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Low-level pathogen infection and geographic location correlate with the skin microbiomes of Columbia spotted frogs (*Rana luteiventris*) in a montane landscape

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ABSTRACT

The skin microbiome of amphibians can influence host susceptibility towards the fungal pathogen *Batrachochytrium dendrobatidis* (Bd), while simultaneously having the potential to be altered by Bd. Severe Bd infections are known to alter the amphibian skin microbiome; however, little is known about microbiome interactions in amphibians with low infection intensity. In addition to disease dynamics, environmental factors may influence the microbiome. To test for patterns in bacterial diversity based on pathogen infection and environmental factors, 399 Columbia spotted frogs (*Rana luteiventris*) were sampled throughout northern Idaho and northeastern Washington across two years. Bd prevalence and intensity were measured in 376 frogs, revealing a prevalence of 69%, but generally low infection intensity (Mean = 127 Bd zoospore equivalents among infected frogs). Skin bacterial communities were characterized in 92 frogs using 16S rRNA gene amplicon sequencing. Our results indicated correlations of decreasing Shannon diversity and evenness as infection intensity increased. Latitude was correlated with bacterial richness and Faith's Phylogenetic Diversity measures, indicating increased diversity in northern locations. Beta diversity (UniFrac) analyses revealed that skin microbiomes were distinct between infected and uninfected frogs, and infection intensity had a significant effect on microbiome composition. Site explained the majority of microbiome variation (weighted UniFrac: 57.5%), suggesting a combination of local habitat conditions explain variation, as only small proportions of variation could be explained by year, month, temperature, elevation, and latitude individually. Bacterial genera with potential for Bd-inhibitory properties were found with differential relative abundance in infected and uninfected frogs, with higher *Stenotrophomonas* and lower *Pseudomonas* relative abundance observed in infected frogs. Further study may indicate if Bd inhibition by members of the skin microbiome is an influence behind the low infection intensities observed and whether low Bd infection intensities are capable of altering skin microbiome composition.

Introduction

Emerging infectious diseases threaten global biodiversity by contributing to population declines and extinctions in a large range of organisms, including amphibians, birds, mammals, invertebrates, and plants (Daszak et al., 2000; Harvell et al., 2002). The fungal skin disease chytridiomycosis, caused by the chytrid fungus *Batrachochytrium dendrobatidis* (Bd), is associated with the greatest documented disease-driven loss in biodiversity and estimated to have caused population declines in hundreds of amphibian species, some of which have been confirmed or presumed extinct (Scheele et al., 2019). Chytridiomycosis poses a threat to amphibians by interfering with amphibian skin processes, creating imbalance in the exchange of respiratory gases, water, and electrolytes (Voyles et al., 2007). However, the amphibian skin microbiome, the community of microbes inhabiting the skin of amphibians, can prevent morbidity and mortality caused by chytridiomycosis (Harris et al., 2009; Lam et al., 2010; Becker et al., 2015a; Holden et al., 2015; Walke et al., 2015), with some bacterial community members producing metabolites that inhibit Bd growth (Brucker et al., 2008; Harris et al., 2009; Becker et al., 2015b;

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Woodhams et al., 2015; Walke et al., 2017). For example, the bacterium *Janthinobacterium lividum* produces the metabolite violacein, which is associated with survival of Bd-infected red-backed salamanders (*Plethodon cinereus*) (Becker et al., 2009) and mountain yellow-legged frogs (*Rana muscosa*) (Harris et al., 2009). Bd-inhibitory bacterial isolates are widely distributed among phyla, though some genera such as *Stenotrophomonas, Aeromonas*, and *Pseudomonas* have high proportions of inhibitory isolates (Becker et al., 2015b). Composition of the skin bacterial community has been associated with survival of infected Panamanian golden frogs (*Atelopus zeteki*), demonstrating the importance of the skin bacterial community in disease susceptibility (Becker et al., 2015a).

While the skin microbiome can inhibit Bd, infection by Bd can disturb the skin microbiome by acting as a selective force, with these effects being able to occur simultaneously (Jani and Briggs, 2014; Walke et al., 2015). Jani and Briggs (2014) used both an observational study following a natural epidemic and an experimental infection testing for a causal relationship between Bd and the skin microbiome to show that the skin microbiome is consistently altered by pathogen infection. A time series analysis in a subsequent study suggested that infection intensity influences the relative abundance of several bacterial taxa, with the relative abundances changing as Bd loads increased over time (Jani and Briggs, 2018). Understanding of both Bd-inhibitory bacteria and the effects of Bd on the microbiome may inform research on the development of conservation strategies such as probiotics, which have had a mixture of successes (Harris et al., 2009; Muletz et al., 2012; Kueneman et al., 2016) and difficulties (Becker et al., 2011; Antwis et al., 2015; Harrison et al., 2020).

Aside from diseases, host and environmental factors may influence the skin microbiome (Walke et al., 2014; Bates et al., 2018; Jani and Briggs, 2018; Medina et al., 2019; Walke et al., 2021). Host factors include the host species or source of the population, while environmental factors include differences in habitat such as the type of water body, temperature, precipitation, and elevation (Bletz et al., 2017a; Jani and Briggs, 2018). Bacterial richness has been correlated with climatic factors on a global scale (Kueneman et al., 2019), and the skin microbiome and/or its metabolite profiles have been shown to vary across elevations (Hughey et al., 2017; Medina et al., 2017). Therefore, we also considered variables related to environment and geography that may be contributors to skin microbiome variation in our study.

In a large regional scale field survey, we quantified Bd prevalence and infection intensity in Columbia spotted frogs (*Rana luteiventris*) sampled from northern Idaho and northeastern Washington state, U.S.A (Lucid et al., 2016). This study aimed to test for relationships between skin bacterial communities and Bd infection, along with environmental, geographic, and temporal factors such as site, temperature, elevation, and sampling month and year. While other studies have analyzed the effects of Bd infection on amphibians under higher levels of infection, this study focuses on Columbia spotted frog populations with relatively low levels of infection, which may provide insight towards how the skin microbiome is shaped in populations with greater tolerance towards Bd over a large geographic area. We hypothesized that we may still observe correlations between infection and skin microbiome diversity, however, at a lesser degree than would be expected with high infection intensities, and that environment, geography, and/or time may contribute to variation in the skin microbiome.

Materials and methods

Study area

The study area consists of a 21,775 km^2 area including the northern Idaho panhandle and a portion of northeastern Washington (Fig. 1). Portions of five mountain ranges are within the study area: the Selkirk, Purcell, West Cabinet, Coeur d'Alene, and Saint Joe Mountains. The topography ranges from broad glacial valleys to mountainous areas with

