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Skin bacterial metacommunities of San Francisco Bay Area salamanders are structured by host genus and habitat quality

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ABSTRACT

Host-associated microbial communities can influence physiological processes of macroorganisms, including contributing to infectious disease resistance. For instance, some bacteria that live on amphibian skin produce antifungal compounds that inhibit two lethal fungal pathogens, *Batrachochytrium dendrobatidis* (Bd) and *B. salamandrivorans* (Bsal). Therefore, differences in microbiome composition among host species or populations within a species can contribute to variation in susceptibility to Bd/Bsal. This study applies 16S rRNA sequencing to characterize the skin bacterial microbiomes of three widespread terrestrial salamander genera native to the western United States. Using a metacommunity structure analysis, we identified dispersal barriers for these influential bacteria between salamander families and localities. We also analyzed the effects of habitat characteristics such as percent natural cover and temperature seasonality on the microbiome. We found that certain environmental variables may influence the skin microbial communities of some salamander genera more strongly than others. Each salamander family had a somewhat distinct community of putative anti-Bd skin bacteria, suggesting that salamanders may select for a functional assembly of cutaneous symbionts that could differ in its ability to protect these amphibians from disease. Our observations raise the need to consider host identity and environmental heterogeneity during the selection of probiotics to treat wildlife diseases.

KEYWORDS

amphibian, chytridiomycosis, microbiome, metacommunity, salamander, 16S rRNA

INTRODUCTION

A fundamental concept in modern biology is that no organism lives in true isolation. Organisms are shaped by numerous biotic and abiotic interactions across many different scales (Bosch and McFall-Ngai 2011; McFall-Ngai et al. 2013). For example, diverse symbiotic microbes inhabit the bodies of multicellular organisms and play critical roles in metabolism, immune function, and hormone production (Costello et al. 2012; Muller et al. 2020). Microbial ecology processes (e.g. dispersal, colonization, and competition) directly influence host-associated microbial communities (i.e. the microbiome), which in turn impacts host fitness (Mihaljevic 2012).

Most microbiome studies use alpha and beta diversity metrics to understand differences in microbial composition and diversity among host individuals or taxa (Goodrich et al. 2014). However, these analyses are often not sufficient to pinpoint biotic and abiotic factors that affect the assembly of microbiomes (Greenspan et al. 2020). Therefore, interdisciplinary approaches that combine tools from community ecology and population genetics may provide increased resolution to explore processes controlling community assembly of host-associated microbiomes, and the metacommunity framework is one such approach. This framework can be used to describe patterns and mechanisms behind the distribution of organisms across patches of habitat (Leibold and Mikkelson 2002; Leibold and Miller 2004) but is most often used to characterize the community structure of macro-organisms. More recently, the metacommunity framework has been applied to understand the role of dispersal, colonization, and competition in shaping microbial community assembly across local (e.g. hosts) and regional (e.g. populations of hosts) scales (Mihaljevic 2012; Christian, Whitaker and Clay 2015; Burns et al. 2017). Applying a hierarchical approach that evaluates symbiont distributions at different ecological scales is critical to understand the extent to which host or environmental factors shape the assembly of host-associated microbiomes (Mihaljevic, Hoye and Johnson 2018).

The skin of amphibians is a particularly valuable environment with which to study bacterial metacommunities due to its importance for host respiration, osmoregulation, and immune function (Campbell et al. 2012). The bacteria that live on the amphibian skin are on the front line of defense against diseases such as *Batrachochytrium dendrobatidis* (Bd), a fungal pathogen that causes chytridiomycosis—one of the deadliest wildlife diseases ever described (Fisher and Garner 2020). Bd infects the amphibian skin and has a notably broad host range, making it a suspect in the declines of over 500 species from around the world (Scheele et al. 2019, 2020; Lambert et al. 2020). Bd's sister species, *B. salamandrivorans* (Bsal), is an emerging pathogen that was found to be associated with die-offs of European salamander populations after likely being introduced from Asia through the pet trade (Martel et al. 2013, 2014; Fitzpatrick et al. 2018). The high levels of susceptibility observed in European salamanders suggest that Bsal is highly virulent when introduced to naïve populations (Yap et al. 2015; Berger et al. 2016). The emergence of Bsal in Europe has raised concerns about its potential threat to salamander biodiversity on other continents.

The structure and function of the amphibian skin microbiome has been shown to play an important role in Bd and Bsal disease dynamics (Rebollar and Harris 2019). Certain cutaneous bacteria found on amphibians are known to produce compounds that inhibit *Batrachochytrium* in-vitro (henceforth referred to as anti-Bd bacteria; Becker et al. 2009; Woodhams et al. 2015). Some bacteria have been shown to modulate host immune responses to chytridiomycosis (Woodhams et al. 2020) and have even been used successfully as probiotic treatments for threatened amphibian species (Harris et al. 2009; Muletz et al. 2012; Bletz et al. 2013;

Kueneman et al. 2016). The overall community composition of the skin microbiome prior to Bd infection relates to disease dynamics in many cases, suggesting that species interactions are playing an important role in disease defense (Walke et al. 2015; Bates et al. 2018; Becker et al. 2019). Because numerous factors can contribute to variation in amphibian skin microbiomes (Kueneman et al. 2014; Jiménez and Sommer 2017), it is essential to holistically evaluate the distribution of key symbionts and the biotic and abiotic factors that influence their distributions (Loudon et al. 2016).

Given the potential for continued spread of Bsal, evaluating patterns of skin microbial symbiont distribution is an important task for vulnerable salamanders outside of the current Bsal range. Californian salamander populations are predicted to be particularly vulnerable to Bsal invasion based on habitat suitability models for the fungus and patterns of legal and illegal salamander trade into the U.S. (Yap et al. 2015; Richgels et al. 2016). This looming threat emphasizes the need to study the microbial ecology of some of California's native salamanders' skin microbiomes (Abarca et al. 2018). We compared the skin microbiota of five different salamander species that are native to the San Francisco Bay Area. Based on laboratory studies, we know that three of these species (*Ensatina eschscholtzii*, *Taricha granulosa*, and *T. torosa*) are vulnerable to Bsal (Martel et al. 2014; Piovia-Scott et al. 2019), while two of these species (*Batrachoseps attenuatus* and *T. rivularis*) have not been tested, though *T. rivularis* is likely susceptible due to its relatedness to the other *Taricha* in this study. We compared patterns of bacterial taxa distribution for the overall skin microbiome as well as for a subset of microbial taxa that have been characterized as anti-Bd across eight localities using 16S rRNA sequencing. In addition to traditional microbiome community analyses, we also characterized various metrics of metacommunity structure in order to evaluate the pattern of symbiont bacteria distribution across all salamanders. We then evaluated how the distribution of these bacteria is influenced by host and environmental factors. Applying these same approaches to potential anti-Bd bacteria allowed us to also evaluate how these potentially important symbionts are influenced by host and environmental factors.

