutes at 20 Hz/seconds, followed by the addition of 60µL of Solution C1. We then incubated each plate for 10 minutes at 65°C. We repeated the TissueLyser step for 12 minutes for 20 Hz/sec. We then followed the standard manufacturer protocol, with the additional step of heating elution buffer C6 to 70°C before use. We used the Quant-It kit (Qiagen) to determine DNA concentrations and standardized all samples to 2.5ng/µL.

Following the protocol of DeCandia *et al*. (2019, 2020, 2021) and Lu *et. al* (2023), we amplified the 16S rRNA V4 region using polymerase chain reaction (PCR) in a 13.2µL total reaction volume composed of: 5µL of 2x MyTaq HS Red Mix, 3.2µL of the forward and reverse primer mix (1.25µM; Caporaso *et al.* 2011), and 1.8µL of template DNA (4.5ng of DNA). We

used distinct combinations of uniquely barcoded forward (n=8) and reverse (n=12) primers (Caporaso *et al.* 2011). An additional positive control, ZymoBIOMICS Microbial Community DNA Standard D6305, was added for PCR. The PCR cycling conditions were: initial denaturation of 94°C for 3 min; 30 touchdown cycles of 94°C for 45 s, 80°C–50°C for 60 s, 72°C for 90 s with 1°C decrease each cycle; 12 cycles of 94°C for 45 s, 50°C for 60 s, 72°C for 90 s; and a final extension of 72°C for 10 minutes. We checked a random subset of eight samples on a 2% agarose gel via electrophoresis to confirm amplification (300-400nt) of the target 16S rRNA V4 region. We pooled equal nanograms of DNA from 96 samples (plate 1) and 69 samples (plate 2) and selected for 300-400nt sized fragments using Agencourt AMPure XP magnetic beads. We then submitted each plate to Princeton University's Lewis-Sigler Institute for Integrated Genomics for Illumina paired-end multiplexed sequencing on the Illumina MiSeq (2x150nt; configuration Miseq v2; Micro 300nt kit). A summary of the high throughput sequence data was generated, including the sequences obtained per sample and the distribution of sequence qualities at each position in our sequence data.

16S rRNA sequencing bioinformatic processing

We sequenced 188 rectal swab samples from 89 unique NES pups and seven negative and six positive controls (Yu *et. al* 2023). We obtained a total of 7,097,399 sequences with lengths ranging from 150-152 nucleotides and %GC content of 49. All sequences had a base call accuracy, a measurement that captures the accuracy of the Illumina sequencing platform, of at least 99%.

We first demultiplexed the raw sequence data and allowed for one nucleotide mismatch using a paired-end, dual barcode splitter in Galaxy (Afghan *et al.* 2018). We then imported demultiplexed reads into *QIIME2* v2022.4 for downstream processing and analysis (Afghan *et al.*

2018; Bolyen *et al.* 2019). We used the *dada2 denoise-paired* plugin to filter and trim low-quality sequences. Using *--p-trim-left-f* and *--p-trim-left-r*, we trimmed 13 bases at the beginning of each read. We then used *--p-trunc-len-f* and *--p-trunc-len-r* to truncate sequences at the 150th base. The paired reads were then merged. We proceeded to organize our sequences into a feature table, where a feature is defined as a unique rRNA sequence (also known as an amplicon sequence variant, or ASV). ASVs were chosen over the more traditional operational taxonomic units (OTUs) since ASVs have a higher level of resolution with similar levels of sensitivity and specificity (Callahan *et. al* 2017). Across 192 samples, we retained 4,845,464 sequences and 1,187 unique ASVs with a median of 20,837 features per individual.

Analysis of our positive and negative controls revealed no evidence of plate-wide contamination. We used the *feature-table filter-samples* function to remove duplicates of the same sample, controls, and samples where *p-sampling-depth* was less than 2,000. A sampling depth of 2,000 was selected to retain the samples with biologically meaningful features. We then used the feature-table *filter-features* function to retain features that were found in at least two samples.

Inclusion criteria

At this point, we set criteria to exclude certain pups before conducting further analyses. To control for age, we only considered pups admitted to rehabilitation in the month of March. Because the peak number of NES births occurs in January each year (Condit *et. al* 2022), we assumed all pups in the study were approximately 3 months of age at admission. We then established the following additional inclusion criteria: initial sample collected 10 days or fewer after admission to rehabilitation, no major medical abnormalities (e.g., lungworm infection), and no medication

administration beyond vitamin supplementation (such as anthelmintics and antibiotics). Our final dataset included 54 unique northern elephant seals.

Alpha and beta diversity metrics

To conduct phylogenetic diversity metrics on our full sample dataset, we utilized the QIIME2 *align-to-tree-mafft-fasttree* pipeline. We conducted multiple sequence alignment using the *mafft* program followed by filtering highly variable nucleotide positions (Katoh & Standley 2013). Using this filtered sequence alignment, we implemented the FastTree program to generate a rooted phylogenetic tree with a midpoint from the longest tip-to-tip distance (Price *et al*. 2009). We fed the rooted phylogenetic tree into the *core-metrics-phylogenetic* function to calculate alpha and beta diversity metrics at a specified sampling depth (4700), which retained the maximum number of samples.

With our full sample dataset, we calculated three different alpha (within sample) diversity metrics: 1) Observed features is a measure of community richness or number of different ASVs in the community; 2) Pielou's evenness is a measure of community equitability (relative abundances) of species in a community; and 3) Shannon's diversity index considers both species richness and evenness. We used the Kruskal-Wallis test to determine significant differences between groups of samples in seven variables of interest: city of origin, county of origin, deceased status, biological sex, experienced trauma, and sequencing plate.

