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Microbial Dynamics in Amphibian Conservation and Disease Ecology

By

SONIA LORRAINE GHOSE

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Population Biology

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OFFICE OF GRADUATE STUDIES

of the

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DAVIS

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Abstract

The emerging fungal disease, chytridiomycosis, caused by the chytrid pathogen *Batrachochytrium dendrobatidis* (*Bd*), has contributed to declines or extinctions of hundreds of amphibian species globally. Recent evidence has elucidated the importance of skin microbiomes to *Bd* resistance, revealing the potential of microbial communities to assist in the conservation of endangered species. However, an understanding of the historical and present distribution of *Bd* is needed to identify where amphibians are at risk. Moreover, amphibian microbiomes require further investigation to determine the extent of their protective power and how it may be harnessed. My dissertation addresses gaps in our knowledge in both of these areas. Chapter One provides evidence for the recent emergence of *Bd* in African amphibians, identifying multiple *Bd* lineages infecting hosts and predicting *Bd* risk across the continent. Next, I present several studies on the skin microbiome of an endangered amphibian, the Sierra Nevada yellow-legged frog (*Rana sierrae*), including investigations of the impacts of restoration efforts for this species. In Chapter Two, I show that skin regions primarily infected by *Bd*, including ventral surfaces and hindfeet, harbor higher relative abundances of putatively *Bd*-inhibitory bacteria compared to the back of frogs, where *Bd* is often absent. In Chapter Three, I evaluate a common microbiome sampling protocol for amphibian skin and demonstrate that rinsing prior to swab collection does not significantly alter microbiomes detected. Chapter Four examines experimental *Bd* inoculation and antifungal treatment, describing short-term impacts on *R. sierrae* microbiomes and providing evidence for legacy effects of *Bd* to captive frog microbiomes post-treatment. In Chapter Five, I compare microbiomes and *Bd* infections of naturally persisting populations and restored populations consisting of frogs directly translocated from natural populations, frogs reintroduced from captivity, and descendants of relocated frogs. I provide evidence of higher *Bd* loads and lower

predicted *Bd*-inhibitory microbiome function in restored populations, which I hypothesize may be related to site parameters and host genotypes present at restored sites. I did not find evidence for associations of microbiomes or infections with history of specific restoration strategy within restored populations (*i.e.*, reintroduction versus translocation, and *Bd* inoculation treatment group post-reintroduction). Together, these microbiome studies suggest that the present environment outweighs individual identity or history in determining microbiome structure and diversity, though impacts of host genetics require investigation. This work also suggests that bacteria in the family Burkholderiaceae are important to *R. sierrae*, because of their dominance on the skin, their responsiveness to *Bd* infection, and their putative *Bd*-inhibitory function contributing to population-level differences in *Bd* loads.

Preface

The work presented in Chapter One of this dissertation has been previously published as a peer-reviewed journal article under a Creative Commons Attribution License (CC-BY). Chapters Two, Three, Four, and Five are original contributions. The reference for Chapter One is listed below, and its content has been modified from its original published form to fulfill formatting requirements.

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Table of Contents

Acknowledgements	ii
Abstract	v
Preface	vii
List of Tables	x
List of Figures	xii
Introduction	1
<i>References</i>	14
Chapter One: Continent-wide recent emergence of a global pathogen in African amphibians	22
<i>Abstract</i>	22
<i>Introduction</i>	23
<i>Methods</i>	27
<i>Results</i>	36
<i>Discussion</i>	40
<i>Acknowledgements</i>	48
<i>References</i>	50
<i>Tables and Figures</i>	59
<i>Supplementary Material</i>	71
Chapter Two: Skin microbiomes of captive Sierra Nevada yellow-legged frogs (<i>Rana sierrae</i>) vary among body regions and individuals	84
<i>Abstract</i>	84
<i>Introduction</i>	85
<i>Methods</i>	88
<i>Results</i>	102
<i>Discussion</i>	114
<i>Acknowledgements</i>	126
<i>References</i>	127
<i>Figures</i>	138
<i>Supplementary Material</i>	144
Chapter Three: To rinse or not to rinse? An evaluation of rinsing frogs prior to microbiome sampling reveals that impacts are largely insignificant	189

<i>Abstract</i>	189
<i>Introduction</i>	190
<i>Methods</i>	192
<i>Results</i>	197
<i>Discussion</i>	199
<i>Acknowledgements</i>	201
<i>References</i>	201
<i>Figures</i>	204
<i>Supplementary Material</i>	206
Chapter Four: Effects of experimental <i>Batrachochytrium dendrobatidis</i> inoculation and itraconazole treatment on the skin microbiome of Sierra Nevada yellow-legged frogs (<i>Rana sierrae</i>)	213
<i>Abstract</i>	213
<i>Introduction</i>	214
<i>Methods</i>	217
<i>Results</i>	231
<i>Discussion</i>	242
<i>Acknowledgements</i>	255
<i>References</i>	255
<i>Tables and Figures</i>	263
<i>Supplementary Material</i>	273
Chapter Five: Characterizing the impacts of restoration efforts on skin microbiomes of Sierra Nevada yellow-legged frogs (<i>Rana sierrae</i>) across restoration histories and sites	291
<i>Abstract</i>	291
<i>Introduction</i>	292
<i>Methods</i>	296
<i>Results</i>	311
<i>Discussion</i>	331
<i>Acknowledgements</i>	349
<i>References</i>	349
<i>Figures</i>	359
<i>Supplementary Material</i>	372