MATERIALS & METHODS

Field Methods

We sampled terrestrial salamanders (*Taricha granulosa*, *T. torosa*, *T. rivularis*, *Batrachoseps attenuatus*, and *Ensatina eschscholtzii* ssp. *xanthoptica* and *oregonensis*) during the fall and winter (rainy season) between October 2018 and December 2018 at the University of California (UC), county, and federal properties throughout the San Francisco Bay Area (Figure 1). We restricted our sampling to terrestrial individuals only to minimize microbiome variation in response to habitat variation. Salamander sampling localities consisted of creek riparian zones composed of a mix of California oak (*Quercus* sp.), California bay (*Umbellularia* sp.) and/or redwoods (*Sequoia sempervirens*). We located salamanders through visual detection and log flipping. Immediately after capture, we placed animals in clean plastic bags and marked each animal's capture location. New gloves were donned before handling each animal to prevent cross-contamination of microbiome samples. At each locality, we sampled up to 21 salamanders of each genus (Figure 1; Table S1).

Before swabbing, we rinsed each animal with approximately 250 mL of sterile distilled water to remove transient microbes (Culp, Falkinham and Belden 2007). We then used a sterile cotton-tipped applicator to swab the dorsal skin surface 30 times, then stored the swab in a sterile 1.5-mL microcentrifuge tube. We used a second swab to swab the dorsal and ventral surface 15

times each and used this swab for another study. Given the ventral, feet, and dorsal communities do not differ in other salamanders and anurans (Hernandez-Gomez et al., 2017; Sabino-Pinto et al., 2016), we used only the dorsal skin surface swabs for the current study. We measured each animal's tail and snout-vent length to the nearest millimeter using calipers and weighed them to the nearest tenth of a gram on a 500 g digital scale. We returned animals to their exact flagged location immediately after processing. We stored the skin swab samples on dry ice until they were transported to the laboratory and stored in a -80°C freezer. We handled all animals using the approved University of California - Berkeley IACUC protocol (AUP-2015-01-7083).

Environmental Data Collection

For each sampling locality, we recorded GPS coordinates at the position where sampling began. Given that we collected salamanders along streams, we used the USGS National Watershed Boundary Dataset to identify the HUC 12-level boundaries overlapping with our sampling locations in QGIS (version 3.2.1). We applied these boundaries to characterize the percentage of land with natural cover using the CALVEG GIS dataset (USDA Forest Service, Remote Sensing Laboratory) and to extract bioclimatic variables from the WorldClim dataset. We used a principal component analysis to transform the bioclimatic variables (highly correlated) into a new set of uncorrelated variables. The sample scores of the first two principal components were retained for subsequent analyses as together these explained 99.85% of the bioclimatic variation captured across our sites. These two principal components exhibited strong correlations with temperature seasonality (r = 0.800; PC1 explained variance = 95.38%) and annual precipitation (r = -0.996; PC2 explained variance = 4.47%). We will refer to these two principal components as temperature seasonality (PC1) and annual precipitation (PC2) hereafter.

Laboratory Methods

We processed skin samples using the protocol outlined in Hernández-Gómez et al. (2017). To isolate microbial DNA from skin swabs, we used the DNeasy PowerSoil Kit (Qiagen N.V., Hilden, Germany). To control for contamination, we included negative extraction controls (i.e. unused swabs) and researcher glove swabs. We amplified the bacterial 16S rRNA gene V4 region using primer pair F515/R806 attached to barcode/sequencer adaptor connector sequences. We ran each sample in triplicate, with each 15-µL reaction containing 5 µL of template DNA, 7.5 µL of 2X MyTaq Master Mix (Bioline, Tauton, MA, USA), 1.0 µL of 1 nM forward and reverse primers, and 1.5 µL of sterile water. PCR conditions consisted of 94°C for 3 min, 30 cycles of 94°C for 45s, 50°C for 60s, and 72°C for 90s, followed by 72°C for 10 minutes. We pooled triplicate PCR products and cleaned them using the Qiagen UltraClean PCR Clean-up kit.

We performed a second PCR on microbiota amplicons to ligate dual-index barcodes paired with Illumina sequencing adaptors to the ends of amplicons. The 15 µL PCR reactions consisted of 5.0 µL clean amplicons, 7.5 µL 2X MyTaq MasterMix, 1.0 µL of 1 nM forward and reverse barcode primers (Hernández-Gómez et al. 2017), and 1.5 µL of sterile water. We quantified the PCR products using a Qubit Fluorometer (Invitrogen Corp, Carlsbad, CA, USA), pooled samples in equimolar amounts, and cleaned the sample pool using the UltraClean PCR Clean-Up kit. The sample pool was submitted to the California institute for Quantitative Biosciences Vincent J. Coates Genomics Sequencing Laboratory and sequenced on a MiSeq platform (Illumina Inc., San Diego, CA, U.S.A.) using the Reagent Kit V3 to produce 300 bp paired-end reads.

Data Filtering and Analysis

We filtered raw reads using Trimmomatic (Bolger, Lohse and Usadel 2014) to remove adaptor sequences, bases below the threshold quality of phred-20 from both ends of the reads, and any resulting reads under 30 bp in length. We then paired the quality-trimmed forward and reverse reads using PANDAseq (Masella *et al.* 2012). We only used reads that paired successfully in the subsequent analyses.

We implemented established sequence read processing pipelines to filter erroneous reads, generate amplicon sequence variants (ASVs - sequencer error-corrected unique DNA sequences), and to create a representative sequence phylogeny and assign taxonomy to ASVs. We processed reads using the Quantitative Insights Into Microbial Ecology version 2.2018.4 (QIIME2) pipeline (Bolyen *et al.* 2019). We used the DADA2 plugin to quality filter, dereplicate, remove chimeras and denoise reads using default settings (clustering at 99% similarity; Callahan *et al.* 2016). We generated a phylogeny using MAFFT aligned representative ASV sequences in FastTree2 (DeSantis *et al.* 2006; Price, Dehal and Arkin 2010). We ran the ASV table through the package *decontam* in R to identify ASVs associated with glove samples/negative extraction controls and removed identified contaminants from the salamander skin swab ASV table (Davis *et al.* 2018). We also filtered out ASVs whose taxonomy matched chloroplast or mitochondria as these were not the target of our sequencing library preparation protocol. To standardize sequencing depth throughout all samples, we rarefied the filtered ASV table to 5038 sequences per sample. All control samples were rarefied out of our analysis.

We transferred the rarefied ASV table and Newick phylogeny to R (version 3.5.1) for further analyses. We calculated three distinct alpha diversity metrics using the R packages *vegan* (Oksanen *et al.* 2020) and *picante* (Kembel *et al.* 2010): community richness (i.e. number of observed ASVs per sample), evenness (i.e. Shannon Diversity Index) and phylogenetic diversity (i.e. Faith's phylogenetic diversity). To evaluate differences in community composition across samples, we used the R packages *GUniFrac* (Chen 2015) and *vegan* to calculate two separate beta diversity metrics: unweighted UniFrac distances and Bray-Curtis dissimilarities. We chose to include these beta diversity metrics as they account for differences in presence/absence of bacterial lineages among samples (unweighted UniFrac) and non-phylogenetic abundance-based differences among samples (Bray-Curtis). In addition, we characterized the core microbiota (ASVs present in at least 70% of individuals) for each species.

We queried each ASV representative sequence using BLAST against a database of 16S rRNA sequences from anti-Bd bacteria (Woodhams *et al.* 2015). ASVs with 100% similarity to the query were used to generate an anti-Bd ASV table. Using the new ASV table, we calculated the anti-Bd community richness (i.e. the number of inhibitory ASVs) and the proportion of anti-Bd reads for each sample.