We then calculated three different beta diversity metrics: 1) Bray-Curtis dissimilarity (quantitative measure for abundance); 2) Unweighted Unifrac (qualitative measure for ASV presence); and 3) Weighted Unifrac (quantitative measure that considers abundance and presence of ASVs). Because different quantitative and qualitative beta measurements on the same dataset

can lead to different results and interpretations, it is best to use a combination of metrics (Lozupone *et al.* 2007). Bray-Curtis dissimilarity measures compositional dissimilarity (i.e., differences in abundance). Unlike both Unifrac measurements, this metric does not make assumptions about phylogenetic relationships. The key difference between the two Unifrac measurements is that Unweighted Unifrac counts each branch length unique to either community, while Weighted Unifrac weights branch length according to relative abundances. As a result, Unweighted Unifrac is effective at detecting changes in the abundance of rare lineages and Weighted Unifrac is more sensitive to common feature changes. We evaluated differences in beta diversity metrics using ADONIS, a method for non-parametric multivariate analysis of variance, as implemented through the QIIME2 plugin. The EMPeror plugin was used to visualize principal coordinate analyses (PCoA) (Anderson 2001; Vázquez-Baeza *et al*. 2013).

Assessing taxonomic composition

We first summed the frequencies of features across groups of male and female NES individuals. We trained a Naïve Bayes classifier using reference sequences from Greengenes 13_8 99% OTU database and then used the *classify-sklearn* function to assign each feature to known taxonomy (Bokulich *et al.* 2018; DeSantis *et al*. 2006). We assessed the taxonomic composition of males and females at the phylum, class, order, family, genus, and species levels.

Differential Abundance Testing

A drawback of taxonomic composition analysis is the compositionality of microbiome datasets. Because taxonomic composition analysis measures taxa as relative abundance estimates, a change in the absolute abundance of a single taxon alters its relative abundance as well as the relative

abundance of all other taxa (Lin & Peddada 2020). A statistical challenge that arises is the identifying which taxa drive significant differences in abundance between samples or conditions. To overcome this challenge, we used analysis of composition of microbes (ANCOM), which detects significant relative abundance changes by calculating the log-ratio between pairwise combinations of taxa in both groups and counting the number of times the null hypothesis (i.e. the average abundance of a taxa is the same in both groups) is violated (Mandal *et al.* 2015). ANCOM assumes that less than 25% of taxa are changing between groups. We applied this method to detect differential abundances between male and female NES on a phylum, class, order, family, genus, and species level.

Longitudinal sampling analyses

To test for differences across time points, we compared samples from a subset of 24 seals that had one sample collected on intake and a second collected approximately one month after admission (Table S2). Following the methods above, we calculated Shannon's diversity index, Observed features, and Pielou's evenness for: 1) all intake samples compared to all second exam samples; 2) male intake samples compared to male second exam samples; 3) female intake samples compared to female second exam samples; and 4) male samples compared to female samples within each time point. We applied a two-tailed paired t-test to assess significant differences in all three alpha diversity metrics between individuals across time points. We applied a two-tailed Welch's t-test to assess significant differences in all three alpha diversity metrics between males and females within each time point. We then calculated Bray-Curtis dissimilarity, Unweighted Unifrac, and Weighted Unifrac for the first time point and the second time point combined, and tested for the influence of plate, sex, and and county of stranding at each time point separately. We

applied ANCOM to detect differential abundances: 1) between time points on a phylum and feature level and 2) between sexes within time points 1 and 2 on a phylum and feature level. Phylum level analysis was conducted to gain insight into the core microbiome. Feature level analysis was conducted to gain insight into the finest level of taxonomic change.

Restriction-site associated DNA sequencing

We randomly selected a cohort of 42 NES pups out of our original 89 for restriction-site associated DNA sequencing (vonHoldt *et. al* 2023). Of these 42 pups, 26 did not meet our inclusion criteria and 16 pups met our inclusion criteria (Table S3). Due to limited blood samples, we performed population genetic analyses on all 42 sequenced pups, including the 26 individuals that were not part of the microbiome analyses.

We extracted genomic DNA from whole blood samples stored in EDTA for restriction-site associated DNA sequencing (RADseq-capture; Ali *et al*. 2016), which were then digested with the SbfI restriction enzyme. We then ligated a unique 8-bp barcoded biotinylated adapter to the fragmented DNA that allowed us to pool equal amounts of up to 48 samples. We sheared the pools in a Covaris LE220 to 400bp fragments, which we then enriched for fragments that contained the adapter using a Dynabeads M-280 streptavidin binding assay. Once enriched, we prepared the pools for Illumina NovaSeq paired-end (2x150nt) sequencing at Princeton University's Lewis Sigler Genomics Institute core facility using the NEBnext Ultra II DNA Library Prep Kit and used Agencourt AMPure XP magnetic beads for any library purification or size selection step.

Bioinformatic processing and SNP analysis

We retained raw sequences where the read (and its pair) contained the expected unique barcode and the remnant SbfI recognition motif using the process_radtags module in *STACKS* v2 (Catchen *et al*. 2013; Rochette *et al*. 2019) and allowed up to a 2bp mismatch and had a quality score ≥10. We next used the clone_filter module to remove PCR duplicates prior to mapping to the NES reference genome (NCBI assembly: ASM2128878v3). We excluded mapped reads with MAPQ<20 from all further processing, and we converted the SAM files to BAM format in *Samtools* v0.1.18 (Li *et al.* 2009).

We implemented the *gstacks* and *populations* modules in *STACKS* v2 following the recommended pipeline for data mapped to a reference genome and constructing a catalog with all polymorphic sites. We further increased the stringency of SNP annotation by using the marukilow model flags --vt-alpha and --gt-alpha with p=0.01. We retained all SNPs discovered per locus and used *VCFtools* v0.1.17 (Danecek *et al.* 2011) for filtering out singleton and private doubleton alleles, to remove loci with more than 90% missing data across all samples, and to remove individuals with more than 60% missing data. We further filtered to exclude loci with a minor allele frequency (MAF<0.03) while allowing up to 20% missingness rate per locus (--geno 0.2) in *PLINK* v1.90b3i (Chang *et al.* 2015). We further filtered for linkage disequilibrium (LD) and Hardy-Weinberg Equilibrium (HWE) to obtain a set of SNPs that were considered statistically unlinked and neutral. We used PLINK's genotype correlation function to remove sites within a 50-SNP window whose genotypes were highly correlated (r^2>0.5; --indep-pairwise 50 5 0.5) and excluded sites that significantly deviated from HWE (--hwe 0.001). This was the SNP set used for all downstream genetic analyses.