List of Tables

Table 1-1. Compiled historical and recent frequency of <i>Batrachochytrium dendrobatidis</i> (<i>Bd</i>) occurrence by African country and region.	59
Table S1-1. Summary of all <i>Bd</i> data for Africa by decade.	74
Table S1-2. Summary of Cameroonian <i>Bd</i> data by decade	75
Table S1-3. Summary of Kenyan <i>Bd</i> data by decade	76
Table S1-4. Histology results	77
Table S1-5. Genotyped <i>Bd</i> swab samples	79
Table S2-1. For which pairwise comparisons between frogs and environment types does alpha diversity differ?	144
Table S2-2. Which frog individuals harbor differences in alpha diversity?	145
Table S2-3. Which frog body regions harbor differences in Shannon diversity?	148
Table S2-4. Do Beta diversity metrics differ among frogs and environment sample types?	150
Table S2-5. For which pairwise comparisons of frogs and environment sample types do Beta diversity metrics differ?	151
Table S2-6. Do frog and environment samples harbor different group dispersions in Beta diversity metrics?	153
Table S2-7. Which pairwise comparisons of frog and environment samples harbor different group dispersions in Beta diversity metrics?	154
Table S2-8. Which metadata factors explain variation in Beta diversity metrics across frog samples?	156
Table S2-9. Which frog individuals harbor differences in Beta diversity?	157
Table S2-10. Which frog body regions harbor differences in Beta diversity?	163
Table S2-11. Do frog individuals harbor differences in group dispersions of Beta diversity?	172
Table S2-12. Which frog individuals harbor different group dispersions in Beta diversity?	173
Table S2-13. Do frog body regions harbor different group dispersions in Beta diversity?	176
Table S2-14. Which pairwise comparisons of frogs and environment sample types harbor different relative abundance of the family Burkholderiaceae?	177
Table S2-15. Which pairwise comparisons of frog and environment sample types harbor different relative abundances of amplicon sequence variants?	178
Table S2-16. Which frog individuals harbor different relative abundances of amplicon sequence variants?	179
Table S2-17. Which frog body regions harbor different relative abundances of amplicon sequence variants?	182
Table S2-18. Is SV1 relative abundance different from SV2 relative abundance within each frog individual?	185
Table S2-19. Is SV1 relative abundance different from SV2 relative abundance within each frog body region?	186
Table S2-20. Which amplicon sequence variants are differentially abundant between ventral body regions and the dorsal back region?	187
Table S3-1. Does mean unrarefied sample depth differ by sample type?	206
Table S3-2. Does observed richness differ by sample type?	207
Table S3-3. Does Shannon diversity differ by sample type?	208
Table S3-4. Does community structure differ by sample type?	209
Table S3-5. Which sample types harbor distinct community structure?	210
Table S3-6. Does community dispersion differ by sample type?	211
Table S3-7. Which sample types differ in community dispersion?	212
Table 4-1. Taxa identified to differ in normalized relative abundance across inoculation status groups	263
Table S4-1. For which pairwise comparisons of inoculation status groups does alpha diversity differ?	273