Statistical Analysis

We performed all statistical analyses using a hierarchical design with the goal of evaluating effects of host and environmental factors on the skin microbiome of salamanders. First, we evaluated how host species and environment shaped skin microbial communities and possibly anti-Bd bacteria among all salamanders sampled. Given that genus identity was predicted to be a strong influencer, we also assessed how the skin microbiota of each genus was influenced by host and environment factors independently. Because *T. torosa* and *T. granulosa* can be difficult to differentiate morphologically and their ranges overlap in the San Francisco Bay Area, we grouped all *Taricha* individuals by genus and used genus assignment for all

statistical comparisons. To account for multiple comparisons, all *p*-values were Bonferroni-corrected.

Effects of host and environment on alpha diversity, beta diversity and ASV distribution

We calculated body condition indices for each salamander by obtaining the least squares regression residuals of log-transformed mass and total body length. This metric has shown to accurately represent estimates of body reserves in amphibians and other vertebrates (Ardia 2005; Schulte-Hostedde *et al.* 2005).

To evaluate how host and environmental variables influenced the diversity, structure, and distribution of ASVs across all salamanders, we used linear mixed models to assess which variables influence alpha diversity metrics in terrestrial salamanders. Either community richness, evenness, or phylogenetic diversity were entered as dependent variables; genus, percent natural cover, temperature seasonality (PC1), and annual precipitation (PC2) as fixed independent variables (we did not include body condition indices in these analyses given the large variation in morphology across the three genera studied); and locality as a random independent variable. We implemented AICc ranking to exclude non-significant fixed variables using the R package *MuMIn* (Barton 2020). We evaluated differences in alpha diversity within each genus in a similar fashion, replacing the genus fixed variable with body condition indices. We used PERMANOVA tests (R package *vegan*) to evaluate how host and environmental variables contribute to community composition overall and at the genus level.

To evaluate differences in composition across all salamanders, we inputted unweighted UniFracs and Bray-Curtis dissimilarities as dependent variables; genus, percent natural cover, temperature seasonality-PC1, and annual precipitation-PC2 as fixed variables; and locality identity as a random factor (i.e. permutations were performed within localities). As in the alpha diversity analysis, we ran genus-specific PERMANOVAS using the same variables, except with body condition indices as a fixed variable instead of genus identity. To visualize community structure, we generated NMDS plots using unweighted UniFracs and Bray-Curtis dissimilarities. Also, to assess for the effect of geographic distance on community composition we ran Mantel tests between site pairwise Euclidean distances and unweighted UniFracs/Bray-Curtis dissimilarities. We repeated the Mantel test using these same distances within each genus.

We analyzed the metacommunity structure of salamander skin communities using the package *metacom* in R (Dallas 2014). We restricted this analysis to ASVs present in greater than 10% of all individuals as recommended by Dallas (2014) to eliminate rare ASVs which may complicate sample/ASV ordination. We ranked ASVs according to their distribution and salamander skin samples according to their composition using reciprocal averaging (i.e. correspondence analysis). Then, we calculated the three elements of metacommunity structure (EMS): coherence, turnover, and boundary clumping (Leibold and Mikkelson 2002; Presley, Higgins and Willig 2010). EMS values are calculated for each dataset, then a permutation test is used to assess the significance of the observed EMS indices. Because we were working with a very large dataset, we restricted permutations to 99 and allowed null matrices to have empty rows and columns as recommended by Dallas (2014) to ease computation time. These three statistical tests are used in a dichotomous key-manner to classify the ranked matrix's metacommunity structure against those of idealized metacommunity structures (Table S3) described in Presley et al. (2010).

Coherence evaluates the number of absences ASVs have within their distribution across the ranked samples and is used to infer the degree that species co-occur (Leibold and Mikkelson

2002). Metacommunities with negative coherence resemble a 'checkerboard' pattern because of mutual exclusion between ASVs, those with a coherence value of zero display a 'random' pattern of absences within ranges, and those with positive coherence display a consistent presence of ASVs within their range. Turnover assesses the rate of ASV replacements that occurs between samples and is used to assess changes in community composition. A metacommunity that displays negative turnover exhibits a loss of ASVs without replacement across the sample gradient resulting in a 'nested' structure and a metacommunity that possesses positive turnover exhibits a change in ASV composition along the sample gradient. Boundary clumping is used to differentiate the patterns of species turnover in metacommunities with positive coherence and positive turnover. The test uses Morisita's overlap index to evaluate whether groups of ASVs share boundaries (Morisita's Index > 0) resulting in discrete communities across the sample gradient, whether ASV boundaries are evenly distributed across the range (Morisita's Index < 0) resulting in gradual composition change along the sample gradient, or whether there is a random pattern in the distribution of ASV boundaries across the range (Morisita's Index = 0). We implemented the default 'r1' to characterize the significance of the EMS and compared them against those of idealized metacommunity structures (Table S3) described in Presley et al. (2010).

To evaluate the effect of host and environmental variables in structuring salamander community ordination, we performed Pearson correlation tests between sample ordination scores and percent natural cover, temperature seasonality-PC1, and annual precipitation-PC2. We ran a Kruskal-Wallis test to evaluate if genus (i.e. a categorical variable) influences sample ordination scores. An identical analysis was performed within each genus; however, we tested for correlations between body condition indices or environmental variables and salamander ordination using Pearson correlations tests.

Effects of host and environment on richness, relative abundance and turnover of possible anti-Bd bacteria

We evaluated how host and environmental variables influence the richness, relative abundance (proportion of reads per sample identified as inhibitory) and distribution of anti-Bd ASVs across all salamanders and within each genus. We used linear mixed models to independently assess which variables influence anti-Bd ASV richness and relative abundance. We tested genus, percent natural cover, temperature seasonality (PC1), and annual precipitation (PC2) as fixed independent variables and locality as a random independent variable. As done before, we repeated this analysis for each genus and added body condition indices as fixed variables. We also used ranked AICc scores to exclude non-significant variables.

We analyzed the metacommunity structure of anti-Bd bacteria across all salamanders and within each genus using the package *metacom* in R as described above. To evaluate effects of host and environmental variables in structuring anti-Bd bacteria distribution across salamanders, we performed Pearson correlation tests between sample ordination scores and percent natural cover, PC1, and PC2. We ran a Kruskal-Wallis H test to evaluate if genus (i.e. a categorical variable) correlates with ordination scores. For the intra-genus comparisons, we evaluated Pearson correlations between sample ordination scores, body condition indices, and the environmental variables.

RESULTS

After quality control and filtering, we used reads from 192 skin samples (n = 92 *Batrachoseps attenuatus*, n = 57 *Ensatina eschscholtzii*, and n = 43 *Taricha* sp). The total number of reads was 4,337,652, with 55,333 unique ASVs (Amplicon Sequence Variants) across all skin samples. We detected between 47 and 770 bacterial ASVs on each salamander. We identified ASVs assigned to *Pseudomonas vidiflava, Variovorax paradoxus,* and *Rhizobium sp.* across all genera of salamanders sampled. The *Taricha* core microbiome was dominated by ASVs assigned to an *Arthrobacter* sp. and *Pseudomonas viridiflava*. The core microbiomes of *Ensatina* and *Batrachoseps* were dominated by *P. viridiflava* and an ASV in the family Aeromonadaceae (Fig. 2). All of these bacteria are classified as anti-Bd besides the ASV in the family Comamonadaceae.