We conducted an unsupervised, non-model based principal component analysis (PCA) with the program FlashPCA v2.1 (Abraham *et al.* 2017) to assess the impact of geography and life

history on genetic similarity. We then completed an unsupervised, maximum likelihood cluster analysis in the program ADMIXTURE (Alexander *et al.* 2009) to assess the likelihood at each genetic partition from K=2-10.

To test for an association between host genetic distance and microbiome dissimilarity using a Mantel test following the pipeline of DeCandia *et al*. (2021). Briefly, we calculated euclidean distance between each pair of samples using our pruned set of SNPs using the *dist* function in the *R* package *adegenet* (Jombart 2008; Jombart & Ahmed 2011). We then used the *R* package *vegan* (Oksanen *et al.* 2019) to implement a Mantel test on the matrices of genetic distance and Bray-Curtis dissimilarity of gut microbiomes. We assessed the correlation with Spearman's rank correlation coefficient (ρ) and a statistical significance threshold of $p < 0.05$.

Results

Samples were collected from 89 unique individuals, of which 54 (female n=28, male n=26) met our inclusion criteria. Our full sample dataset included 54 unique NES pups representing a total of 1,512,187 microbiota features (342 unique ASVs) with a median of 24,694.5 features per individual. All 54 individuals were in poor body condition indicating malnutrition at admission and three had minor traumatic injuries (e.g., laceration or small abscess). The majority were ultimately released (n=49), while four individuals were euthanized and one died naturally. The symptoms leading attending veterinarians to euthanize NES included chronic vomiting, weight loss, and progressive electrolyte abnormalities. Although these symptoms may interfere with the gastrointestinal tract and thus affect the gut microbiome, we decided to include these individuals for two reasons: 1) samples with biological noise could be considered representative of rehabilitated NES and 2) these individuals form a small proportion of our total sample set. Of the

54 individuals, 24 had paired samples collected at admission and approximately one month later; we used these individuals for a longitudinal analysis.

Microbial Diversity and Composition

Significant differences ($p<0.05$) for sequencing plates were observed across all three alpha diversity metrics (Table 1). There were no significant differences observed across any alpha diversity metrics for all other factors, including sex and county (Table 1).

Significant factors ($p<0.05$) driving differences in beta diversity were identified using univariate analysis (ADONIS, Table S4) and used in a multivariate analysis (PERMANOVA, Table 2). All three multivariate beta diversity analyses indicated plate and sex as significant factors. Our multivariate Bray-Curtis and Unweighted Unifrac analysis also yielded significant results for the county of stranding. When multivariate Bray-Curtis values are plotted on the same PCoA, PC2 loosely correlates with plate (Fig. 2A), clearly correlates with sex (Fig. 2B), and does not correlate with county (Fig. 2C).

A Mantel test showed that genetic distance among 16 individuals was not significantly correlated with microbiome dissimilarity ($\rho=0.119$, $p=0.262$) (Fig. S1). Despite the sequencing plate being a significant contributor to beta diversity variation, it does not mask significance results of other variables in our multivariate analysis.

Taxonomic Composition and Differential Abundance of Microbes

On a phylum level, taxonomic compositions were similar between females and males but with different total number of features and abundance levels (Fig. S2A). Males (798,662 features and eight phyla) had a greater number of total features and one less phylum compared to females

345 (713,525 features and nine phyla). The top four dominant phyla were the same between males and
346 females but were found with different relative abundances. These four phyla included
347 Proteobacteria (44.62% male, 55.99% female), Firmicutes (26.66%, 28.25%), Bacteroidetes
348 (17.17%, 10.35%) and Fusobacteria (7.70%, 2.12%). All other phyla encompassed 0.14% in male
349 individuals and 0.15% in female individuals.

350 ANCOM results revealed differential abundances for unidentifiable taxa on a phylum, and
351 species level and identifiable taxa on a class, order, and genus level (Table 3). On a class level,
352 there were significantly more *Coriobacteriia* in males than females. On an order level, there were
353 significantly more *Bacillales* in females than males. On a genus level, there were significantly
354 more *GW-34* and *Tissierella* (*Soehngenia*) in females than males.

355

356 *Longitudinal patterns during rehabilitation and detection of host genetic effects*

357 Only 24 individuals (males=17, females=7) had paired samples taken at intake and roughly one
358 month later. We found a significant increase in Shannon's diversity index in the samples collected
359 during the second exam compared to those collected at intake (Paired t-test, ρ=0.03, df=23) (Fig.
360 3A), which increased by an average of 0.572 between time points. We did not find a significant
361 increase in Pielou's evenness and Observed features across time points (Paired-t test, Table S5).

362 When sexes were analyzed separately across time points, which decreased total sample
363 size, males (Paired t-test, ρ=0.12, df=16) and females (Paired t-test, ρ=0.61, df=7) did not show a
364 significant increase in Shannon's diversity (Fig. 3A). We also did not find a significant increase
365 with Pielou's evenness and Observed features (Paired t-test, Table S5). Within each time point,
366 Shannon's diversity index did not differ between males and females (Fig. 3B). We also found no
367 difference between sexes for Pielou's evenness and Observed features (Welch's test, Table S5).

When time points were analyzed together, we first identified significant factors ($p<0.05$) that drive differences in beta diversity using univariate analysis (ADONIS, Table S4) and used these factors in a multivariate analysis (PERMANOVA, Table 2). All multivariate beta diversity metrics yielded significant results for time point, plate, and sex. Multivariate Unweighted Unifrac analysis indicated significant results for the county of stranding as well. When first and second time point samples are plotted on the same PCoA using Bray-Curtis values, PC1 clearly correlates with time point (Fig. 4A). In addition, the PCoA shows greater variation among the intake samples, while the second exam samples are more tightly clustered.