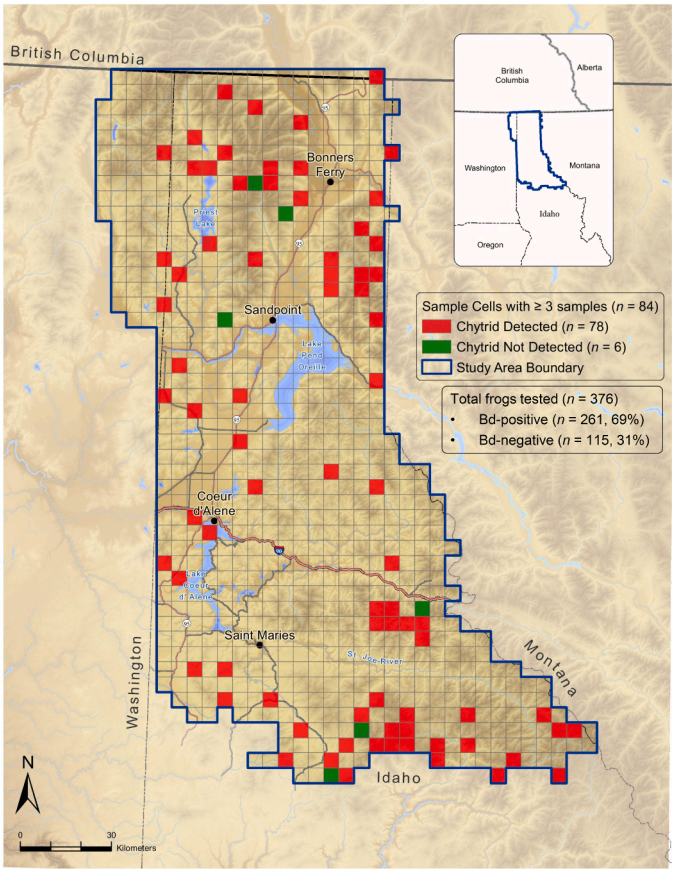

Fig. 1. Map of northern Idaho/northeastern Washington study area visualizing Bd presence by 5 × 5 km cells. At the individual level, 69% (261 frogs) of the 376 frogs tested were determined to be Bd-positive, demonstrating that Bd was highly prevalent in northern Idaho during 2013 and 2014.

moderate to substantial relief, and elevation ranges from 530 to 2,350 m. Land uses are primarily managed temperate forest, but also include urban, rural developed, agricultural, and relatively pristine areas. The heavily forested area is characterized as supporting inland temperate rainforest (DellaSala, 2011) and is dominated by a diverse mix of conifer species. Coniferous trees dominated the vast majority of survey sites. There are five pond-breeding amphibians native to our study area, and one non-native species (American bullfrog, *Rana catesbeiana*) occurs in this area. Northern leopard frogs (*Lithobates pipiens*), historically very common, were extirpated from the study area in the 1980s (Lucid et al., 2016). Western toads (*Anaxyrus boreus*) may be in a current population decline, while other native species do not appear to be in the midst of demographic shifts (Lucid et al., 2022).

Climate in the study area is characterized by wet and moderately cold winters, wet and cool springs, and warm and dry summers. Winter snowpack is highly variable based on elevation, ranging from non-existent to persisting for several months at lower elevations, while higher elevations are characterized by deeper snowpacks that persist into early summer. Over the past century, several climatic trends have been documented in the study area. For example, minimum daily temperature has increased 1.6°C, average annual stream run off has increased by 33% (in part, due to a shift in timing from earlier snowpack melt), and 'March 1st snowpack' at lower elevations has decreased by 30% (Tinkham et al., 2015).

Sampling overview

Our study focuses on the Columbia spotted frog (*Rana luteiventris*), which is an abundant and highly aquatic species with IUCN status of

Least Concern (LC). The species has a wide distribution across elevations in the western US, ranging from British Columbia south to Nevada and Utah (Bos and Sites Jr, 2001). Within our study area, populations do not appear to be under population decline (Lucid et al., 2022). Columbia spotted frogs were sampled as part of a landscape level amphibian survey from April 22 through September 17 in 2013 and 2014 (n = 399 frogs). Adult Columbia spotted frogs were sampled for Bd at a total of 153 ponds (hereafter 'site'), defined as a lentic water body ≤500 m in diameter, with up to three frogs being sampled in each site (Lucid et al., 2016; Lucid et al., 2020). Each site was sampled only once. Skin swab samples were collected using sterile buccal swabs (MW fine-tipped plastic DrySwab; Medical Wire and Equipment, Wiltshire, England) to swab each appendage, and dorsal and ventral sides of the frog back and forth 15 times (30 total swipes). A new pair of vinyl gloves was used to handle each frog. Swab samples were placed in sterile 1.5 mL microcentrifuge tubes containing 95% ethanol in the field and stored at room temperature at a climate-controlled storage unit until DNA extraction. Because frogs were not rinsed prior to swabbing in this survey, there may be inclusion of transient environmental microbes in microbiome samples (Lauer et al., 2008). However, the dominant taxa in our samples are consistent with other amphibian skin microbiome studies that include rinsing (Walke et al., 2014; Hughey et al., 2017; Ellison et al., 2019a).

Detection of Batrachochytrium dendrobatidis (Bd)

To screen for prevalence and intensity of Bd infection, swabs were sent to Amphibian Disease Laboratory, San Diego Zoo Wildlife Alliance where DNA was extracted using PrepMan Ultra (Bletz et al., 2015) and amplified with a real-time Taqman quantitative Polymerase Chain Reaction (qPCR) assay as described in Boyle et al. (2004). Ethanol removal was performed as described in Hyatt et al. (2007). Samples were run in triplicate on an Applied Biosystems 7900HT thermocycler using 384 well plates with an exogenous internal positive control labeled with VIC (Applied Biosystems) for each sample to detect PCR inhibitors. Quantification standards were created by growing Bd isolate JEL 197 on 1% tryptone agar and harvesting zoospores by rinsing plates with 1X PBS. Standard curves were generated with ten-fold serial dilutions of extracted Bd DNA (range 1×10^{6}–1×10^{-2} zoospores). Replicate zoospore equivalent values were averaged for each sample and multiplied by 100 to account for dilutions.

Infection prevalence was defined as the proportion of individuals where Bd was detected, while infection intensity was defined as the number of Bd zoospore equivalents, a measure that estimates the number of zoospores present on an individual based on the number of gene copies found during qPCR. After exclusion of 23 Bd samples for equivocal results, Bd intensity and prevalence were calculated using 376 frogs.