Effects of host and environment on alpha diversity, beta diversity and ASV distribution

Genus significantly predicted skin richness, Shannon Diversity indices, and Faith's phylogenetic diversity indices at a global scale (Table 1). Across all salamanders, our model selection pipeline resulted in models that did not indicate any environmental effects on alpha diversity across the landscape. Post-hoc analyses showed higher alpha diversities in *Ensatina* salamanders compared to *Batrachoseps* and *Taricha* (Fig. 3). Within *Ensatina*, all variables were dropped for richness and Shannon diversity indices and annual precipitation (PC2) had a negative effect on Faith's phylogenetic diversity indices. Within *Batrachoseps* and *Taricha*, all environmental variables were removed during variable selection or had no significant associations with alpha diversities.

Genus also explained the largest portion of the variation observed in Bray-Curtis dissimilarities across all salamanders followed by temperature seasonality (PC1; Table 2). We detected a marginal effect from environmental variables on unweighted UniFrac dissimilarities, signifying that the effects of environmental variables on community composition were specific to the turnover of rare bacteria. Within *Batrachoseps*, *Ensatina* or *Taricha*, none of the environmental or host-specific variables significantly explained the grouping of samples. When visualizing all salamanders together, we observed loose clustering by genus identity in the NMDS plots generated from unweighted UniFracs and Bray-Curtis dissimilarities (Figure 4); but *Taricha* salamanders were amongst the most conserved of the three (Figure 4B). Still, Genus-specific NMDS plots displayed a pattern of clustering by location among the first or second axis across all beta diversity metrics used (Figure 5). In addition, Euclidean geographical distances correlated with skin microbiota Bray-Curtis distances in our global comparison (including all salamander genera: $r = 0.086$, $p = 0.008$) and within the two plethodontid salamanders only (*Batrachoseps*: $r = 0.114$, $p = 0.012$; *Ensatina*: $r = 0.238$, $p = 0.012$). We did not detect correlations between geographic Euclidean geographical distances and unweighted UniFrac distances.

Across all salamanders, skin microbial communities displayed a Clementsian structure (positive, significant coherence, positive, significant turnover, positive (>1), significant boundary clumping; Table 3). In addition, percent natural cover ($r = 0.49$, $p < 0.001$), temperature seasonality (PC1; $r = 0.68$, $p < 0.001$), annual precipitation (PC2; $r = -0.37$, $p < 0.001$) and genus identity $X2 = 98.74$, $df = 2$, $p < 0.001$) all correlated with the ordination scores of salamanders. However, salamanders were ordered into family clusters by the reciprocal averaging protocol (Figure S1), suggesting a strong effect of host phylogeny on the distribution of bacteria. The metacommunity structures within each salamander genus displayed positive coherence, negative turnover, and positive boundary clumping, aligning with the idealized nested Clementsian

structure (Table 3; Figure S1). *Batrachoseps* salamanders experienced correlations between sample ordination scores and annual precipitation (PC2; r = -0.40, p < 0.001) and body condition indices (r = -0.25, p = 0.0175). Percent forest cover (r = 0.53, p < 0.001) was significantly associated with the ordination of *Ensatina* salamanders, and Taricha ordinations correlated with annual precipitation (PC2; r = 0.50, p 0.003; Figure S1). Together, these patterns suggest that skin microbial communities among salamander genus are discrete; however, within each genus there is a clumped loss of ASVs among groups of salamanders that depends on environmental factors.

Effects of host and environment on anti-Bd bacterial richness, relative abundance and metacommunity structure

None of the tested fixed variables were associated with the richness of anti-Bd bacteria across all salamanders. Within each salamander, we only observed a significant negative effect of percent natural cover on the richness of *Ensatina* anti-Bd bacteria ($F_{1,6} = 13.05$, p = 0.0448). Among the three salamander genera, *Ensatina* (mean ± SE; 12.49 ± 0.71) possessed a greater number of predicted anti-Bd bacteria on skin microbiomes compared to *Batrachoseps* (11.21 ± 0.48) and *Taricha* (11.32 ± 0.48). Genus was the only variable significantly associated with anti-Bd bacteria relative abundance across all salamanders ($F_{2, 178} = 6.08$, p = 0.0112). The microbiome of *Taricha* possessed the highest total relative abundance of anti-Bd bacteria (0.14 ± 0.016), followed by *Ensatina* (0.11 ± 0.011) and *Batrachoseps* (0.085 ± 0.008). Considering each salamander genus separately, we did not find associations between host or environmental factors and the relative abundance of anti-Bd bacteria.

Anti-Bd bacteria metacommunity structure was also characterized as Clementsian (positive, significant coherence, positive, significant turnover, and positive (>1), significant boundary clumping; Table 3). Percent natural cover (r = 0.29, p < 0.001) and genus identity (X^2 =19.40, df = 2, p < 0.001) both correlated with sample ordination scores. These patterns are confirmed by our visualization of metacommunity structure (Figure 6), where a salamander family gradient can be observed along the salamander community axis. Within each genus (Table S4) we detected either a Clementsian structure (*Batrachoseps*) or quasi-Clementsian structures (*Ensatina* and *Taricha*). Quasi-Clementsian structure is associated with positive boundary coherence, positive turnover (n.s.) and positive boundary clumping. Quasi-Clementsian structures are similar to the idealized Clementsian structure, except the range of turnover is indistinguishable from random. Among *Batrachoseps*, salamander ordination scores did not correlate with environmental factors or body condition. The ordination scores of *Ensatina* correlated with percent forest cover (r = - 0.35, p = 0.029) only, while none of the individual and environmental variables correlated with the ordination of *Taricha* samples.

DISCUSSION

Incorporating community ecology into microbiological studies is useful for identifying factors that shape the distribution of key microbes in different habitats. Applying this approach to host-associated microbiomes presents us with an opportunity to simultaneously explore host and environmental factors that may influence key symbionts. In this study, we assessed the composition and distribution of bacteria that live on the skin of three genera of salamanders from nine sampling localities in California. We found that the core microbiomes of *Taricha*, *Ensatina*, and *Batrachoseps* are almost exclusively composed of bacterial amplicon sequence variants (ASVs) that match with strains that inhibit Bd *in vitro*. Of the three salamander genera, *Ensatina*

microbiomes had a higher skin bacterial alpha diversity than *Batrachoseps* and *Taricha* and bacterial community composition of the skin microbiotas clustered primarily by genus. Finally, metacommunity structure analysis allowed us to identify host identity as a major boundary in the distribution of bacterial ASVs across our salamander samples. When each species' metacommunity was considered separately, the ordination scores of the salamanders correlated with one or more environmental variables.