Table S4-2. Does community structure differ among subgroups of pre-treatment frog samples?	274
Table S4-3. Does community dispersion differ between pre-treatment time points?	275
Table S4-4. Does community structure differ between survival groups of post-infection frog samples?	276
Table S4-5. Does community structure differ among subgroups of post-treatment frog samples?	277
Table S4-6. Does community dispersion differ among subgroups of post-treatment frog samples?	278
Table S4-7. For which pairwise comparisons of post-treatment subgroups does community structure differ?	279
Table S4-8. Does community structure differ among inoculation status groups?	280
Table S4-9. For which pairwise comparisons of inoculation status groups does community structure differ?	281
Table S4-10. Does community dispersion differ among inoculation status groups?	282
Table S4-11. Which inoculation status groups harbor differences in community dispersion?	283
Table S4-12. What is the relative importance of treatment factors to variation in community structure?	284
Table S4-13. Differentially abundant amplicon sequence variants across inoculation status groups	285
Table S5-1. Wild frog captures and sample counts	372
Table S5-2. Several metrics do not differ significantly among unique frog individuals	373
Table S5-3. Frog-associated microbiome structure does not differ among unique frog individuals	374
Table S5-4. Differences in host-associated metrics among site types and sites	375
Table S5-5. Differences in host-associated metrics among restoration types	377
Table S5-6. Frequency of <i>Bd</i> occurrence across wild-collected frog samples	378
Table S5-7. <i>Bd</i> status does not differ among field site types and sites	380
Table S5-8. <i>Bd</i> loads differ by field site type and site	381
Table S5-9. Correlation of wild-captured frog <i>Bd</i> loads with other factors	382
Table S5-10. Correlation of wild-captured frog <i>Bd</i> loads (across <i>Bd</i> statuses) with other factors	383
Table S5-11. <i>Bd</i> status does not differ by frog restoration type	384
Table S5-12. <i>Bd</i> loads differ by frog restoration type	385
Table S5-13. Microbiome structure differs between frogs and water for all sites	386
Table S5-14. Microbial community dispersion differs between frogs and water for most sites	387
Table S5-15. PERMANOVA models for frog microbiome structure	388
Table S5-16. Frog microbiome structure differs in most pairwise comparisons of site types and sites	389
Table S5-17. Frog microbiome dispersion differs by site type and site	390
Table S5-18. Frog microbiome dispersion differs in few pairwise comparisons of site types and sites	391
Table S5-19. PERMANOVA models for water-associated microbiome structure	392
Table S5-20. Water-associated microbiome structure differs in most pairwise comparisons of site types and sites	393
Table S5-21. Water-associated microbial community dispersion differs by site type and site	394
Table S5-22. Water-associated microbial community dispersion largely does not differ in pairwise comparisons of site types and sites	395
Table S5-23. PERMANOVA model for wild-captured frog microbiome structure	396
Table S5-24. Frog microbiome structure differs by restoration type	397
Table S5-25. Differences in frog microbiome structure in pairwise comparisons of restoration types	398
Table S5-26. Frog microbiome dispersion differs by restoration type	399
Table S5-27. Frog microbiome dispersion largely does not differ in pairwise comparisons of restoration types	400
Table S5-28. Frog microbiome structure differs by <i>Bd</i> inoculation treatment group in captivity but not after reintroduction	401
Table S5-29. Captive frog microbiome dispersion differs between <i>Bd</i> inoculation treatment groups	402
Table S5-30. Differences in water-associated microbial metrics among site types and sites	403
Table S5-31. Taxonomy of predicted anti- <i>Bd</i> community members	404
Table S5-32. Anti- <i>Bd</i> prediction metrics do not differ between <i>Bd</i> inoculation treatment groups in captivity or after reintroduction	407

List of Figures

Figure 1-1. Distribution and frequency of <i>Batrachochytrium dendrobatidis</i> (<i>Bd</i>) occurrence in Africa 1852-2017.	64
Figure 1-2. Genotyping of <i>Batrachochytrium dendrobatidis</i> (<i>Bd</i>) lineages	67
Figure 1-3. Predicted <i>Bd</i> disease risk in Africa	70
Figure S1-1. Images of histological samples	81
Figure S1-2. Unique alleles from newly genotyped <i>Bd</i> swab samples	83
Figure 2-1. Diagram of <i>Rana sierrae</i> body regions sampled	138
Figure 2-2. Does alpha diversity vary across sample groupings?	139
Figure 2-3. Does beta diversity vary across sample groupings?	141
Figure 2-4. How do mean relative abundances of top represented bacterial families vary across samples?	142
Figure 2-5. How do mean relative abundances of two dominant frog-associated taxa vary across frog samples?	143
Figure S2-1. Mean relative abundance of Burkholderiaceae taxon identified to be differentially abundant between body regions	188
Figure 3-1. Within-sample diversity is not affected by rinsing frogs with sterile water and varies between frogs and tank water	204
Figure 3-2. Community structure does not differ between unrinsed and rinsed frog samples but is distinct between tank water and frog samples	205
Figure 4-1. Experimental design and microbiome sample collections	265
Figure 4-2. Images of <i>Rana sierrae</i> at the San Francisco Zoo before and after treatments highlight impacts to skin coloration	267
Figure 4-3. Within-sample diversity differs among inoculation status groups	268
Figure 4-4. Microbial community structure within and among experimental phases	269
Figure 4-5. Patterns of differential relative abundance for amplicon sequence variants across inoculation status groups	271
Figure S4-1. Alpha diversity of pre-treatment frog samples does not differ due to treatment category or time point	287
Figure S4-2. Alpha diversity of post-infection frog samples does not differ due to survival	289
Figure S4-3. Within-sample diversity of post-treatment frogs does not differ between treatment categories	290
Figure 5-1. Study design	359
Figure 5-2. Mean <i>Bd</i> loads of wild-captured frog samples across sites and restoration types	361
Figure 5-3. Microbiome structure across sites and restoration types	362
Figure 5-4. Microbiome diversity across sites and restoration types	364
Figure 5-5. Frog-associated anti- <i>Bd</i> predictions across sites and restoration types	366
Figure 5-6. Relative abundance of bacterial phyla and families across captive, reintroduced, and new recruit frog samples	368
Figure 5-7. Changes in microbial communities over time for reintroduced frogs relative to natural variation	370
Figure S5-1. Rarefaction curves across sample categories	408
Figure S5-2. Generating scaled mass indices (SMIs)	409
Figure S5-3. Scaled body mass index (SMI) across sites and restoration types	411
Figure S5-4. Microbial community structure of sample categories (frog vs. water) by site	412
Figure S5-5. Environmental data by site types and sites	414
Figure S5-6. National maps of study sites in the Desolation Wilderness	416