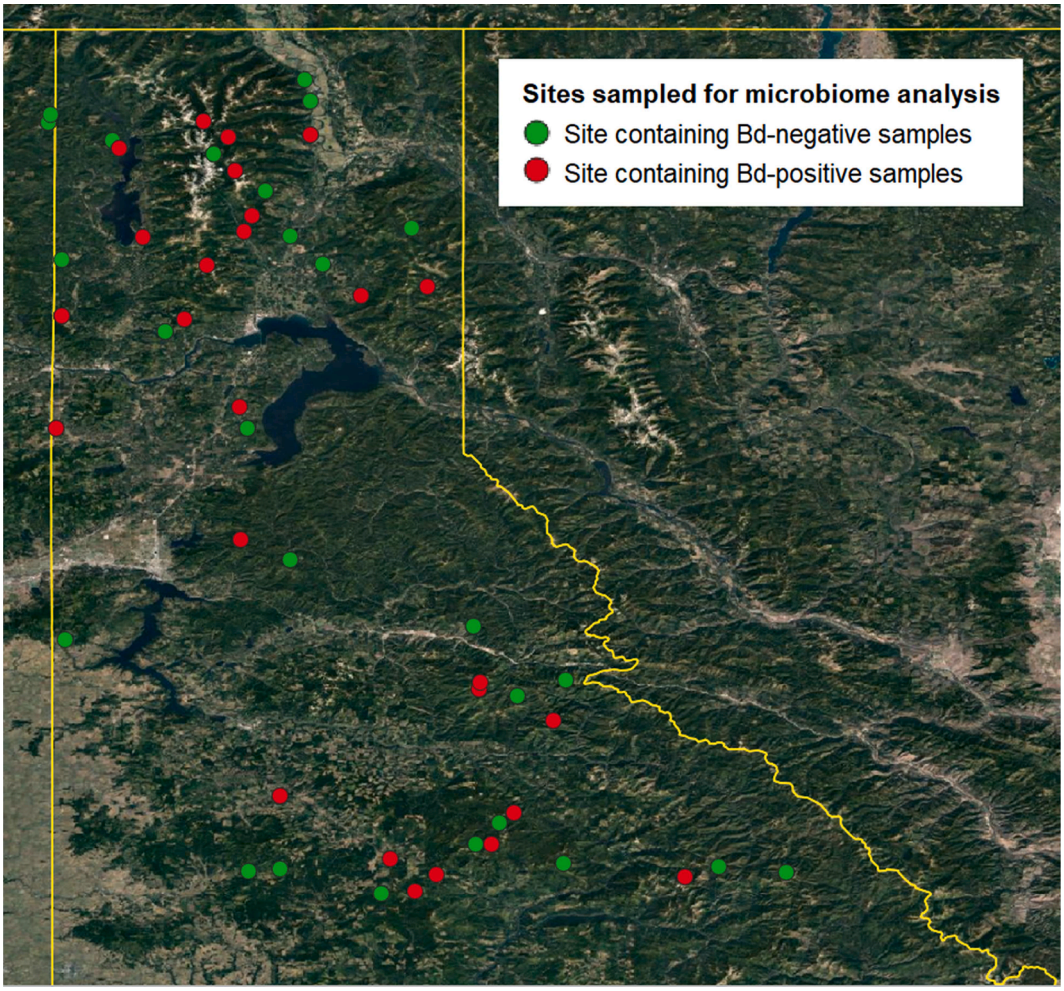

Fig. 2. Locations of Idaho/Washington 2013 and 2014 wetland sites selected for microbiome analysis, subsampled from sites tested for Bd presence (Fig. 1). Color of points indicates infection status (red = Bd-positive or green = Bd-negative) of samples gathered at the site. The selected sites cover a large geographic distribution in the northern Idaho panhandle. Image from QGIS.

Sample selection procedure for skin bacterial community analysis

Extracted DNA was processed for bacterial community analysis in the Walke Lab at Eastern Washington University. To determine if there was a correlation between Bd infection and skin bacterial communities, a subset of 92 samples (47 infected, 45 uninfected) were selected for 16S rRNA gene amplicon sequencing. A stratified process was used to create similar geographic distributions between the infected and uninfected samples to avoid differences in geography influencing comparisons between infected and uninfected frogs. This process was completed by first listing sites with two or more uninfected frogs, then narrowing down these sites by randomly choosing sites at different geographic locations (visualized by coordinates in open-source QGIS Geographic Information Systems software). These sites containing uninfected frogs were matched with sites in close geographic proximity (within 20 km and shorter distances prioritized) containing infected frogs (Fig. 2).

Bacterial community analysis

The skin bacterial community composition was characterized using amplicon barcoded sequencing of the V4-V5 regions of the 16S rRNA gene with primers 515F (barcoded) and 926R (Quince et al., 2011; Caporaso et al., 2012; Parada et al., 2016). PCR for each sample was performed in triplicate plus a negative control (all reagents excluding DNA). Volumes of reagents per 25 μl reaction were: 11.75 μl Qiagen UltraClean PCR grade H2O, 10 μl QuantaBio 5 Prime Hot Master Mix, 0.25 μl of 10μg/μl Bovine Serum Albumin (BSA), 0.5 μl of 10μM Illumina Forward primer + barcode 515F, 0.5 μl of 10μM Illumina Reverse primer 926R, and 2 μl of DNA from the sample (or PCR water for negative controls). Reactions were run in a thermocycler set for the following settings: 1) 94°C for 3 min, 2) 94°C for 45 sec, 3) 50°C for 1 min, 4) 72°C for 1.5 min, 5) 72°C for 10 min, and 6) hold at 4°C. Steps 2 to 4 were repeated for 35 cycles. Amplification of bacterial DNA fragments was confirmed using 1.5% agarose gel electrophoresis. DNA concentrations of samples were quantitated using a Qubit 4.0 fluorometer (Invitrogen™). Samples were pooled at equimolar concentrations, and the pooled product was cleaned using the Qiagen QIAquick PCR Purification Kit. The multiplexed sample was sequenced on the Illumina MiSeq platform using a single-end, 250bp approach at the Molecular Biology Core Facilities at Dana-Farber Cancer Institute at Harvard University. Sequences are available in NCBI's SRA database under BioProject ID PRJNA757404.

Bioinformatics

Microbiome bioinformatics were performed on sequencing data using QIIME 2 (Quantitative Insights Into Microbial Ecology) version 2019.10 up to the taxonomic assignment step and 2023.7 for all remaining steps (Bolyen et al., 2019). The QIIME 2 code utilized is available on GitHub (https://github.com/BenficaPhil/qiime-2-skin-microbiome). Single-read, demultiplexed sequence data were quality filtered using the q2-demux plugin and denoised using DADA2 via q2-dada2 (Callahan et al., 2016) at a quality threshold of Q25. Taxonomy was assigned using q2-feature classifier (Bokulich et al., 2018) with the classify-sklearn naïve Bayes taxonomy classifier against the SILVA release 138 99% Operational Taxonomic Units (OTUs) from 515F/926R region of sequences (Quast et al., 2012). Using q2-phylogeny, all amplicon sequence variants (ASVs) from DADA2 were aligned with mafft (Katoh et al., 2002) and used to construct a phylogeny with fasttree2 (Price et al., 2010).

The DADA2 feature table was filtered using q2-taxa to remove mitochondria, chloroplasts, eukaryotes, and unassigned sequences. After this step, the total number of sequences from all 92 samples was 2,362,289, with an average of 25,677 sequences per sample and range of 5134–60,643 sequences per sample. The total number of ASVs across all 92 samples was 25,393, with an average of 570 ASVs in each sample and a range of 86 to 1,578 ASVs per sample. Samples were rarefied to 12,165 sequences per sample to create an equal number of sequences per sample. The number of sequences was determined by using a rarefaction plot (Supplementary Fig. 1) to visualize the number of sequences typically required to represent the diversity of bacteria on an individual sample. Samples with fewer than 12,165 sequences were dropped, resulting in 84 (91.30%) of the 92 samples remaining in the subset. Alpha-diversity metrics, beta-diversity metrics, and Principal Coordinates Analysis (PCoA) were estimated using q2-diversity. Climatic data (mean, minimum, and maximum temperatures and precipitation at 4 km resolution) and elevation data used for diversity analyses were obtained from PRISM Climate Group (https://prism.oregonstate.edu/) (Prism Climate Group, 2014) by inputting each sample's coordinates and collection date.

Alpha diversity metrics

Alpha diversity metrics included Shannon's diversity index, richness (observed features, or ASVs), Faith's Phylogenetic Diversity (Faith PD), and Pielou's evenness. Shannon's diversity index is a measure that increases with increasing community richness and evenness. Observed features measures community richness with counts of ASVs. Faith PD is measure of community richness that incorporates phylogenetic relationships between the features (Faith, 1992). Evenness is a measure of how equal in number each ASV was in the sample. The non-parametric Wilcoxon signed-rank test was performed using q2-diversity to test for significant differences in alpha diversity between Bd infected and uninfected frogs. To test for correlations between alpha diversity metrics and the continuous variables of Bd infection intensity (zoospore equivalents), latitude, and longitude, the Spearman's rank correlation coefficient was used using q2-diversity.