Host identity is a strong predictor of microbiome structure and symbiont distribution in terrestrial salamanders

Previous studies have assigned either host taxonomy (Jani and Briggs 2014; Kueneman et al. 2014; Walke et al. 2014; Belden et al. 2015; Sabino-Pinto et al. 2016; Prado-Irwin et al. 2017; Abarca et al. 2018) or environmental variation (Bird et al. 2018; Muletz Wolz et al. 2018; Varela et al. 2018; Ellison et al. 2019; Kueneman et al. 2019) as the predominant factor associated with amphibian microbiome variation. In our study, genus identity strongly correlated with the structuring of skin microbial communities and the distribution of ASVs among *Batrachoseps*, *Ensatina* and *Taricha* followed by climatic variables and land cover. We also noted differences in ASV distribution patterns between the salamander genera. For example, plethodontid salamanders experienced a decay in skin microbiome similarity with distance, whereas this pattern was not evident in *Taricha*. Temperature seasonality and annual precipitation different among our sites, and within each genus, we found correlations between climatic variables, natural cover and ASV distribution.

There are numerous ecological and physiological characteristics that could contribute to skin microbiome assembly differences among communities of sympatric salamanders. The three genera we included represent two families of salamanders (Plethodontidae and Salamandridae) that have diverged for at least 150 million years (Zhang and Wake 2009). These salamander families tend to use similar microhabitats for at least part of the year and are therefore exposed to a similar microbial pool. Thus, variation in skin physiology, immune function, behavior, and/or reproductive modes between these taxa could result in different selection pressures among individuals (Stebbins and McGinnis 2018 AmphibiaWeb 2020). Of particular interest is the highly conserved microbiome observed in *Taricha*, and what the high prevalence of bacteria such as *Pseudomonas viridiflava* mean for individual fitness. Similarities in microbiota composition throughout the range may be related to *Taricha*'s life history, as all species of Pacific newts are highly vagile aquatic-breeders that migrate between land and aquatic environments to mate (AmphibiaWeb 2020). It is also possible that *Taricha* retain associations with critical symbiotic bacteria that contribute to other crucial physiological functions (e.g. toxicity). Tetrodotoxin (TTX), a powerful neurotoxin found on the skin of *Taricha*, has been quantified in bacterial cultures derived from their skin (Vaelli et al. 2020). If TTX is generated solely by symbiotic bacteria, co-dependence between *Taricha* and one or more TTX-producers could result in commonalities in core bacteria across the range of the species, as has been observed in other systems, where symbiotic bacteria that facilitate physiological functions comprise a significant part of their core microbiome (Ainsworth et al. 2015). While microbial community functionality is out of the scope of our study, our results suggest the need for future investigations into the importance of conserved microbiomes on pathogen and predator defenses in amphibians.

Distribution of anti-Bd bacteria across sympatric salamanders is tied to host identity

The few microbiome papers that have incorporated metacommunity theory when evaluating patterns of microbial community composition have mostly been performed outside of the amphibian realm (Van Der Gucht et al. 2007; Costello et al. 2012; Burns et al. 2017; Toju, Tanabe and Sato 2018; Cleary et al. 2019; Brown et al. 2020, but see Hernández-Gómez et al. 2017). The present study provides additional insight into the use of this analysis to track the distribution of important symbionts across three sympatric genera of terrestrial salamanders. Although many ASVs were found in all three genera, skin microbial metacommunities exhibited a high degree of turnover along the sample gradient for both overall and anti-Bd bacteria, meaning that individuals at both extremes of the ordination possessed very different community assemblies (Figures 6 and S1). In our dataset, this compartmentalization correlated strongly with the two families of salamanders. Because our salamander families were sympatric, and often found using the same microhabitats (e.g. under the same log), our results show heterogeneity in the association between salamander families and anti-Bd bacteria. Given the contribution of anti-Bd bacteria to disease resistance, divergent anti-Bd bacteria between salamander families could translate into different mechanisms and magnitudes of pathogen protection (Becker et al. 2015), or differences in the maintenance of microbial symbionts through the production of antimicrobial peptides (AMPs; Küng et al. 2014; Flechas et al. 2019). While our methodology cannot distinguish between these two probable mechanisms, our study highlights the need to continue to evaluate co-evolutionary associations between hosts and relevant members of their natural microbiotas.

Relative abundance, richness and distribution of putatively anti-Bd bacteria within salamander species are linked to environmental factors

Our metacommunity structure analysis allowed us to evaluate host and environmental factors that may shape the distributions of anti-Bd bacteria within each genus. Since the microbiome composition on the skin of these animals may protect them against *Batrachochytrium*-induced chytridiomycosis (Loudon et al. 2014; Becker et al. 2015), nested and quasi-Clementsian assemblies of bacteria within each genus could indicate the presence of multiple types of "functional assemblies" of bacterial taxa. None of the environmental variables we used correlated with the ordination of *Ensatina* salamanders. However, percent natural cover had a significant effect on the distribution of bacteria among *Taricha*, and *Batrachoseps* was significantly affected by temperature seasonality. These observations imply that different anti-Bd bacteria could provide protection across a species range, and that the assembly of these communities is sensitive to habitat characteristics. It is important to note that temperature seasonality and annual precipitation can also have an impact on the prevalence of Bd in the landscape and this could also influence pathogen burden on the populations (Longo and Zamudio 2017). Moving forward, characterizing how various anti-Bd bacterial assemblies contribute to host immunity and pathogen growth is crucial for understanding the dynamics associated with microbiome-derived pathogen resistance across the landscape.

Conclusion

In the event of future amphibian chytrid fungus outbreaks in California, our study can serve as a valuable reference of the distribution of functionally important bacteria among these native salamanders (West et al. 2019). Quantifying the distribution of putative anti-Bd bacteria on the skin across different populations allows us to infer whether there are natural defenses already present on their skin that may give these salamanders a chance against the potential

threat of Bsal and whether any of these naturally associated bacterial taxa could be augmented using cutaneous probiotics, which have been successfully used to protect other amphibian species from chytridiomycosis (Harris et al. 2009; Muletz et al. 2012; Kueneman et al. 2016; Woodhams et al. 2016). The application of metacommunity structure analysis allowed us to compare the effects of host-specific and habitat-specific characteristics on ASV and anti-Bd bacteria distribution. More importantly, we were able to quantify and visualize the compartmentalization between sympatric terrestrial salamanders across a certain degree of habitat heterogeneity. Our results demonstrate that the appropriate selection and application of probiotics may depend on specific factors such as genus and even specific locality. This will be important if the goal is to bolster the functional assemblies that are already present on the skin of these ecologically important amphibians (Bletz et al. 2013; Woodhams et al. 2016).

Our observations come with the caveat that we used 16S rRNA sequences to infer specific functions that are not related to our genetic marker; thus, future work in this system should consider measuring whether predictions of anti-Bd functionality using 16S rRNA data correlate with fungal inhibition in vitro or in vivo as in Rebollar et al. 2019. This is particularly important given the recent push to develop probiotics using anti-Bd bacteria to manage chytridiomycosis (Bletz et al. 2013; Kueneman et al. 2016; Mckenzie, Kueneman and Harris 2018). Our study raises the need for future work to continue describing how differences in richness and frequency of beneficial bacteria influence host health as this may be an important precursor to the design of successful probiotic regimens (Bletz et al. 2013; Harrison et al. 2020).