When each time point was analyzed separately, significant factors ($p<0.05$) driving differences in beta diversity were first identified using univariate analysis (Table S4). Based on our univariate results, multivariate Weighted Unifrac was not conducted for either time point and multivariate Unweighted Unifrac was not conducted for time point 1. Multivariate beta diversity metrics conducted did not find county of origin to be a significant factor driving differences within both time points. Multivariate Bray-Curtis analysis showed that both plate and sex was a significant factor in gut microbial composition in the intake samples and continued to be a significant factor that explained a greater amount of variation in the samples taken after a month of rehabilitation at TMMC. Multivariate Unweighted Unifrac showed that plate and sex were only significant at the second time point.

We calculated the genetic distance of seven male pups from the longitudinal sample set for which we generated RADseq data. A Mantel test showed that genetic distance did not correlate with microbiome dissimilarity calculated from swabs collected at intake (ρ=-0.014, p=0.529) (Fig. 4B). However, after a month of rehabilitation, genetic distance was significantly and positively correlated with microbiome dissimilarity (ρ=0.618, p=0.0375) (Fig. 4C).

Our taxonomic analysis revealed that at time point 1 and time point 2, males and females exhibited similar top four phyla (Fig. S2B). Within time point 1, there were significantly greater *Firmicutes* in females and greater *Actinobacteria* in males (Table S6). Within time point 2, there were significantly more *Tenericutes* and *Actinobacteria* in females and more *Bacteroidetes*, *Firmicutes*, and *Fusobacteria* in males (Table S6). Across time points, there was a decrease in *GN02, Tenericutes, Deferribacteres, Proteobacteria, and Firmicutes* and increase in *Bacteroidetes*, *Actinobacteria,* and *Fusobacteria* (Table S6). Our differential abundance analysis showed significant changes in five species across time points (Table 3). There was significant decrease in *Psychrobacter sanguinis* and a significant increase in *Winkia neuii* or *uncultured Actinomyces*, *Photobacterium damselae*, *Actinobacillus delphinicola*, and *Paeniclostridium sordellii*. Within both time point 1 and time point 2, there were significantly more unidentifiable features in females than males.

Lack of genetic signature of geographic structure in stranded seals

We successfully constructed a SNP catalog of 149,225 loci discovered across 17 genome scaffolds of 42 NES pups with an average of 11.8 (s.d.=3.9) depth of sequence coverage. After initial filtering, we excluded two seals with high levels of missingness (Table S3). We retained 7,166 variants meeting all filtering thresholds, with a subset of 3,298 SNP loci identified as neutral and unlinked. We found no genetic clustering as a function of California county of stranding or individual sex (Fig. 5A, B). We found further support that the stranding location and sex were not crucial in driving genetic patterns with the model-based clustering (Fig. S3), whereas larger species-level patterns may be revealed given a much larger geographic survey. California counties themselves also lacked any distinct and private signature of genetic variation (Fig. 5C).

Discussion

The gut microbiome is a complex ecosystem that can be affected by a wide variety of intrinsic and extrinsic factors, including host diet, genetics, sex, and environment. Due to a population bottleneck from overhunting, NES have very low genetic variation (Abadía-Cardoso *et. al* 2017; Hoelzel *et. al* 2002), representing one of the few natural systems in which non-genetic intrinsic and extrinsic factors can be assessed on a background of minimal genetic variability. Furthermore, NES rehabilitated at TMMC presents a unique opportunity to study these factors in a controlled manner. NES are highly sexually dimorphic (Kienle *et al.* 2022) but the uniform diet and shared environment at TMMC control for behavioral and ecological sex differences that might exist in the wild. Here, we used a metabarcoding approach to investigate the gut microbiome of 54 NES at a single time point shortly after rescue and found that county of origin and biological sex were significant factors in shaping microbial composition. We then took a cohort of 24 seals with samples at two time points, shortly after rescue and then after a month rehabilitating at a rescue facility, and found an overall increase in alpha diversity (Shannon's diversity index) and decrease in beta diversity (Bray-Curtis dissimilarity).

Host Genetics

Our RADseq analysis showed no significant population structure among our samples, despite animals originating from a several hundred-kilometer span of coastal California. This pattern suggests that little population genetic structure exists along the NES rookeries of California, consistent with high dispersal estimates in this species (Condit *et al*. 2022) and a prior study using microsatellites that found extremely low F_{ST} estimates between rookeries in northern and southern California (Abadía-Cardoso *et al.* 2017). The lack of genetic structure in our dataset therefore

shows that the influence of county of origin on microbiome composition derives from environmental and not genetic factors. However, we also found that when environment and diet were shared during a month of rehabilitation at TMMC, genetic distance between a pair of individuals was strongly correlated with the Bray-Curtis dissimilarity of their gut microbiomes. This correlation suggests that in northern elephant seal pups, as in other species, genotype influences gut microbiome composition (Bonder *et al.* 2016; Suzuki *et al.* 2019).

Environment

In our full sample dataset, we found that the county of stranding explained the greatest variation in beta diversity, as seen through our Bray-Curtis dissimilarity and Unweighted Unifrac analysis. Given these results, there were likely significant compositional differences in phylogenetically similar, low abundance features but not in phylogenetically dissimilar, high abundance features. The pups included in these analyses were found stranded in March, at an estimated 3 months of age (Condit *et al.* 2022). Given that the pups were admitted primarily for malnutrition, they were likely separated from their mothers before gaining sufficient weight to survive the extended post-weaning fast. Significant differences relating to the county of stranding therefore likely reflect how microbial communities on different beaches or in different marine habitats shaped the microbial colonization of these pups' gut microbiomes. Previous research on the microbiomes of weaned NES only examined animals on a single beach in Baja California (Stoffel *et al.* 2020) and could not explore how the local environment affected microbiome development. Our results suggest that the local environment is an important factor in the early microbiome of NES, with differential exposure possibly originating from differences in local environmental microbial communities and not from local adaptations of the host (see below).