Beta diversity metrics

To investigate differences in bacterial community composition between samples, the unweighted and weighted UniFrac beta diversity metrics were used. The UniFrac distance matrix is a technique developed for comparison of microbial communities in a phylogenetic context (Lozupone et al., 2007), with the unweighted UniFrac considering presence and absence of ASVs and the weighted UniFrac considering the relative abundance of ASVs. Permutational multivariate analysis of variance (PERMANOVA), a non-parametric statistical test that allows for analysis of non-normal multivariate data (Anderson, 2014), was used to determine whether microbiome profiles (based on UniFrac matrices) were distinct between infected and uninfected frogs. Principal Coordinates Analysis (PCoA) ordination was used to visualize the distances between samples. To test for differences in multivariate dispersion of community composition among frogs (comparing infected to uninfected), we used the PermDISP method as part of the q2-diversity beta-group-significance function. To analyze what proportion of variation could be explained by Bd zoospore equivalents, time, environment, and geography, the adonis2 function from vegan package version 2.6 (Anderson, 2001; Oksanen et al., 2007) was used in R, version 4.2.1 (R Core Team, 2022) to run a PERMANOVA incorporating a UniFrac distance matrix and the following formula: zoospore equivalents + year + month + mean temperature + maximum temperature + minimum temperature + precipitation + elevation + latitude + longitude + site.

Bacterial abundance analysis

To identify bacterial taxa that differed in relative abundance in infected and uninfected frogs, Linear Discriminant Analysis Effect Size (LEfSe) was used through the Galaxy web application (https://huttenhower.sph.harvard.edu/galaxy/), using the default parameters and an LDA effect size cutoff of >2.0. LEfSe is an algorithm for biomarker discovery which first uses the non-parametric Kruskal-Wallis test to detect

features with significant differential abundance. Features with statistical significance in the Kruskal-Wallis test ($p < 0.05$) are then analyzed using Linear Discriminant Analysis to estimate the effect size of each feature (Segata et al., 2011).

To investigate correlations between the relative abundance of individual bacterial taxa and infection intensity among Bd infected frogs, the Spearman's rank correlation coefficient test was used via the cor.test() function in the vegan R package. The top 10 most abundant taxa overall were analyzed to understand how abundance of dominant members of the microbiome may be influenced by Bd infection intensity. Visualizations of alpha diversity, beta diversity, PCoA ordination, LEfSe, and abundance correlations were produced using ggplot2 and dplyr functionality from the R package tidyverse 1.3.1 (Wickham et al., 2019).

Results

Bd prevalence and intensity

Bd was highly prevalent on Columbia spotted frogs across the study area. Of the 376 spotted frogs tested for Bd presence, 69% (261 frogs) tested positive and 31% (115 frogs) negative. Despite the relatively high prevalence, the infection intensity on individuals was typically low, with Bd zoospore equivalents ranging from 0 to 5,023 (All samples: Mean = 81, Median = 1; Bd-positive samples only: Mean = 127, Median = 5; Bd-positive samples in microbiome analysis subset: Mean = 382, Mean = 17).

Alpha diversity

Bd infection intensity (zoospore equivalents) was negatively correlated with Shannon diversity (Spearman's rank correlation coefficient, r_s = -0.22, p = 0.04, Fig. 3A) and bacterial evenness (r_s = -0.22, p = 0.048, Fig. 3B) among all sampled frogs, such that frogs with higher infection intensities tended to have lower bacterial diversity. No significant correlations with Bd infection intensity were found for richness, or observed features (r_s = -0.13, p = 0.24) and Faith PD (r_s = -0.16, p = 0.15).

There were no significant differences in alpha diversity between infected and uninfected frogs for all four metrics: Shannon diversity (Wilcoxon signed-rank test, H = 3.28, p = 0.07, Fig. 4A), observed features (H = 1.38, p = 0.24), Faith PD (H = 2.44, p = 0.12), and evenness (H = 3.03, p = 0.08, Fig. 4B).

As latitude increased, some measures of amphibian skin bacterial diversity also increased. Latitude was significantly positively correlated with observed features (r_s = 0.27, p = 0.01, Fig. 5A) and Faith PD (r_s = 0.28, $p < 0.01$, Fig. 5B). No significant correlation was found between latitude and Shannon diversity (r_s = 0.18, p = 0.10) or evenness (r_s = 0.11, p = 0.33). No significant correlations were found between longitude, elevation, minimum temperature, mean temperature, maximum temperature, and alpha diversity for all four metrics (all $p > 0.05$).

Beta diversity

The skin microbiomes of infected and uninfected frogs were distinct based on weighted UniFrac distance (PERMANOVA, pseudo-F = 1.83, p = 0.04, Fig. 6), but not based on unweighted UniFrac distance (pseudo-F = 1.21, p = 0.08). Dispersion did not differ between skin microbiomes of infected and uninfected frogs for both weighted (p = 0.9) and unweighted (p = 0.08) UniFrac metrics. Variation in skin bacterial communities could be explained, in part, by the following variables based on weighted UniFrac distance (Table 1): Bd zoospore equivalents (PERMANOVA, R^2 = 0.03, $p < 0.01$), year (R^2 = 0.03, $p < 0.01$), month (R^2 = 0.07, p = 0.01), mean temperature (R^2 = 0.02, p = 0.02), minimum temperature (R^2 = 0.02, p = 0.02), elevation (R^2 = 0.01, p = 0.02), latitude (R^2 = 0.02, $p < 0.01$), and site (R^2 = 0.58, $p < 0.01$). Maximum temperature, precipitation, and longitude did not significantly contribute to variation (all $p > 0.05$). For unweighted UniFrac (Table 2), variation was explained by: Bd zoospore equivalents (R^2 = 0.01, p = 0.03), year (R^2 = 0.02, $p < 0.01$), month (R^2 = 0.06, $p < 0.01$), mean temperature (R^2 = 0.01, p = 0.02), maximum temperature (R^2 = 0.01, p = 0.02), elevation (R^2 = 0.02, p = 0.02), latitude (R^2 = 0.02, $p < 0.01$), and site (R^2 = 0.56, $p < 0.01$). Minimum temperature, precipitation, and longitude did not significantly contribute to variation (all $p > 0.05$).