Future studies can increase our understanding of the microbiomes of amphibians and other wildlife by incorporating techniques that characterize community functionality (e.g. metagenomics, transcriptomics), conducting range-scale and fine-scale microbiome surveys (Presley, Higgins and Willig 2010) studying how species characteristics (e.g. skin structure, immune responses, behavioral differences) shape skin microbiomes, or by evaluating the effects of environmental disturbances (e.g. fires) on host-associated microbiota.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSEC online. This dataset has been deposited in the NCBI Sequenced Read Archive (SRA; accession number PRJNA731185). R code is available in the supplemental material.

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Figure Legends

Figure 1: Sampling localities throughout the San Francisco Bay area. Locality colors are consistent with subsequent figures. The circle for Tilden Regional Park is split because there are two separate sampling locations within the park.

Figure 2: Core bacterial ASVs (Amplicon Sequence Variants) present in 70% or more of the samples from each genus of host salamander. Each color represents a different class of bacteria (blue (top) – Actinobacteria, violet – Alphaproteobacteria, pink – Betaproteobacteria, orange – Gammaproteobacteria, yellow (bottom) - Thermoleophilia), and each shade represents a distinct ASV. *Batrachoseps* exhibited a less conserved group of core microbiota across sampling localities.

Figure 3: Dot plots showing three alpha diversity metrics - (A) community richness, (B), Shannon Diversity Index, and (C) Faith's Phylogenetic Diversity. Mean values (long horizontal lines) and standard errors (short horizontal lines) are displayed. Significant groupings from pairwise comparisons are labeled using lowercase letters within each plot.

Figure 4: NMDS plots of terrestrial salamander bacterial communities built using (A) unweighted UniFrac (stress = 0.125645), and (B) Bray-Curtis (stress = 0.1735216) dissimilarities. Each point corresponds to the bacterial community on an individual, and distance between points correlates with dissimilarity among samples. In all three cases, variation with genus is observed.

Figure 5: NMDS plots of *Batrachoseps* (A & D), *Ensatina* (B & E) and *Taricha* (C & F) skin bacterial communities built using unweighted UniFrac (A, B & C) and Bray-Curtis dissimilarities (D, E and F). Points correspond to each community, and distance between points correlates with dissimilarity among samples.

Figure 6**:** Distribution of anti-Bd bacteria (columns) detected on the skin of individual salamanders (rows), where rows and columns are ordinated based on marginal probabilities. Sidebars display salamander genus, locality, site annual precipitation (PC1), temperature seasonality (PC2), body condition index, and percent natural cover (white – 50%, dark green – 100%). Significant associations between salamander ordination scores and sidebar variables are denoted with an asterisk at the top of the sidebar. (A) The microbiomes of salamanders from all genera exhibited a Clementsian metacommunity structure (positive and clumped inhibitory ASV replacements). (B) *Batrachoseps* exhibited Clementsian structure. (C) *Ensatina* exhibited Clementsian structure. (D) *Taricha* exhibited quasi-Clementsian structure.

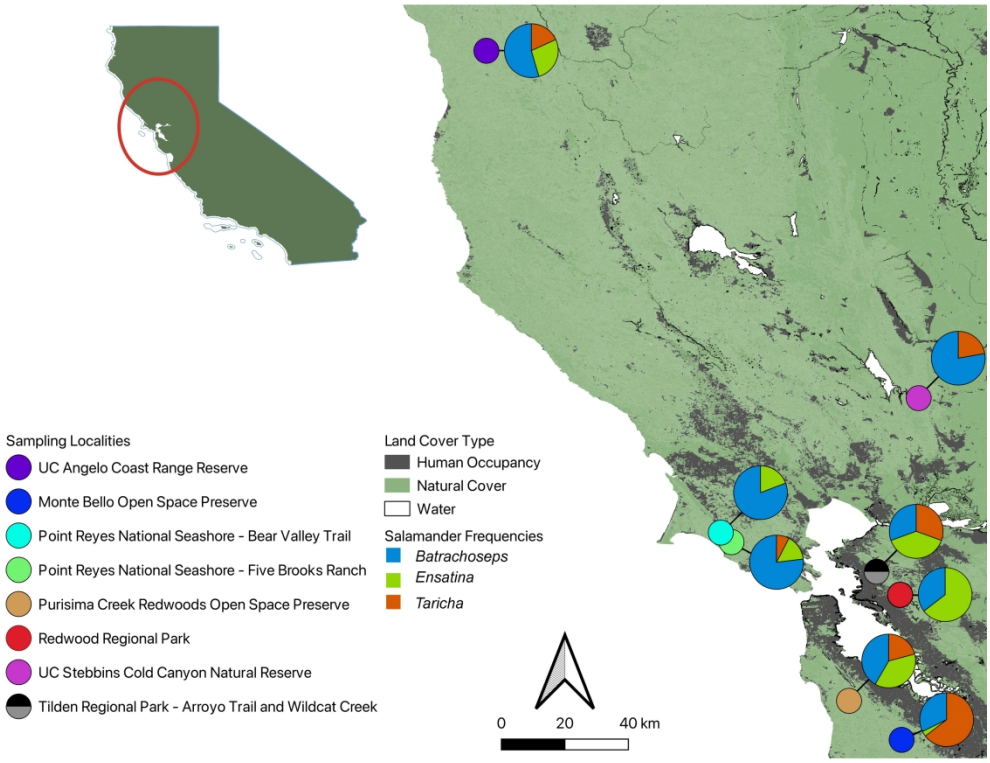

Figure 1

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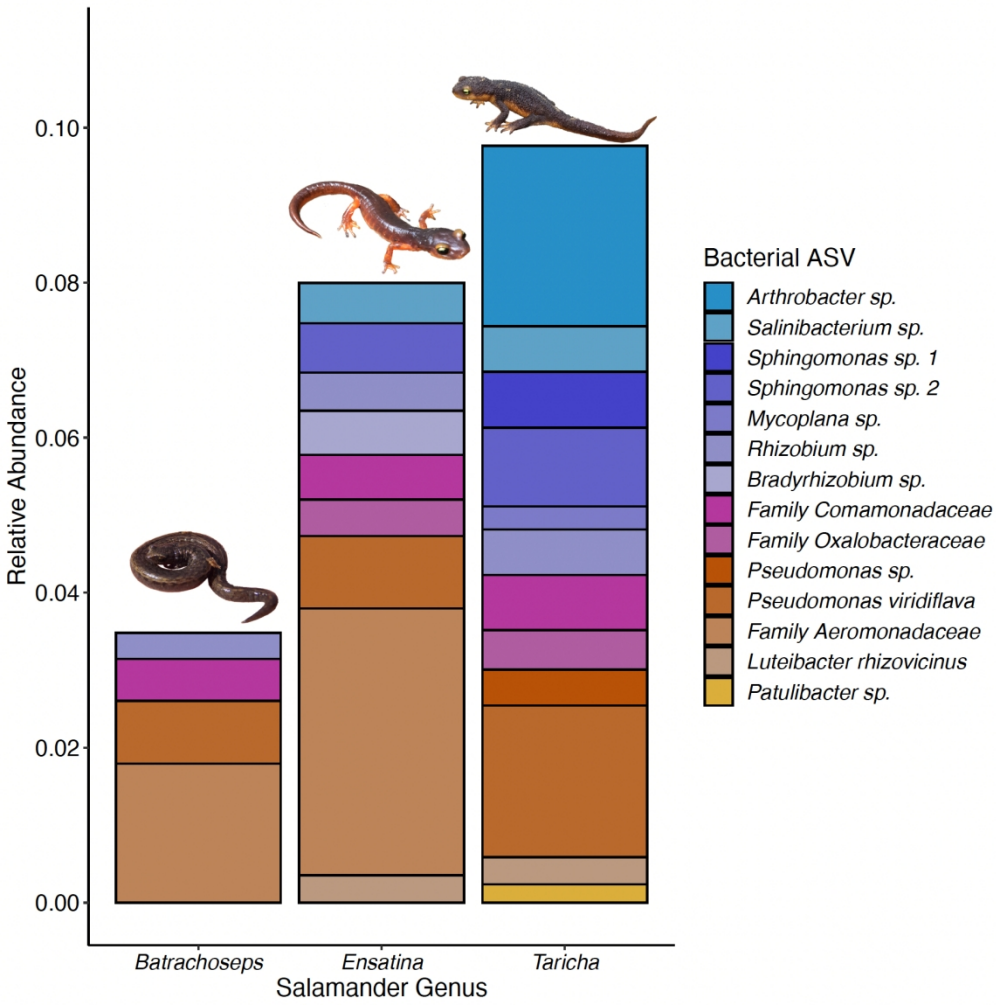