Figure S5-7. Microbiome structure of experimental <i>Bd</i> inoculation treatment groups for captive and reintroduced frogs	418
Figure S5-8. Within-sample diversity of sample categories (frog vs. water) by site	419
Figure S5-9. Putative anti- <i>Bd</i> richness compared between sample categories (frog vs. water) by site	421
Figure S5-10. Putative anti- <i>Bd</i> relative abundance compared between sample categories (frog vs. water) by site	422
Figure S5-11. Water-associated anti- <i>Bd</i> predictions among sites	423
Figure S5-12. Correlation of frog-associated anti- <i>Bd</i> and SV1 relative abundances	425
Figure S5-13. Mean relative abundance of bacterial phyla and families across site types, sites, and restoration types	426
Figure S5-14. Mean relative abundance of bacterial phyla and genera across site types, sites, and restoration types	428
Figure S5-15. Relative abundance of bacterial phyla and families across experimental <i>Bd</i> inoculation treatment groups	430
Figure S5-16. Sample values of microbial metrics for natural frogs, captive frogs, and reintroduced frogs across time intervals	432

Introduction

This dissertation is composed of five chapters that I completed in Jonathan Eisen's laboratory at the University of California, Davis. Prior to and early on in graduate school, I had several experiences that ignited my interests related to host-pathogen-microbiome interactions and their role in the conservation of endangered species. These led me to develop the questions that I address within my thesis. My first chapter describes the emergence of a fungal pathogen in African amphibians. My second and third chapters characterize putative microbial defenses of an endangered amphibian species and consider microbiome sampling methods. Finally, my fourth and fifth chapters examine impacts of restoration efforts for this species to their associated microbiomes and fungal infections. The background and main findings for these studies are described below.

Emerging infectious diseases

In the spring of 2009, as an undergraduate at Occidental College, I studied abroad in a field-based tropical biology program in Costa Rica. I was enamored by the incredible diversity of amphibians we observed in forests throughout the country. Each night, I was soothed by their cacophony of calls. However, when we traveled to a montane cloud forest in Monteverde, I remember noting a marked reduction in frog sightings and in their nighttime refrains. It was there that I attended a lecture by Alan Pounds, a scientist at the Monteverde Cloud Forest Preserve. Pounds told us about the disease chytridiomycosis, caused by the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*), that was decimating amphibians globally. For example, in Monteverde, extinction of endemic golden toads (*Incilius periglenes*) in 1989 was most likely

driven by *Bd* (Cheng et al., 2011; Pounds et al., 2006). *Bd* infects the skin of amphibians, an organ they use to mediate gas exchange, electrolyte balance, and water homeostasis. Chytridiomycosis, therefore, causes cascading physiological impacts that often result in mortality (Berger et al., 1999, 1998; Voyles et al., 2009). Pounds explained that *Bd* most often has the greatest impacts at mid-high altitudes with cooler climates, contributing to why Monteverde had experienced such dramatic losses (Pounds et al., 2006). However, there were also non-montane species affected even at sea level, making predictions of which species would be most threatened quite complicated (Fisher et al., 2009).

Chytridiomycosis is an example of an emerging infectious disease (EID). Diseases are considered emerging if their distribution or the virulence of causative pathogens has recently increased, and/or if disease is found in novel host species and populations (Olsen et al., 2011). There are many causes of EIDs, and there are examples of EIDs across most animal and plant systems (Olsen et al., 2011). Most studied EIDs are caused by bacterial and viral pathogens, however emerging fungal pathogens in particular are especially problematic. It has been proposed that fungal pathogens are more deadly than other causative agents of disease because they are not constrained by the same evolutionary dead ends; many fungal pathogens of animals can survive in the environment, grow on other substrates, or can go dormant, so driving host organisms to extinction is not selected against (Olsen et al., 2011).

Prediction and control of fungal pathogens is quite difficult, in large part because the diversity of fungi is not well studied. For example, the first observations of *Bd*-driven amphibian declines were in Australia and central America in the 1970's and 1980's, respectively (Berger et al., 1998; Cheng et al., 2011; Laurance et al., 1996). However, *Bd* was not identified and described until 1999 (Longcore et al., 1999). *Bd* is in the little-known phylum Chytridiomycota, and within

that phylum, *Bd* is unique (Longcore et al., 1999). *Bd* and *Batrachochytrium salamandrivorans* (*Bs*, a closely related pathogen primarily impacting salamander species) are the only chytrid fungi known to infect keratinized skin of living amphibians (Fisher et al., 2009; Martel et al., 2013). Further, only one other chytrid fungus is known to be pathogenic to vertebrates (Berger et al., 1998; Fisher et al., 2009).