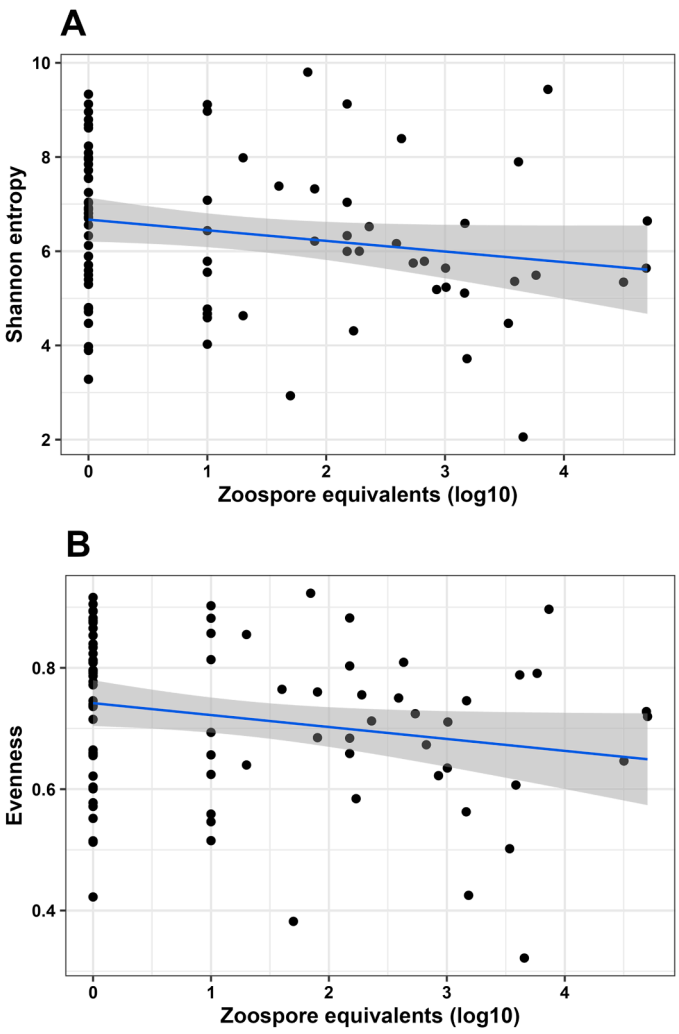

Fig. 3. Correlations between Bd infection intensity (visualized by log scale of zoospore equivalents) and skin microbial community (A) Shannon diversity (r_s = -0.22, p = 0.04) and (B) evenness (r_s = -0.22, p = 0.048). Generally, samples were found with low infection intensities, showing that even low levels of infection may possibly affect alpha diversity. However, there were no significant correlations when only Bd infected frogs were analyzed, meaning that the presence of the infection likely influences the microbiome, as opposed to the degree of the infection.

Relative abundances of bacterial taxa between infected and uninfected frogs

At the genus level, 7 bacterial genera had significantly higher relative abundance on infected frogs compared to uninfected frogs, while 24 genera had significantly higher relative abundance on uninfected frogs compared to infected frogs (Fig. 7). LDA effect size was greatest ($\geq$ 3.0) in infected frogs for *Stenotrophomonas* (Kruskal-Wallis, H = 4.54, p = 0.04), indicating increased abundance of *Stenotrophomonas* in infected frogs. LDA effect size was greatest in uninfected frogs for *Pseudomonas* (H = 5.14, p = 0.02), *Pantoea* (H = 3.95, p = 0.02), and *Sphingomonas* (H = 4.01, p = 0.03), indicaing increased abundance of these taxa in

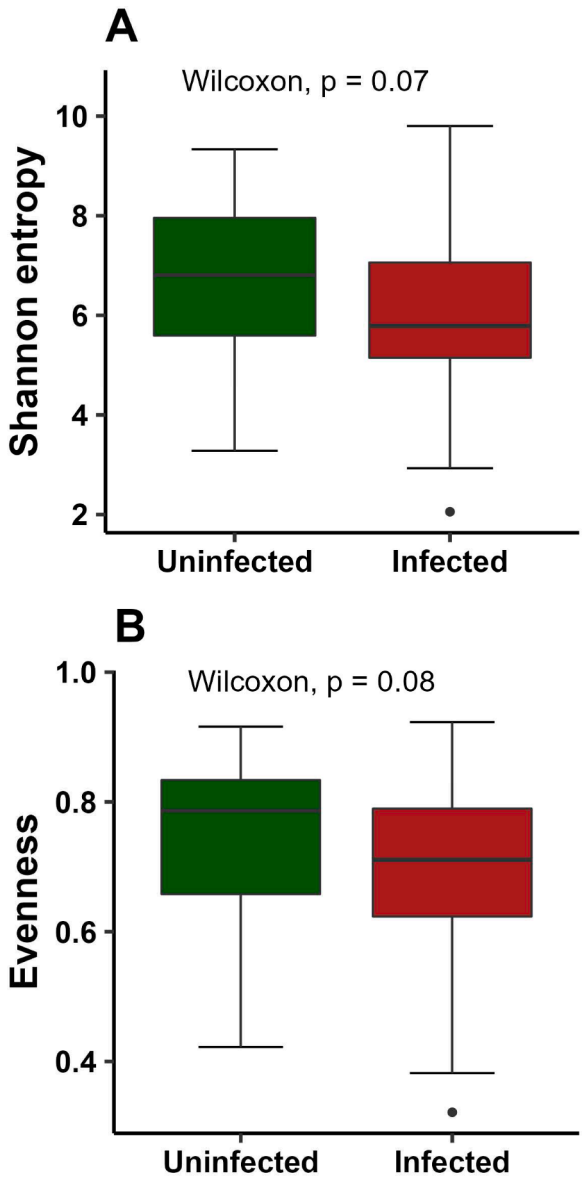

Fig. 4. Comparisons of alpha diversity between the microbiomes of infected and uninfected frogs. There was a trend of A) greater Shannon diversity (Spearman's rank correlation coefficient, H = 3.51, $p = 0.07$) and B) greater evenness (H = 3.55, $p = 0.08$) in uninfected frogs.

uninfected frogs.

Correlations between dominant taxa and infection intensity

The ten most abundant taxa overall at the genus level were an unclassified genus of Comamonadaceae, *Pseudomonas, Stenotrophomonas, Sanguibacter, Acinetobacter*, unclassified Enterobacteriaceae, *Streptococcus, Bacteroides*, unclassified Yersiniaceae, and *Bacillus* (Fig. 8). Furthermore, these taxa were analyzed for potential correlations with Bd infection intensity among infected frogs. Unclassified Comamonadaceae was significantly positively correlated with infection intensity ($r_s = 0.45$, $p < 0.01$, Fig. 9A), while unclassified Enterobacteriaceae was significantly negatively correlated (r_s = -0.35, $p = 0.02$, Fig. 9B). No significant correlations were found between infection intensity and the remaining dominant taxa among infected frogs: *Pseudomonas, Stenotrophomonas, Sanguibacter, Acinetobacter, Streptococcus, Bacteroides*, unclassified Yersiniaceae, and *Bacillus*.

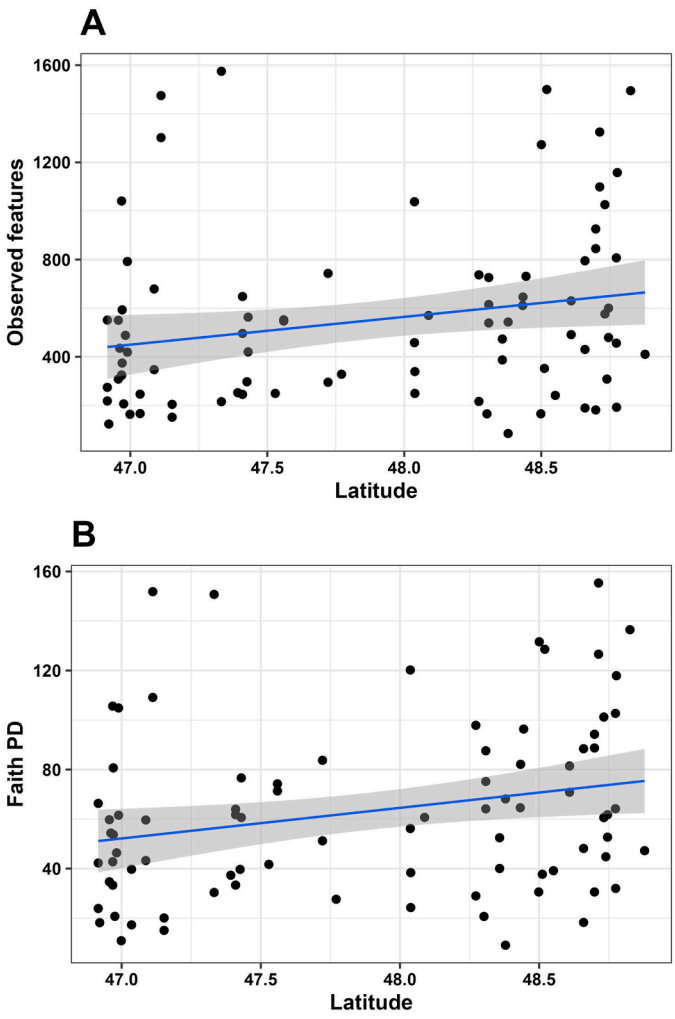

Fig. 5. Correlations between latitude and skin microbial community (A) Observed features (Spearman's rank correlation coefficient, $r_s = 0.27$, $p = 0.01$) and (B) Faith's Phylogenetic Diversity ($r_s = 0.28$, $p = 0.01$).