Figure 2

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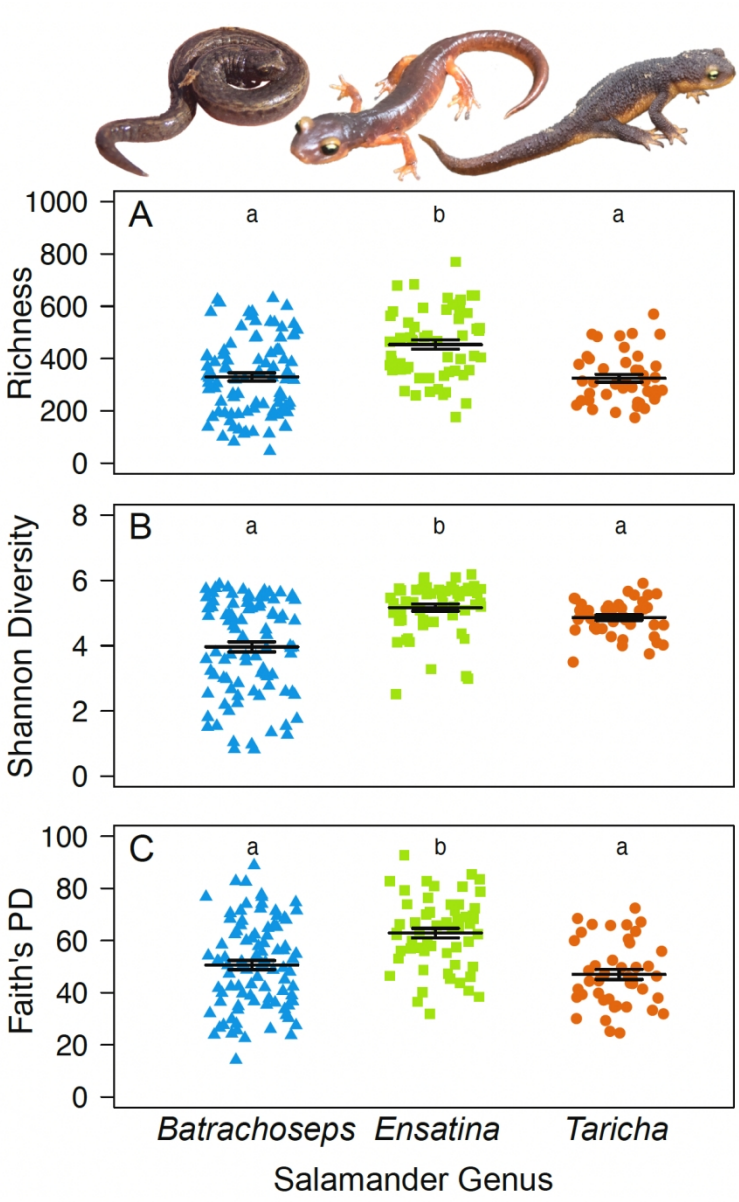

Figure 3

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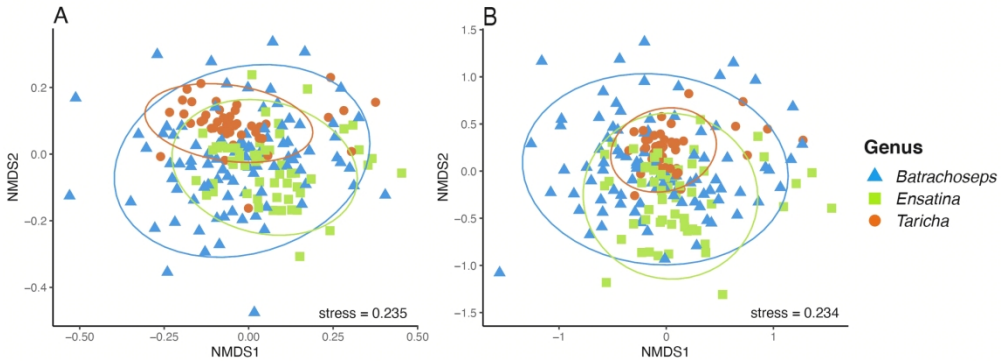

Figure 4

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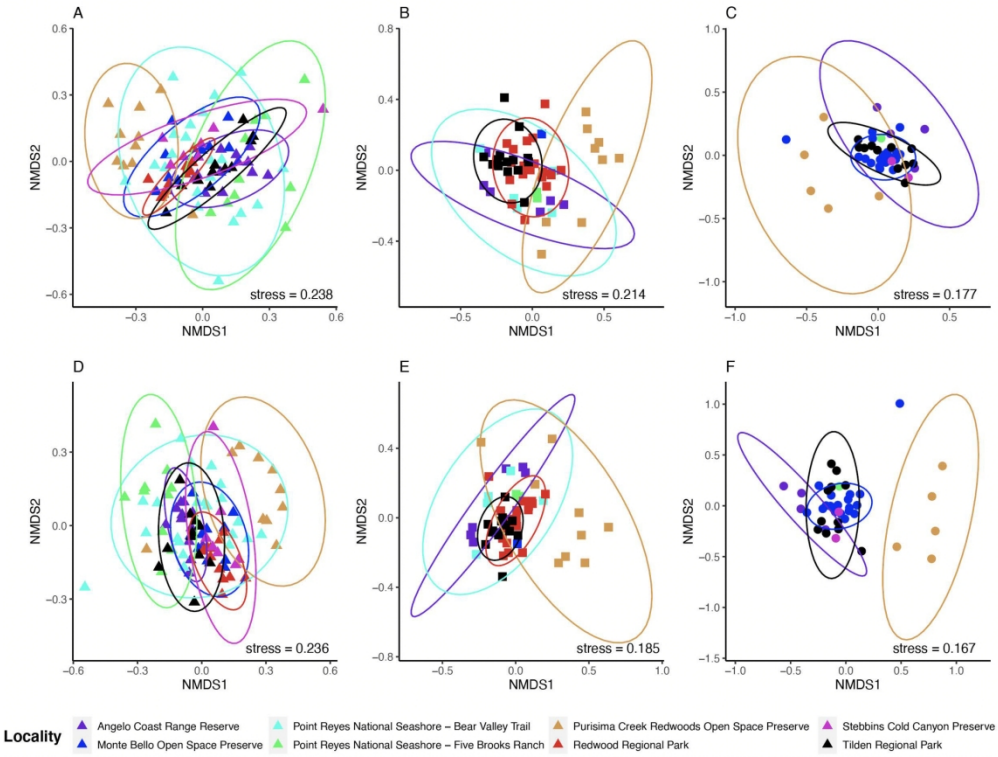