In contrast to our cross-sectional results, the county of stranding was not associated with significant differences in longitudinal microbiome diversity. We were consequently not able to test how TMMC environment affected the county of stranding's influence on the gut microbiome over time. Given the decrease in sample size from our cross-sectional to longitudinal study (n=54 to n=24), we suspect that our discrepancy in results is an issue of statistical power. Future work with larger sample sizes that track individuals over time can establish how founding microbiomes are influenced across multiple environments throughout an individual's lifetime.

Our longitudinal analysis found an overall increase in Shannon's diversity, a metric that accounts for both microbial richness and evenness. Given that higher microbial diversity in the gut microbiome is a well-established signal for healthy states in humans (Hills *et. al* 2019; Mosca *et. al* 2016), we suspect that the lower alpha diversity evident during intake can be explained by the pups' malnourished state from stranding. We hypothesize that the overall increase in alpha diversity could be due to dietary transitions – post-wean fasting to gruel with mashed fish to whole fish – as part of TMMC's standard rehabilitative care. Studies in humans have provided evidence that diet diversity is positively correlated with microbial diversity (C. Xiao *et. al* 2022; Heiman & Greenman 2017). A study investigating wild pup gut microbiomes during the transition from maternal dependency to independent foraging can elucidate the effect of a natural diet on microbial diversity. Understanding these natural alpha diversity trends, in turn, can illuminate if rehabilitated pup's overall increase in alpha diversity over time can be attributed to TMMC diet.

While our longitudinal analysis indicated the continued development of a typical mammalian core microbiome on a phylum level, we found significant differences in composition on a finer species level. We identified a decrease in *Psychrobacter sanguini*, which is commonly found in marine environments (Bowman *et. al* 1997; Maruyama *et. al* 2000) but was also noted to

be the cause of post neurosurgical meningitis in a human case study (Le Guern *et. al* 2014). We also identified significant increases of *Winkia neuii*, *Photobacterium damsalae*, and *Paeniclostridium sordellii* from time point 1 to time point 2. *Winkia neuii* are commensal bacteria commonly found in the oral cavity, gastrointestinal tract, and female genital tract (Ávila *et. al* 2015; Petrova *et. al* 2015). These bacteria are also noted to be rare human pathogens capable of causing infections when tissue barriers are disrupted (Ávila *et. al* 2015; Gomez-Garces *et. al* 2010). *Photobacterium damsalae* is a well-established deadly pathogen in a variety of marine animals – fish, crustaceans, molluscs, and cetaceans – and humans (Morick *et. al* 2023; Rivas *et. al* 2013). *Paeniclostridium sordellii* is a bacterial pathogen that causes human uterine infections (Vidor *et. al* 2019) and has been associated with intestinal illnesses in horses (Nyaoke *et. al* 2020). Our metadata (Table S1) indicate that 23 of the 24 NES pups included in our longitudinal analysis were ultimately deemed by TMMC veterinary staff, in consultation with NOAA fisheries, to be healthy enough to be released back into their natural habitats following achievement of good body condition and demonstrated ability to forage. It is possible that NES can possess differentially abundant potential pathogens in their gut microbiomes without these pathogens causing disease states. Understanding of the mechanisms of potentially pathogenic bacteria in diverse mammalian hosts is an important area for future research.

Sexual dimorphism

Previous research (Stoffel *et al.* 2020) found evidence of the sexual dimorphism of the microbiome during a unique time period in which NES remained in their natal colonies and fasted, which minimized environmental and nutritional variation. Our study illustrates that even after this time period, during a transition period from maternal dependence to independence, sexual dimorphism of the gut microbiome is significant. Existing studies in moderately sexually

dimorphic animals, namely humans and mice, have illustrated that environmental factors overshadow intrinsic factors such as sex (Valeri & Endres *et. al* 2021; Y.S. Kim *et. al* 2020). Here, in a controlled environment, we found evidence that the extreme sexual dimorphism of NES extends beyond behavior, body size, and physiology to include the gut microbiome. Our results also provide the first evidence of gut microbiome sexual dimorphism beyond eight weeks of age in NES.

Our longitudinal analysis supports that differences in gut microbiome across biological sex persist for at least 1 month in a shared environment at a rehabilitation hospital. Previous studies on wildlife living in captivity have illustrated a convergence of the gut microbiome (Y. Xiao *et. al* 2019; Zhou *et. al* 2022), likely due to dietary overlap. More recently, a study on rehabilitated harbor seals' gut microbiomes from the period shortly after maternal separation through weaning illustrated an overall increase in dissimilarity and strong resemblance to age-matched local wild harbour seal gut microbiomes (Switzer *et. al* 2023). In contrast, the pups in this study experienced a convergence of gut microbiome. Still, microbial variation was present and primarily explained by sex as well as host genetics (as outlined above). In the rehabilitation setting, NES are hosted in shared pens and fed the same diet, which greatly reduces environmental variability compared to the natural environment. This uniformity minimizes the impact of sex differences in behavior, foraging ability, or other traits on our observed gut microbiome dimorphism.

Our study identified microbiome compositional differences between NES sexes on various taxonomic scales. While the top four phyla in pups reflected the typical marine mammal core microbiota (Bik *et. al* 2016; Nelson *et. al* 2013) and showed no relative differences in abundance between males and females, we found significant differences in gut microbiome composition at finer taxonomic levels. For example, there was a greater abundance of *Coriobacteriia* in NES

males. The few studies on *Coriobacteriia* found that this class encompasses three families of bacteria with vastly different metabolic abilities when breaking down carbohydrates (Hoyles 2019). In contrast, there was a greater abundance of *Bacillales, GW-34,* and *Tissierella (Soehngenia)* in female seals. *Bacillales* are gram-positive bacteria speculated to produce a wide range of antimicrobial compounds of unknown functions (Zhao & Kuipers 2016). Antimicrobial compounds are generally associated with decreases in the age of mortality due to infection and could therefore increase the potential for lifetime reproductive success (Burney *et al*. 2019). While *Tissierella (Soehngenia)* are one of the dominant genera in healthy human esophagi (Gillespie *et. al* 2021), they are also speculated to be pathogenic due to their increased presence after acute myocardial infarctions in rats (Wu *et. al* 2017). *GW-34* was significantly increased in the gut microbiomes of old laying hens fed with supplements demonstrated to inhibit pathogenic bacteria (Li *et. al* 2022) and human breast cancer survivors when compared to healthy controls (Caleça *et. al* 2023). Given only the relative abundances of these taxa, it is difficult to predict whether the presence of these compounds ultimately benefit its host by boosting the innate immune response or harm its host by killing both beneficial and commensal microbes. As more insight is gained into bacterial functions as well as the NES gut microbiome at different ages, future research can reveal the functional significance of sex-specific microbial patterns.