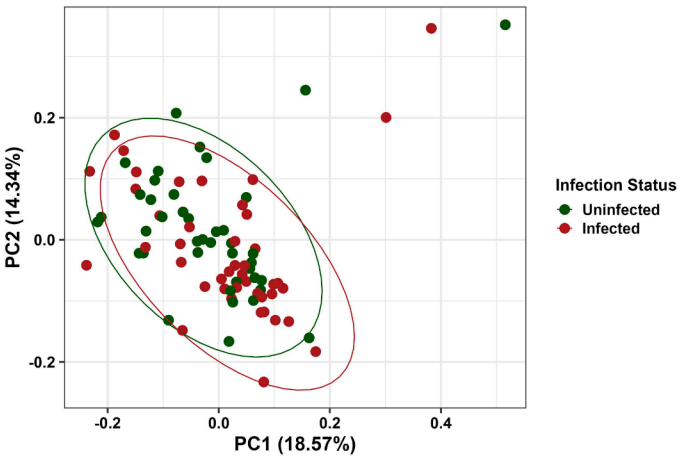

Fig. 6. Principal Coordinates Analysis (PCoA) ordination on weighted UniFrac distance matrix comparing the microbiome profiles of infected and uninfected Columbia spotted frogs. Microbiomes were distinct between infected and uninfected frogs (PERMANOVA, pseudo-F = 1.83, $p = 0.04$), however, there was no separate clustering of the two groups as displayed in the PCoA. A reduced model demonstrated other variables, primarily site, contributed to variation in the microbiome in our study.

Table 1
Adonis analysis (PERMANOVA under reduced model) results using a weighted UniFrac distance matrix and a formula with the following explanatory variables: zoospore equivalents, year, month, mean temperature, maximum temperature, minimum temperature, precipitation, elevation, latitude, longitude, and site. Site explained the majority of variation, while zoospore equivalents, year, month, mean temperature, minimum temperature, elevation, and latitude each explained small proportions of variation.

Variable	Df	SumOfSqs	R2	F	Pr(>F)
Zoospore equivalents	1	0.3562	0.01386	1.416	0.025 *
Year	1	0.4327	0.01684	1.7203	0.003 **
Month	4	1.4511	0.05648	1.4422	0.001 ***
Mean temperature	1	0.3682	0.01433	1.4638	0.02 *
Max. temperature	1	0.3779	0.01471	1.5025	0.017 *
Min. temperature	1	0.2771	0.01079	1.1016	0.18
Precipitation	1	0.3169	0.01233	1.2597	0.079
Elevation	1	0.3931	0.0153	1.5629	0.019 *
Latitude	1	0.5779	0.02249	2.2972	0.001 ***
Longitude	1	0.3092	0.01204	1.2292	0.095
Site	44	14.2904	0.55624	1.2911	0.001 ***
Residual	26	6.5402	0.25457		
Total	83	25.6909	1		

Table 2
Adonis analysis (PERMANOVA under reduced model) results using an unweighted UniFrac distance matrix and a formula with the following explanatory variables: zoospore equivalents, year, month, mean temperature, maximum temperature, minimum temperature, precipitation, elevation, latitude, longitude, and site. Site explained the majority of variation, while zoospore equivalents, year, month, mean temperature, maximum temperature, elevation, and latitude each explained small proportions of variation.

Variable	Df	SumOfSqs	R2	F	Pr(>F)
Zoospore equivalents	1	0.1851	0.02726	3.6156	0.001 ***
Year	1	0.2176	0.03205	4.2505	0.001 ***
Month	4	0.4962	0.07308	2.423	0.003 **
Mean temperature	1	0.1116	0.01643	2.1789	0.02 *
Max. temperature	1	0.0901	0.01327	1.7602	0.055
Min. temperature	1	0.1056	0.01555	2.0623	0.022 *
Precipitation	1	0.0499	0.00734	0.9739	0.451
Elevation	1	0.099	0.01458	1.9337	0.022 *
Latitude	1	0.1434	0.02111	2.8001	0.005 **
Longitude	1	0.0546	0.00804	1.0665	0.4
Site	44	3.9062	0.57526	1.734	0.001 ***
Residual	26	1.3311	0.19603		
Total	83	6.7904	1		

Discussion

Bd prevalence and intensity in northern Idaho and northeastern Washington

Within our study area, Bd was found to be highly prevelant but in generally low infection intensity among Columbia spotted frogs. Zoospore equivalent (ZE) values typically ranged from 1-1,000 ZEs, with only 7 samples recording over 1000 ZEs. Studies investigating populations with high infection intensities often observe individuals with around 10,000 ZEs (Becker et al., 2011; Vredenburg et al., 2010; Kinney et al., 2011), though standards for measuring zoospores can differ across different studies. One possible explanation for the observed low infection intensities is a strong immune response, as seen in highly resistant American bullfrogs (*Rana catesbeiana*), where adaptive immune genes and genes for antimicrobial peptides are more highly expressed compared to susceptible species (Eskew et al., 2018). Columbia spotted frogs appear to have been persisting with Bd for at least a decade prior to our study, with a 2004 survey in Latah County of north Idaho recording 60% or greater prevalence in all seasons sampled (spring, summer, and fall) (Russell et al., 2010), similar to the 69% found in our study. Outside of our study area, Bd has also been detected on Columbia spotted frogs in Oregon and Olympic National Park (Washington) in 2007 (Pearl et al., 2007), the Heber Valley population of Utah in 2002 (Wilson and Olsen, 2002), and Yellowstone National Park in northwestern Wyoming in 2000 (Green, 2004). Generally, Bd can persist at the same location for many years (Briggs et al., 2010; Lam et al., 2010; Phillott et al., 2013), allowing innate host defenses to evolve over time after initial outbreaks for populations to avoid declines or extinction (Briggs et al., 2010; Bates et al., 2018). In addition to genetically expressed immune responses, Bd inhibition by skin bacterial communities are another potential contributor to defenses.

Relationships between Bd infection and skin microbiome diversity

We hypothesized that bacterial diversity may correlate with infection intensities in Columbia spotted frogs, but that results would be limited because of the low infection intensities observed. Despite the low infection intensities, we observed correlations where Shannon diversity and evenness decreased as Bd ZE measurements increased. Richness metrics were not significantly correlated, suggesting evenness contributed to the pattern in Shannon diversity. In comparison between infected and uninfected frogs, alpha diversity metrics did not significantly differ, though we observed trends of decreased Shannon diversity and evenness in infected frogs which are consistent with the correlations observed with ZEs. Our beta diversity (weighted UniFrac) results suggest skin microbiomes were distinct between infected and uninfected frogs, and infection intensity had a significant effect on microbiome composition, although infection accounted for only 2.7% of variation. A manipulative study would be required to pinpoint the mechanisms behind these correlations, as both the skin microbiome and Bd are capable of affecting the other. One possibility is that higher evenness increased the likelihood of limiting zoospore loads, such as if higher evenness was beneficial for maintaining high proportions of Bd-inhibitory bacterial taxa (Walke et al., 2017; Ellison et al., 2019a). Another possibility is that Bd infection can still disturb the skin microbiome even at low intensities, as seen in a manipulative study on highly infected *Rana sierrae* (Jani and Briggs, 2014).