There have been significant increases in fungal EIDs in the 20th century and beyond due to changes in climate, weather events, and human mediated movement. All of these factors have contributed to reduced constraints on pathogens and to susceptibility of naive hosts (Olsen et al., 2011). For example, in amphibians, virulence of *Bd* is inversely related to temperature, possibly in a species-dependent manner (Fisher et al., 2009; Walker et al., 2010). Therefore, climate change may have played a role in its success (Pounds et al., 2006).

Trade and movement of humans have caused movement of pathogens for thousands of years, however globalization in the 20th century increased the magnitude and speed at which this occurred (Olsen et al., 2011). For example, the fungal-like oomycete associated with sudden oak death (*Phytophthora ramorum*) was introduced to North America via trade of ornamental plants (Olsen et al., 2011). *Pseudogymnoascus destructans* (*Pd*), the causative agent of white-nosed syndrome, was introduced to North American bats via movement by humans (Leopardi et al., 2015; Wibbelt et al., 2010). We also know that *Bd* was introduced globally via anthropogenic trade and movement (O’Hanlon et al., 2018). Trade of African clawed frogs (*Xenopus laevis*), a *Bd*-resistant amphibian species that acts as a reservoir, began in the 1930’s and has increased since then, moving *Bd* around the world with it (Olsen et al., 2011; Weldon et al., 2007, 2004). From 1998-2004, >70,000 animals were exported out of South Africa to 30 countries around the world (Weldon et al., 2007). Another resistant reservoir species, *Lithobates catesbiana* (the North

American bullfrog), is a part of the global food trade along with over 200 other amphibian species, and high proportions of traded *L. catesbiana* have been shown to harbor *Bd* (Olsen et al., 2011; Schloegel et al., 2009). Further, there is evidence of anthropogenic involvement in movement of the pathogen on a regional scale. For example, in the Sierra Nevada of California, *Bd* invaded areas more highly trafficked by humans (e.g., in Yosemite National Park), decades earlier than less accessible areas (e.g., in Sequoia-Kings Canyon National Parks; Vredenburg et al., 2019).

Major questions across many systems impacted by fungal EIDs are where the causative pathogen came from, where it is spreading, and where it may cause further problems. For example, these were the early questions associated with white-nosed syndrome upon its appearance in North American bat populations in 2006. In that case, the fungal pathogen *Pd* was found to have originated in Europe (Leopardi et al., 2015; Olsen et al., 2011). For *Bd*, one early hypothesis was that it originated in Africa and had been introduced globally via *X. laevis* exports (Weldon et al., 2004). However, information on the past and present distribution of *Bd* in Africa was incomplete and had not been fully synthesized. Further, *Bd* actually consists of several divergent lineages, including *Bd-GPL* (associated with most amphibian declines and extinctions), *Bd-CAPE* (thought to have originated in South Africa), multiple lineages from Asia, and others (Byrne et al., 2019; O’Hanlon et al., 2018), and genetic evidence showed that *L. catesbiana* in the eastern United States harbored *Bd* lineages with higher genetic diversity than *X. laevis* associated *Bd* (James et al., 2009). This evidence suggested that Africa was not the origin. It took nearly two decades from the discovery of *Bd* to determine that the ancestral lineage of all extant *Bd* lineages likely originated on the Korean peninsula, based on high levels of diversity in *Bd* lineages found there (O’Hanlon et al., 2018).

Prior to that discovery, as a post-bac in 2012, I began working on a project to describe the historical and recent distribution of *Bd* in African amphibians for David Blackburn (at the time, a curator at the California Academy of Sciences) and Vance Vredenburg (professor at San Francisco State University). We sought to assess whether *Bd* was endemic or emerging on the African continent. In 2014, I went to UC Davis to work on topics related to the amphibian-*Bd* system for my thesis. While in graduate school, I had the opportunity to lead a collaborative effort to expand on the African *Bd* project, which is presented in **Chapter One: “Continent-wide recent emergence of a global pathogen in African amphibians.”** Together, we published this work in *Frontiers in Conservation Science* (Ghose et al., 2023). The main findings were that (1) *Bd* recently emerged after the year 2000 in African amphibians throughout much of the continent, (2) we found multiple lineages of *Bd* infecting African amphibians, including *Bd-GPL*, *Bd-CAPE*, and possible hybrid lineages, and (3) we suggested that *Bd-CAPE* may be associated with documented declines of amphibian species in Cameroon (Ghose et al., 2023; Hirschfeld et al., 2016). Our work also includes predictions of *Bd* risk to amphibians across the African continent.