Conclusion

Here, we studied the gut microbiome of pups in a species in which adults show extreme sexual dimorphism. Our study included a unique population of NES pups that were found stranded in multiple northern and central California counties and brought to a common environment for rehabilitation. We conducted a microbial diversity and compositional analysis on a dataset of 54

pups shortly after rescue followed by a longitudinal analysis of a sub cohort of 24 pups with samples taken shortly after rescue and after about a month acclimating to TMMC.

NES are a useful model natural system that allows us to assess the impact of sex and environmental factors on a background of minimal genetic structure. We were able to demonstrate that when this natural system is in a rehabilitative center that minimizes environmental factors, host genetics can be introduced as a significant factor that explains differences in microbial composition despite pups' incredibly low genetic variation. Furthermore, although pups of this age exhibit minimal phenotypic and anatomical dimorphism, biological sex was a consistent and significant factor that explains microbial variation. Our study ultimately offers insight into the nature of sexual dimorphism in NES by illuminating that sex driven differences in the gut microbiome precedes sex-driven divergence in morphology and behavior.

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Data Accessibility

16S sequencing data and RADseq data analyzed are publicly available through the NCBI Sequence Read Archive under BioProject PRJNA1007377 and BioProject PRJNA1007380, respectively.

Benefit-Sharing

Benefits from this research accrue from the sharing of our data in the NCBI databases above.

Author Contributions

ALG, BVH, EY, and SJG secured research funding. CF and EW cared for the patients, collected rectal samples, and provided metadata. ALD, BVH, EY, and SJG designed the study, carried out experiments, and performed statistical analyses and interpretations. BVH, EY, SJG wrote the manuscript. ALD, ALG, BVH, CLF, ERF, EY, SJG revised the manuscript.

Main Figures and Tables

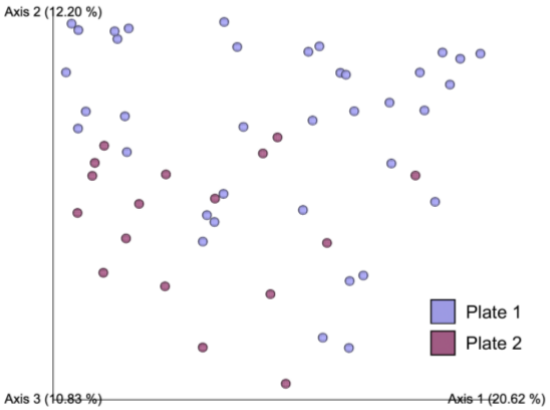
Figure 1. Map of California **(A)** where stranding locations of rescued northern elephant seal (NES) pups in six counties of central and northern California are shaded in gray. Counties from top to bottom: Marin, San Francisco, San Mateo, Santa Cruz, Monterey, and San Luis Obispo. Vapor (ES4686) **(B)** is a rescued, malnourished NES pup undergoing rehabilitation at The Marine Mammal Center. Photo is taken by Bill Hunnewell © The Marine Mammal Center (n.d.).

A.

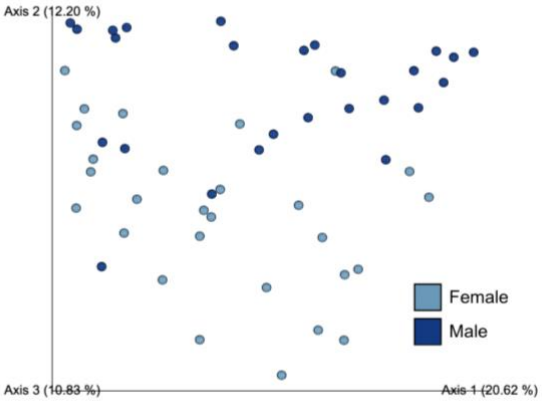
B.

Figure 2. PCoA constructed from full sample Bray-Curtis dissimilarity matrices. Plate **(A)**, sex **(B)**, and county of stranding **(C)** were investigated for their contributions to beta diversity variation along Axis 1 (20.62%) and Axis 2 (12.2%).

A.



B.



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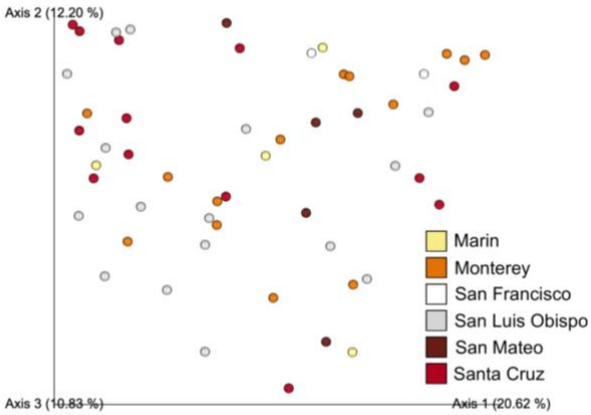
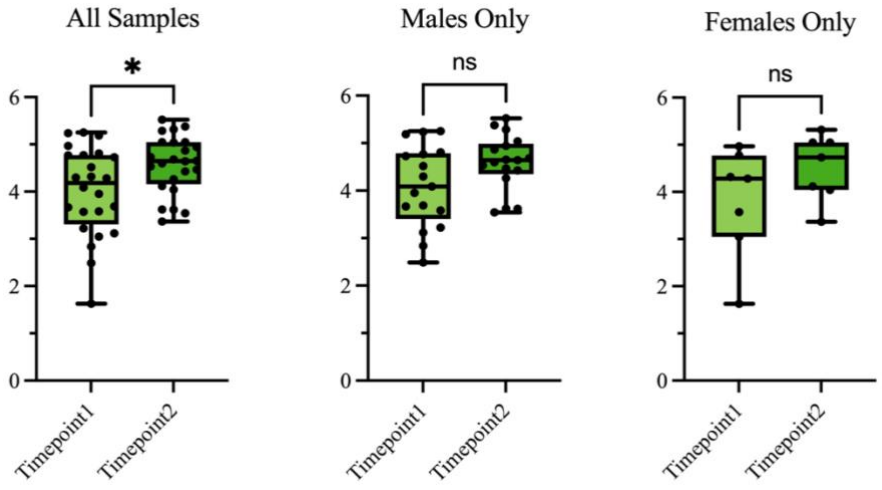