Relative abundances of potentially Bd-inhibitory bacteria differed between infected and uninfected frogs. Two notable differences were higher relative abundance of *Pseudomonas* in uninfected frogs and higher relative abundance of *Stenotrophomonas* in infected frogs. *Pseudomonas* was the second highest genus in overall relative abundance in our study and is known to include species that inhibit growth of Bd (Brucker et al. 2008; Becker et al. 2015b, Familiar López et al. 2017; Muletz-Wolz et al. 2017; Jiménez et al. 2019). It should be noted strain level variation in anti-Bd properties within genera of bacteria, even bacteria with 100% identical 16S rRNA sequences, may affect interactions of bacteria with Bd (Brucker et al., 2008; Walke et al., 2017; Kruger, 2020), making it uncertain what degree of anti-Bd properties characterizes our group of *Pseudomonas*. Nonetheless, the high *Pseudomonas* abundance could partly contribute to the low infection intensities observed. In Jani and Briggs (2014), where Bd infection was shown to disturb the microbiome, *Pseudomonas* was found to decrease in experimentally Bd-infected frogs and was negatively correlated with Bd load in frogs from the wild. Therefore, the higher relative abundance of *Pseudomonas* in uninfected frogs could be explained by one of two reasons or a combination of both: (1) its anti-Bd properties increased the likelihood of frogs remaining uninfected, and/or (2) disturbance of the microbiome by Bd resulted in decreased relative abundance among infected frogs. *Stenotrophomonas*, also known for potential in anti-Bd properties, was found in greater relative abundance in infected frogs on average, which interestingly was the opposite result than observed in the Jani and Briggs study. Multiple explanations are again possible, such as differences in Bd inhibition effectiveness or differing competition dynamics among bacteria due to the low infection intensities in our study.

The bacteria with greatest overall relative abundance in our study was an unclassified genus of Comamonadaceae, within the order

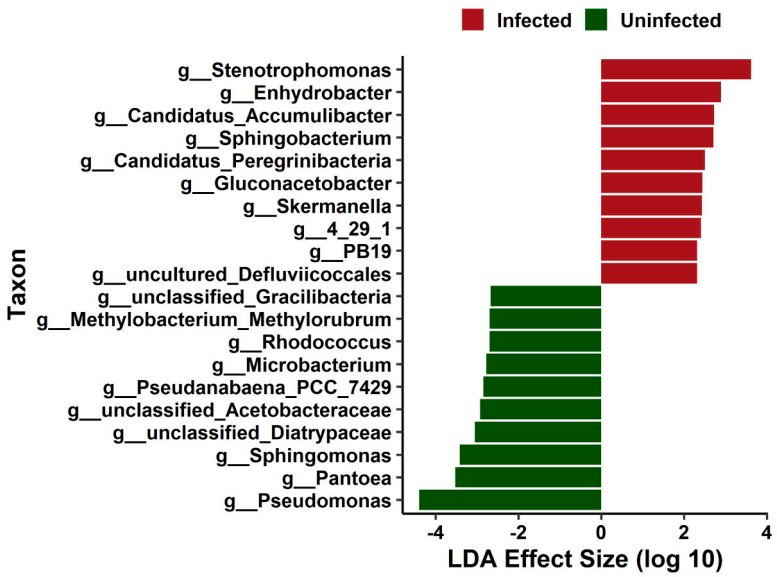

Fig. 7. LEfSe (Linear Discriminant Analysis Effect Size) plot displaying effect size of differentially abundant bacteria at the genus level. Up to 10 bacterial genera with the greatest effect sizes in each group (infected and uninfected) are listed. Most notably, *Stenotrophomonas* was greater in relative abundance in infected frogs on average, and *Pseudomonas* was greater in relative abundance in uninfected frogs on average, both of which are genera with potential of having antifungal properties.

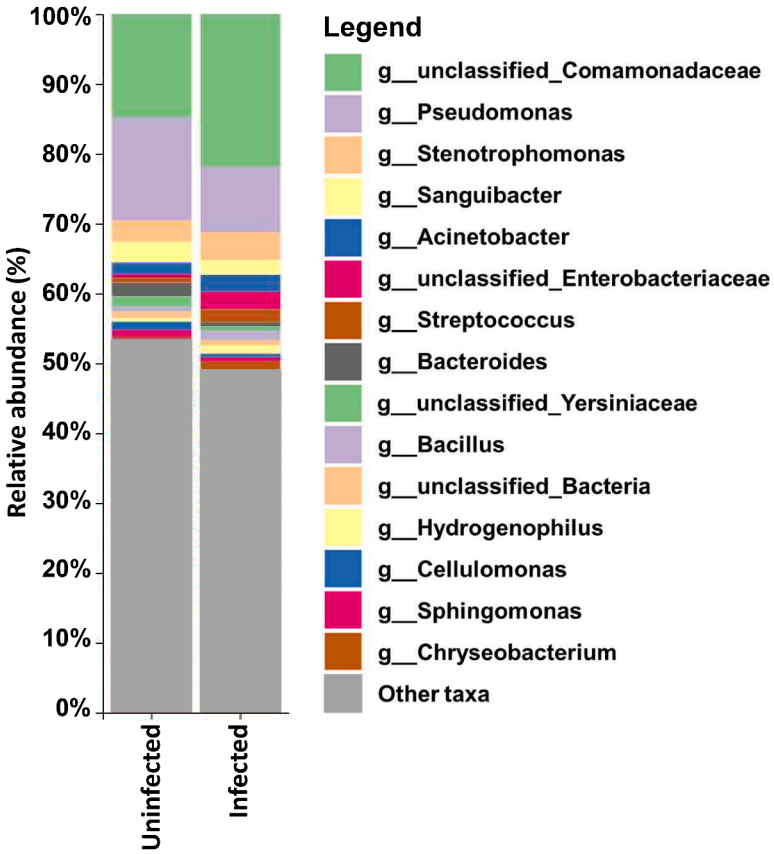

Fig. 8. Relative abundance barplot at the genus level comparing mean relative abundance of skin bacteria on uninfected (left bar, $n = 45$) and infected (right bar, $n = 47$) Columbia spotted frogs. The legend displays the 15 most abundant taxa overall, while all remaining taxa were colored in gray. As seen in LEfSe results (Fig. 7), *Pseudomonas* (lavender portion of bar) was significantly higher on average in relative abundance in uninfected frogs.