Microbial defenses

In 2011, before working on the African *Bd* project at the California Academy of Sciences, I volunteered in Vance Vredenburg’s lab at San Francisco State University to assist on other various studies of *Bd* and amphibian declines. One project was related to the recent finding that a bacterium naturally associated with certain amphibian species, *Janthinobacterium lividum*, was able to inhibit *Bd* growth (Harris et al., 2009a). Vredenburg applied this knowledge to the only population of the endangered Sierra Nevada yellow-legged frog (*Rana sierrae*) remaining in Dusy Basin (Sequoia-Kings Canyon National Parks, CA). Using *J. lividum* isolated from a frog at the

Dusy population, they inoculated 80% of individuals in 2010 (Vredenburg et al., 2011). I assisted on a field trip in 2011 to determine whether treatment had improved survival after *Bd* invaded the previous year. Finding that surviving frogs had received *J. lividum* treatment was a turning point in my interests related to this disease system. I became fascinated by host-pathogen-microbiome interactions, and later joined Jonathan Eisen's lab to work on these interactions for my thesis.

Microbiomes in many systems are known to contribute to host health and defense against pathogens (Cho and Blaser, 2012; Lee and Hase, 2014; Oever and Netea, 2014). One mechanism is that specific microbes can inhibit pathogens and control infection loads via production of antifungal metabolites, like in the case of *J. lividum* and *Bd* (Harris et al., 2009a). There are many examples of this in other host-pathogen systems. For example, several soil fungi have been found to inhibit growth of *Phytophthora infestans*, the causative agent of late blight in potatoes, and the bacterium *Pseudomonas fluorescens* has been shown to inhibit *Pd*, the causative agent of white-nosed syndrome in North American bats (Hoyt et al., 2015; Subhani, 2016). However, when applied to hosts, probiotics can be effective for certain host species and not others. For example, *J. lividum* was not protective in Panamanian golden frogs (*Atelopus zeteki*), possibly because it is not found on those frogs naturally (Becker et al., 2011). This emphasizes the need to characterize microbiomes of specific host species to determine which microbes will effectively inhibit target pathogens.

Further, the structure of the whole microbiome is related to health and resistance to disease. One study found that the structure of the amphibian skin microbiome prior to *Bd* infection predicted whether individuals would survive or succumb to disease (Becker et al., 2015). Similar examples exist in other systems, such as in humans, where functional signatures of microbiomes can be used to predict disease (Armour et al., 2019; Liu et al., 2024). In gnotobiotic mice, the host

immune system does not develop properly without the gut microbiome intact (Robinson et al., 2010). This indicates that there are direct impacts of the microbiome on the ability of the host to respond to pathogens (Robinson et al., 2010).

The mechanisms involved in whole-microbiome defenses against pathogens are somewhat enigmatic, as microbiomes include hundreds to thousands of community members, making interactions quite complex. Though mechanisms likely involve a combination of impacts to host immune systems, inhibition of pathogens via microbially produced metabolites (*i.e.*, interference competition), synergistic effects of multiple community members that control infection, and direct competition for space among community members and pathogens (Fraune et al., 2014; Hibbing et al., 2010; Mittelbach, 2012; Robinson et al., 2010). Describing host-associated microbiomes is an important step to elucidating their interactions with hosts and pathogens, which could be applied to manipulating their structure and function for the conservation of endangered wildlife.

I, therefore, sought to characterize the skin microbiomes of captive adult Sierra Nevada yellow-legged frogs (*Rana sierrae*) housed at the San Francisco Zoo. *R. sierrae* is a highly endangered federally listed species in the Sierra Nevada of California, where declines and population extinctions have been driven by a combination of introduced fish and *Bd* invasions (Vredenburg et al., 2010, 2007). Jessie Bushell (Director of Conservation at the San Francisco Zoo) reared frogs to adulthood at the Zoo as part of the restoration efforts for the species. My studies of these captive-reared frogs contribute to a baseline understanding of their associated microbiomes, and to methods used to sample their microbiomes.

In Chapter Two: “Skin microbiomes of captive Sierra Nevada yellow-legged frogs (*Rana sierrae*) vary among body regions and individuals,” I characterized microbiomes of ten distinct body regions of captive-reared frogs. The main findings of this work were that (1)

microbiomes of body regions known to be primarily infected by *Bd* had significantly different community structure with increased relative abundances of putatively *Bd*-inhibitory bacteria relative to body regions that are most often uninfected by *Bd* (Berger et al., 2005, p. 200; North and Alford, 2008; Pessier et al., 1999; Woodhams et al., 2015), and (2) individual variation in the microbiome was primarily associated with differences in their local environment (*i.e.*, most differences were between individuals housed in distinct tank aquaria).

In Chapter Three: “To rinse or not to rinse? An evaluation of rinsing frogs prior to microbiome sampling reveals that impacts are largely insignificant,” I investigated the importance of including a frog rinsing step prior to microbiome sample collection (applied to remove “transient” bacteria; Culp et al., 2007; Lauer et al., 2007). The main finding of this study was that the rinsing step does not significantly alter microbial community structure or diversity detected in swab samples, suggesting that it is not necessary to include rinsing in frog skin microbiome sampling protocols.