Figure 5

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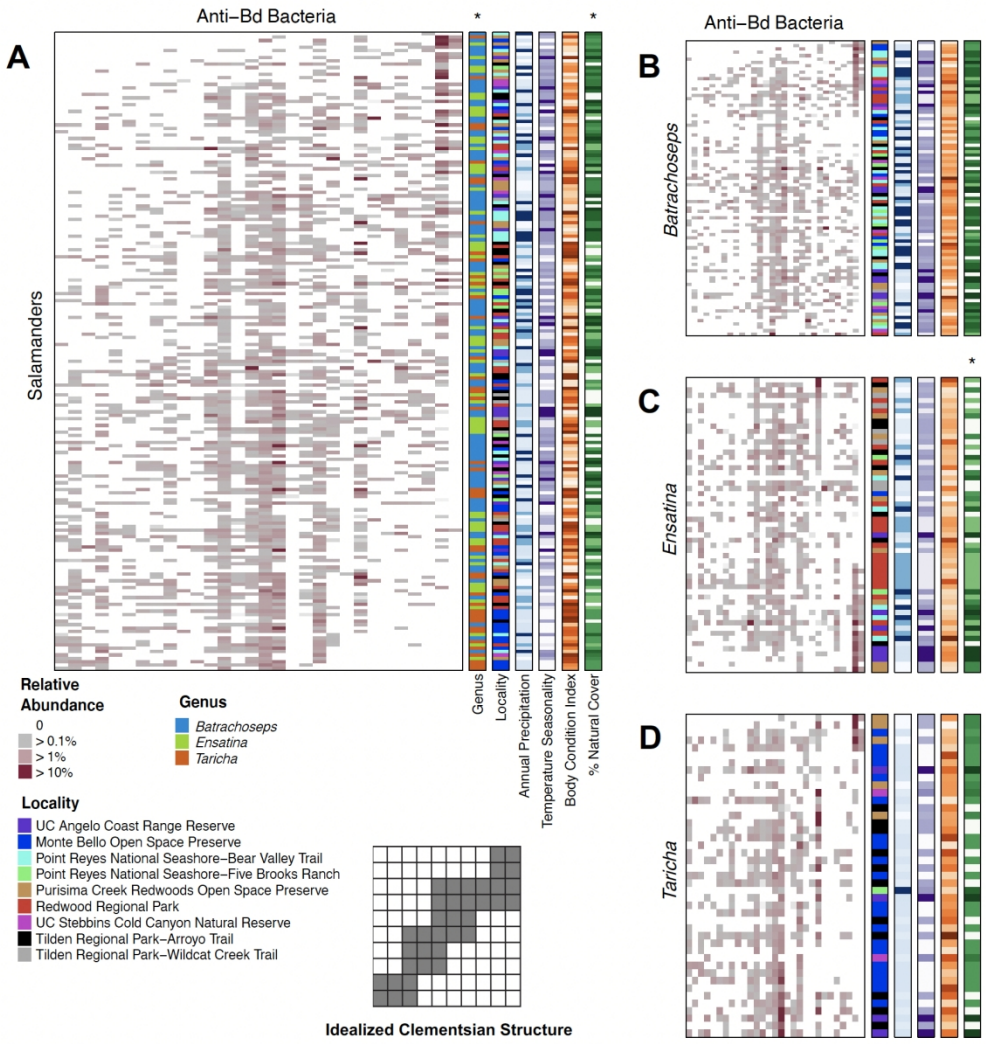

Figure 6

325x341mm (144 x 144 DPI)

Table 1: Linear mixed models selected using ranked AICc selection. The models evaluate the effects of temperature seasonality (Temp - PC1), annual precipitation (Prec - PC2), land use (% natural cover), and genus on alpha diversity metrics. To control for locality, we included it as a random variable.

Alpha Diversity Metric (Gaussian Distribution)	**Variables Dropped**	**Variable(s) Kept**	**AICc of Final Model**	**ANOVA Results**
Richness	% natural cover Temp - PC1 Prec - PC2	Genus	2411.6	Genus: $F_{2,182} = 8.72$, **$p = 0.001$**
Shannon Diversity Index	% natural cover	Genus Prec - PC2 Temp - PC1	574.3	Genus: $F_{2,182} = 13.84$, **$p < 0.001$** Temp - PC1: $F_{1,5} = 4.19$, $p = 0.385$ Prec - PC2: $F_{1,5} = 2.91$, $p = 0.595$
Faith's Phylogenetic Diversity	% natural cover Temp - PC1	Genus Prec - PC2	1574.5	Genus: $F_{2,182} = 10.04$, **$p < 0.001$** Prec - PC2: $F_{1,6} = 2.45$, $p = 0.674$

Table 2: PERMANOVA (adonis2) tests using a similar mix of fixed and random variables as described in the alpha diversity analyses. Distance matrices include unweighted UniFrac and Bray-Curtis dissimilarities. The model was stratified by locality.

Distance Metric	**Variables**	**pseudo-F**	**R**	***p*-value**
Unweighted UniFrac	Genus	3.77	0.037	**0.004**
	Prec - PC2	3.79	0.019	1
	% natural cover	2.50	0.012	1
	Temp - PC1	2.01	0.010	1
Bray-Curtis	Genus	10.08	0.088	**0.004**
	Temp - PC1	9.67	0.042	**0.008**
	% natural cover	7.45	0.032	1
	Prec - PC2	6.15	0.026	1

Table 3: Metacommunity structure analysis results of the overall and anti-Bd ASVs recovered from the skin of San Francisco Bay Area salamanders. The elements of metacommunity structure (EMS) interpretations are presented in the rightmost column.

	Coherence	**Turnover**	**Clumping**	**Interpretation**
Overall Skin Bacteria	**Positive** Embedded absences: 6.04×10^4 Simulated mean: $6.57 \times 10^4 \pm 103.53$, ***p* < 0.001**	**Positive** Replacements: 2.67×10^7 Simulated mean: $1.47 \times 10^7 \pm 1.22 \times 10^6$, ***p* < 0.001**	**Positive** Morisita's index: 3.71, df = 189, ***p* < 0.001**	**Clementsian** - distribution of ASVs exhibits turnover with clumped boundaries across hosts
Anti-Bd Bacteria	**Positive** Embedded absences: 6.14×10^3 Simulated mean: $7.62 \times 10^3 \pm 132.82$, ***p* < 0.001**	**Positive** Replacements: 6.41×10^5 Simulated mean: $3.77 \times 10^5 \pm 8.19 \times 10^4$, ***p* = 0.001**	**Positive** Morisita's index: 4.07, df = 186, ***p* < 0.001**	**Clementsian** - distribution of ASVs exhibits turnover with clumped boundaries across hosts