Figure 3. Box plot comparing longitudinal changes in Shannon's diversity metric in all samples, males only, and females only **(A)**. Box plot comparing sex differences within both time points, time point 1 only, and time point 2 only **(B)**. Significance is where $p < 0.05$.

A.



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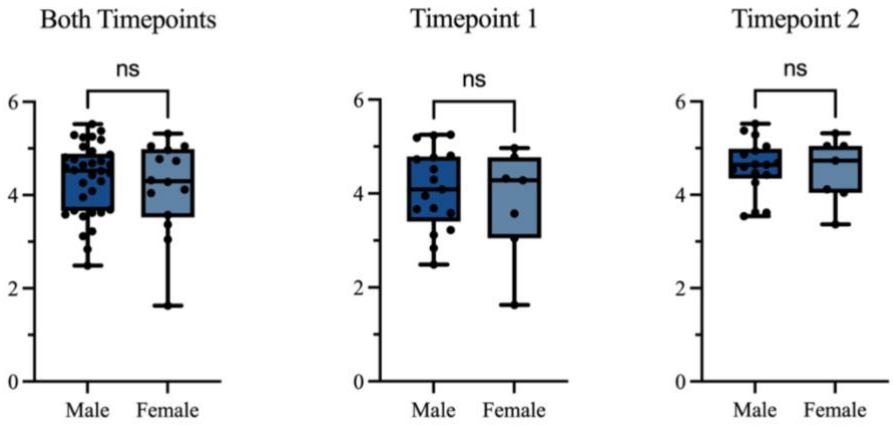
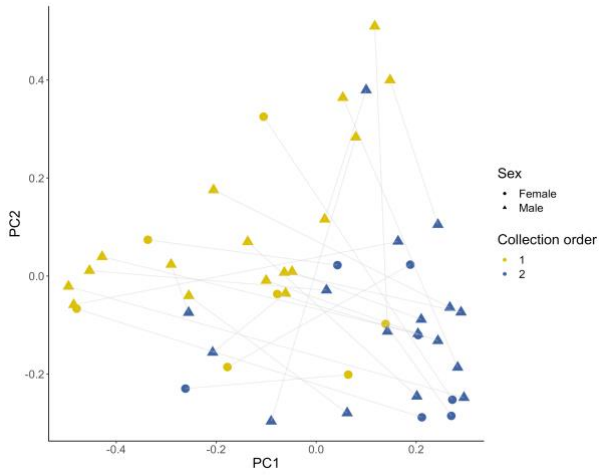
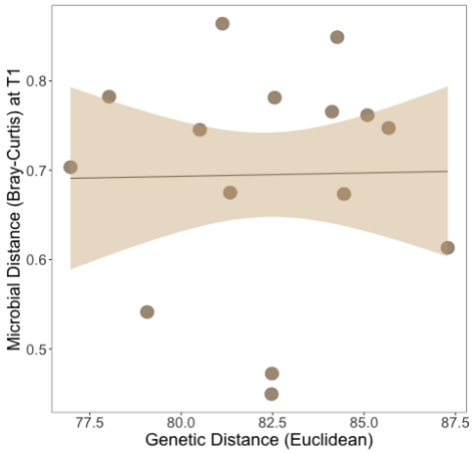


Figure 4. Bray-Curtis dissimilarity PCoA when time points are analyzed together **(A)**. Scatter plots of pairwise genetic distance and microbial dissimilarity (Bray-Curtis) for seven individuals with samples collected at intake **(B)** and after about a month at TMMC **(C)**. Mantel test showed no significant correlation at intake, but a significantly positive correlation after rehabilitation (p=0.0375).

A.



B.



C.

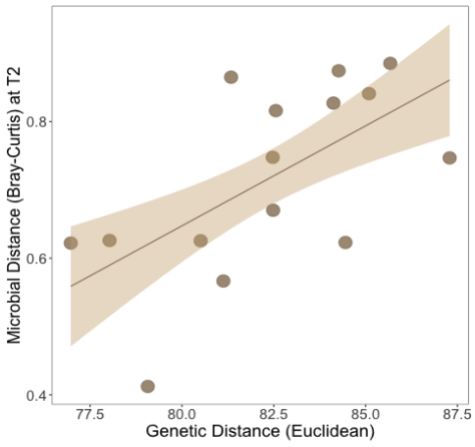


Figure 5. Non-model clustering of individuals reveals a lack of geographic influence **(A)** and sex **(B)** on genetic variation in northern elephant seals. Proportion of probability assignments per genetic partition (K) across the six California counties included in this study **(C).**