Restoration and the microbiome

While collecting samples for my second and third chapters at the San Francisco Zoo, I learned about the ongoing efforts to restore wild populations of *Rana sierrae*. While >93% of *R. sierrae* populations previously experienced local extinctions, remaining populations managed to persist with high *Bd* prevalence, enigmatically maintaining *Bd* loads below the threshold associated with mortality (Kinney et al., 2011; Vredenburg et al., 2010, 2007). Roland Knapp and the Mountain Lakes Research Group, in collaboration with Jessie Bushell at the San Francisco Zoo, were implementing efforts to repopulate sites in the species historic range utilizing remaining natural populations as a frog source. While introduced fish had been removed from these

restoration sites, *Bd* would still be a threat, as it can survive in environments long after susceptible amphibian hosts have disappeared (Brannelly et al., 2018; Johnson and Speare, 2003; McMahon et al., 2013). Therefore, Knapp implemented multiple restoration approaches to improve the odds that restored populations would survive and persist (Mountain Yellow-legged Frog Interagency Technical Team, 2018).

Over the last several decades, efforts to restore populations and species have become a central focus in the field of conservation biology (Novacek and Cleland, 2001; Svenning, 2020). We are currently experiencing an anthropogenically-driven biodiversity crisis that is likely just the beginning of the sixth mass extinction on earth (Cowie et al., 2022; Wake and Vredenburg, 2008). Amphibians are currently the most threatened group of vertebrates, with 41% of species considered threatened with extinction (“The IUCN Red List of Threatened Species,” 2023).

As discussed in previous sections, emerging fungal diseases have contributed to declines and extinctions in many systems of wildlife. *Bd* in amphibians and *Pd* in North American bats are two examples of emerging fungal pathogens that have caused exceptional devastation (Fisher et al., 2020). *Bd* has caused unprecedented impacts for a single pathogen, contributing to the decline of hundreds of species and to the confirmed or presumed extinctions of 90 species in the wild (Scheele et al., 2019; Skerratt et al., 2007). *Pd* has killed millions of hibernating North American bats across 38 U.S. states and seven Canadian provinces (Fisher et al., 2020). In response to the rise of EIDs in wildlife, common approaches to mitigate their effects have been investigated across systems. However, research on this topic is ongoing and most wildlife systems do not have existing infrastructure to combat EIDs (Fisher et al., 2020).

Fungicidal treatments have been widely used in agriculture to protect cash crops from EIDs. For example, fungicide use is the primary control measure against late blight in potatoes

(Olsen et al., 2011; Subhani, 2016). In wildlife, however, fungicides have been used on a smaller scale. For example, their use in treatment of sudden oak death in oaks and tanoaks infected by *P. ramorum* has been limited due to economic feasibility (Olsen et al., 2011; Rizzo et al., 2005). Further, research on application of antifungal volatiles to improve survival of North American bats infected with *Pd* is still ongoing (Gabriel et al., 2022; Padhi et al., 2018). In amphibians, fungicidal treatment of *Bd* infections in captive and wild populations has proven effective in several studies (Bosch et al., 2015; Fisher et al., 2020; Hardy et al., 2015; Knapp et al., 2022). Though a concern with fungicidal treatments of animals is that they may negatively impact host organisms (Loyau et al., 2016; Olsen et al., 2011). Further, fungicide usage in nature can negatively impact surrounding ecosystems (Ghose et al., 2014; Zubrod et al., 2019).

Vaccinations have been successful in many wildlife systems; however, they often provide waning or only partial immunity (Barnett and Civitello, 2020). In North American bats, work on vaccinations against *Pd* is ongoing (Cornwell et al., 2019; Rocke et al., 2019). In amphibians, *Bd* inoculation has been found to reduce future *Bd* loads (McMahon et al., 2014). Though more research is needed on the short- and long-term benefits in diverse amphibian species.

Captive breeding/rearing and reintroduction represent another method used to conserve wildlife threatened by disease. The Smithsonian's Conservation Biology Institute (SCBI) has performed successful captive breeding and reintroduction of many endangered species (Olsen et al., 2011). For example, in restoring populations of black-footed ferrets, a species for which disease contributed to previous extinction in the wild (Olsen et al., 2011; Woodroffe, 1999). The Amphibian Ark is an association of captive breeding programs around the world, and much of their work has involved safeguarding genetic diversity *ex situ* until a time when they can be reintroduced; persistence of *Bd* in habitats of many of these endangered species, however, has

meant that reintroductions have not yet been possible in most cases (Harding et al., 2016; Zippel et al., 2011). Further, a major concern with captivity and reintroduction is the loss of host diversity, as populations must maintain sufficient genetic diversity to ensure presence of pathogen resistance genotypes (Smith et al., 2009).