Burkholderiales, which made up over 25% of the microbiome in 30 individuals and reaching as high as 94.5% relative abundance in one individual. Among infected frogs in our study, the relative abundance of Comamonadaceae positively correlated with an increase in infection intensity. Other studies have found the order Burkholderiales to be capable of dominating the skin microbiome, especially in populations under high infection intensities (Jani and Briggs, 2014; Ellison et al., 2019a). In *Rana sierrae* frogs, Burkholderiales made up 90% of bacterial

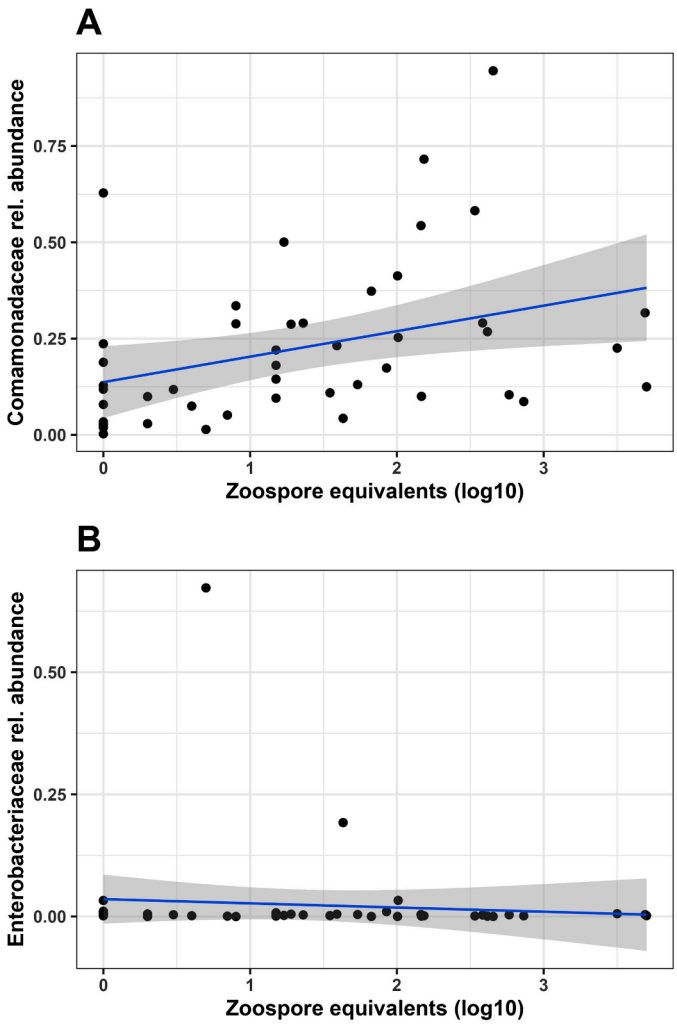

Fig. 9. Correlations between Bd infection intensity (visualized by log scale of zoospore equivalents) and relative abundance of (A) unclassified genus of Comamonadaceae ($r_s = 0.45$, $p < 0.01$) and (B) unclassified genus of Enterobacteriaceae ($r_s = -0.35$, $p = 0.02$).

DNA sequences in highly infected frogs, compared to only 54% in frogs with lower infection levels (Ellison et al., 2019a). Some bacteria within Burkholderiales are suspected to act as opportunistic colonists (Jani and Briggs, 2014), growing in abundance as the numbers of other taxa decrease. Although our dataset lacks highly infected frogs, our results indicate even low Bd infection levels could lead to a pattern of increase in Burkholderiales. In Ellison et al. (2019a), species richness and species evenness decreased as a result of other bacterial taxa decreasing in relative abundance as Burkholderiales dominated. Our results showed correlations of decreasing Shannon diversity and evenness as infection intensity increased, possibly indicating the growing relative abundance of Comamonadaceae as infection intensity rises is one factor influencing the overall diversity in our samples.

Influence of time, environment, and geography on the skin microbiome

Bd infection explained a small percentage of variation in weighted Unifrac distance (2.7%), showing that other factors played a role in shaping microbiome composition such as year (3.2%) and month (7.9%). Shannon diversity and evenness also varied temporally, being higher in 2013 compared to 2014. Temporal variation has been observed in the amphibian skin microbiome previously, with changes in climate (Bletz et al. 2017b; Familiar López et al. 2017) and horizontal transmission (Kruger and Roth, 2022) suggested as possible mechanisms. Interestingly, Bletz et al. (2017b) suggested that temporal variation observed in newts did not affect proportions of potentially Bd-inhibitory taxa. Environmental and geographic variables, including mean temperature (1.6%), minimum temperature (1.5%), elevation (1.5%), and latitude (2.1%) each accounted for significant, but small proportions of variation in weighted Unifrac distances. Latitude was also correlated with bacterial species richness (both observed features and Faith PD), where richness appeared to increase at higher latitudes. At a global scale, bacterial richness of amphibian skin microbiomes have also been positively correlated with latitude, with latitude correlated with climatic variables such as temperature and precipitation (Kueneman et al., 2019). More broadly, marine bacterial diversity has been found to peak at high latitudes in the winter (Ladau et al., 2013), showing that cold temperatures can be an influence on bacterial diversity. However, temperature and precipitation did not appear to be correlated with richness or evenness in our study.

Site explained the largest percentage of microbiome variation (57.5%) in our study, perhaps indicating that local habitat differences that could not be quantified to climatic or geographic variables had the greatest influence on microbiome composition. Although our per site sample size was limited, our results are in agreement with other studies determining site or habitat play an important role in skin microbiome variation (Bletz et al., 2017a; Bird et al., 2018; Jani and Briggs, 2018; Muletz Wolz et al., 2018; Ellison et al., 2019b; Walke et al., 2021). This variation is thought to result from transmission of environmental microbes, but this mechanism is not yet fully understood. Studies comparing amphibian skin microbiomes to environmental microbes from soil, pond water, or pond substrate have suggested that the skin microbiome differs from the environment, but may acquire rare environmental microbes (Walke et al., 2014; Bird et al., 2018). Our study focused on Columbia spotted frogs sampled from ponds; therefore, the water source may be an important factor in microbiome variation (Jani and Briggs, 2018). Within our study area, microclimate, topography (mountain vs. valley), and elevation can be highly variable (Lucid et al., 2021), which may contribute to habitat variation.

Conclusions

Bd was prevalent at low infection intensities across a 21,775 km^2 study area encompassing northern Idaho and northeastern Washington, and even with the relatively low infection intensities, we observed correlations of decreasing diversity and evenness with increasing Bd infection intensities on Columbia spotted frogs. Variation in microbiome profiles was explained by multiple factors, with Bd infection, time period, temperature, and elevation each explaining a small percentage of variation, while site accounted for the majority of variation. Further research should explore the directionality of the Bd-microbiome relationship and whether Bd infection can alter the microbiome at various intensities, or if the correlations observed are due to resistances to Bd (either innately or through the skin microbiome) in Columbia spotted frogs. Our results showing higher relative abundance of *Pseudomonas* in uninfected frogs and higher relative abundance of *Stenotrophomonas* in infected frogs indicate the possibility of Bd-inhibitory bacteria playing a role in resistance and limiting infection intensity, but further testing to confirm the function of these bacteria is required. Other studies have understandably investigated effects of Bd infection in susceptible amphibian populations facing decline to develop conservation strategies such as probiotics, however, populations with greater tolerance or resistance such as those in our study may also inform understanding of beneficial taxa and microbiome compositions that are stable under infection.

CRediT authorship contribution statement

Philip M. Campos: Conceptualization, Methodology, Investigation,

Formal analysis, Data curation, Writing – original draft, Visualization, Funding acquisition. **Michael K. Lucid:** Conceptualization, Methodology, Investigation, Resources, Writing – review & editing, Visualization, Supervision, Funding acquisition. **Shannon Ehlers:** Methodology, Investigation, Visualization. **Jenifer B. Walke:** Conceptualization, Methodology, Resources, Writing – review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Links and Accession Numbers for data and code are available in the manuscript text.

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Supplementary materials

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