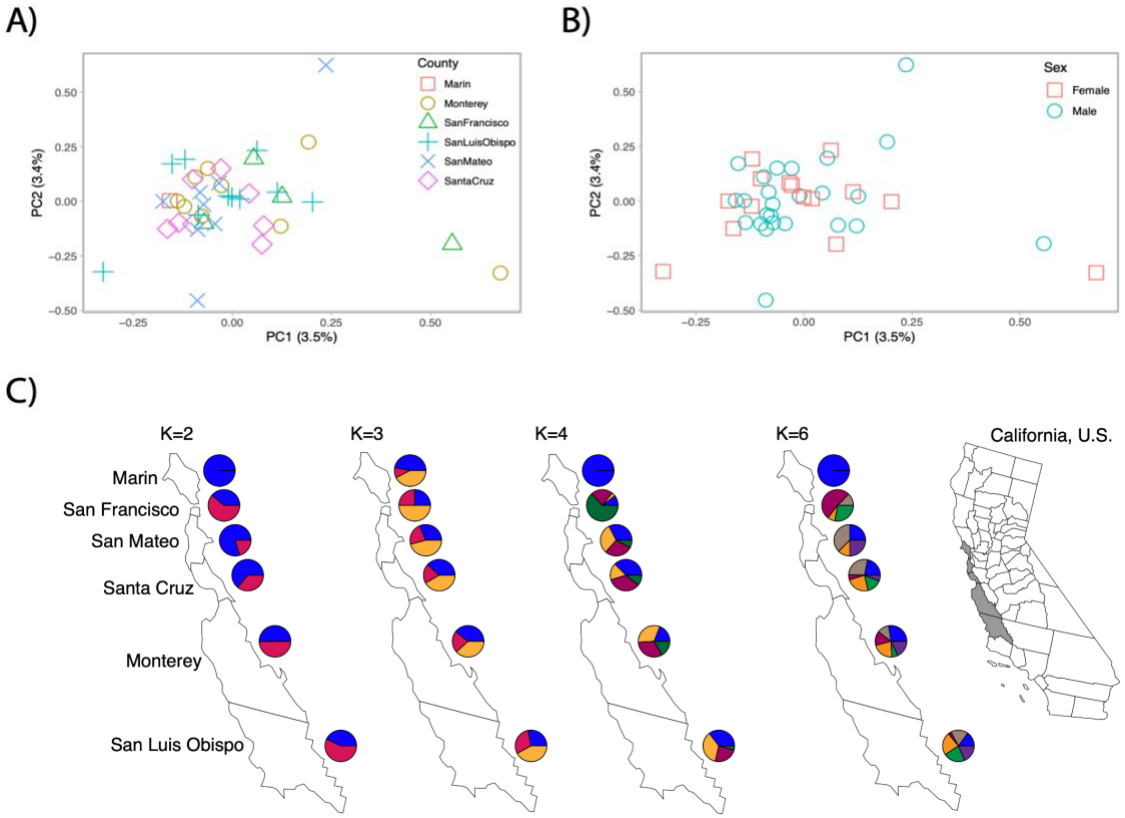


Table 1. Alpha diversity metrics, with bolded values indicative of statistical significance (p<0.05) as measured by their respective test.

Diversity Metric	Statistical Test	Test Statistic	California City of Rescue	California County of Rescue	Sequencing plate	Sex (male or female)	Status (released, euthanized, or resident)	Evidence of physical trauma
Observed Features	Kruskal-Wallis (all groups)	H	22.85	3.90	32.39	1.80	3.18	2.08
		p	0.30	0.56	**<0.001**	0.18	0.37	0.15
Pielou's Evenness Metric		H	16.30	6.43	5.73	0.84	5.29	0.08
		p	0.70	0.27	**1.67×10^{-3}**	0.36	0.15	0.78
Shannon's Diversity Index		H	20.27	7.76	7.96	1.15	5.60	0.93
		p	0.44	0.17	**4.78×10^{-3}**	0.28	0.13	0.34

Table 2. Degrees of freedom and correlation coefficient values are given (df, R^2) for multifactor PERMANOVA analyses of Unweighted Unifrac, Bray-Curtis dissimilarity, and Weighted Unifrac distance matrices. Bolded values indicate significance (p<0.05).

Analysis	Plate	County	Sex	Trauma	Time point	Residuals	Total
Full dataset							
Bray-Crutis	**1, 0.11**	**5, 0.13**	**1, 0.05**	-	-	52, 0.71	59, 1
Unweighted Unifrac	**1, 0.20**	**5, 0.10**	**1, 0.05**	1, 0.01	-	51, 0.64	59, 1
Weighted Unifrac	**1, 0.05**	-	**1, 0.07**	-	-	57, 0.88	59, 1
Time point 1							
Bray-Crutis	**1, 0.10**	-	**1, 0.08**	-	-	21, 0.73	23,1
Time point 2							
Bray-Crutis	**1, 0.15**	-	**1, 0.12**	-	-	21, 0.82	23,1
Unweighted Unifrac	**1, 0.27**	-	**1, 0.11**	-	-	21, 0.61	23,1
Combined time points							
Bray-Crutis	**1, 0.09**	-	**1, 0.08**	-	**1, 0.11**	44, 0.73	47, 1
Unweighted Unifrac	**1, 0.19**	**5, 0.12**	**1, 0.05**	-	**1, 0.07**	39, 0.57	47, 1
Weighted Unifrac	**1, 0.07**	-		-	**1, 0.07**	44, 0.80	47, 1

Table 3. Differential abundance testing by an analysis of composition of microbes. The *W* statistic measures the number of times the null hypothesis that average abundance of taxa between two groups (sexes or time points) are the same is rejected. The *clr* metric measures the effect each individual feature has with respect to the rest of the community within the samples. Positive *clr* values indicate changes in females or time point 2, while negative values represent males or time point 1.

Taxonomic Level	ID	clr	W
Between sexes			
Class	*Coriobacteriia*	-2.16	12
Order	*Bacillales*	3.04	26
Genus	*GW-34*	3.66	115
	Tissierella (Soehngenia)	3.49	112
Longitudinal analysis of time points			
Species	*Psychrobacter sanguinis*	-4.48	421
	Winkia neuii or uncultured Actinomyces	4.33	408
	Photobacterium damselae	4.24	400
	Actinobacillus delphinicola	4.09	398
	Paeniclostridium sordellii	4.01	393