As recent evidence has elucidated the importance of host-associated microbial communities to host health across many systems, the importance of taking microbiomes into account when planning and executing wildlife restoration efforts has now been recognized as well (Redford et al., 2012; Trevelline et al., 2019). However, research on probiotic therapies for endangered wildlife is still in its infancy, and standards for development and use have yet to be fully defined (Garcias-Bonet et al., 2024). There has been research related to probiotics therapies in many systems including various plants, honey bees, corals, bats, and amphibians with the goal of improving conservation efforts (Garcias-Bonet et al., 2024). For example, findings that soil fungi can inhibit fungal and fungal-like pathogens of plants have led to the development of effective bio-agent formulations shown to reduce incidence of potato late blight and tomato wilt (diseases caused by *P. infestans* and *Fusarium oxysporum*, respectively; Subhani, 2016). In North American bats infected by *Pd*, application of the inhibitory bacterium *Pseudomonas fluorescens* was shown to effectively promote host survival in captivity and in the field (Cheng et al., 2017; Hoyt et al., 2019).

In amphibians, while probiotics have been effective in several studies (Becker et al., 2009; Harris et al., 2009a, 2009b; Kueneman et al., 2016), a major issue is that applied microorganisms fail to colonize the community long-term (Becker et al., 2015; Knapp et al., 2022; Woodhams et al., 2012). This is an issue with probiotics in many wildlife systems, though it has been proposed that colonization by the probiotic microorganisms is not required for efficacy in all systems

(Garcias-Bonet et al., 2024). However, amphibian skin probiotics have not always proven effective, and it appears likely that probiotic bacteria that can colonize the skin of hosts long-term are needed (Becker et al., 2021, 2015, 2011; Knapp et al., 2022; Woodhams et al., 2020, 2012).

In part because of mixed results from attempts to utilize microbiomes towards conservation, currently applied restoration work (*i.e.*, outside of academic research) has largely failed to incorporate microbiome research (Trevelline et al., 2019). For example, the interagency conservation strategy for the federally listed mountain yellow-legged frog species complex (*Rana sierrae* and *Rana mucosa*) discusses the potential of probiotic therapies to increase the abundance of *Bd*-inhibitory microbes, referred to as bioaugmentation, but states that more research is needed before this can be widely applied (Mountain Yellow-legged Frog Interagency Technical Team, 2018). However, there had been no published studies of microbiomes associated with mountain yellow-legged frog restoration protocols when I began my studies on the topic in 2016. Roland Knapp and Jessie Bushell's efforts for restoration of *R. sierrae* have involved (1) captive-rearing coupled with *Bd* inoculation and antifungal treatment to boost host immune responses, followed by reintroduction to restoration sites, and (2) direct translocations of frogs from remaining natural populations to restoration sites. If we could better understand host-pathogen-microbiome interactions in this system, we may be able to better harness the protective power of microbial communities. This knowledge could improve conservation of these frogs, other amphibians, and more broadly of other wildlife systems threatened by pathogens. A logical first step would be to characterize how microbiomes are impacted by existing restoration protocols. This was the framework for my last two chapters.

In Chapter Four: “Effects of experimental *Batrachochytrium dendrobatidis* inoculation and itraconazole treatment on the skin microbiome of Sierra Nevada yellow-

legged frogs (*Rana sierrae*),” I examined microbiomes from captive frogs undergoing *Bd* inoculation and anti-fungal treatment with an azole antifungal drug, itraconazole. The main findings from this work were that (1) impacts of *Bd* and itraconazole to the skin microbiome were greater in magnitude than natural temporal variation alone, (2) both infection and antifungal treatment were associated with changes in microbiome structure and in normalized relative abundances of bacterial community members, and (3) we detected legacy impacts of *Bd* infection to frog microbiomes after antifungal treatment.

In Chapter Five: “Characterizing the impacts of restoration efforts on skin microbiomes of Sierra Nevada yellow-legged frogs (*Rana sierrae*) across restoration histories and sites,” I studied microbiomes and *Bd* infections of *R. sierrae* from (1) natural populations persisting with *Bd* that were used as frog sources for restoration of previously extirpated populations, (2) the captive-reared population at the SF Zoo that had undergone experimental *Bd* inoculation and antifungal treatment, and (3) restored populations consisting of frogs reintroduced from the Zoo, frogs directly translocated from source populations, and new recruit frogs born at restored sites. The main findings from this work were that (1) similarities in microbiomes between distinct restored site populations sourced from the same natural population were consistent with a role of host genetics, (2) *Bd* loads and certain microbiome features generally differed between natural and restored populations, with restored populations harboring higher *Bd* loads and lower putative anti-*Bd* function (Woodhams et al., 2015), and (3) history of specific restoration protocol (*i.e.*, reintroduction versus translocation, and *Bd* inoculation treatment group) was not associated with detectable long-term differences in microbiomes or infections, suggesting that history was less important than current environment and host genetics to determining host-microbiome-pathogen interactions.

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Chapter One:

Continent-wide recent emergence of a global pathogen in African amphibians¹

Abstract

Emerging infectious diseases are increasingly recognized as a global threat to wildlife. Pandemics in amphibians, caused by the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*), have resulted in biodiversity loss at a global scale. Genomic data suggest a complex evolutionary history of *Bd* lineages that vary in pathogenicity. Africa harbors a significant proportion of global amphibian biodiversity, and multiple *Bd* lineages are known to occur there; yet, despite the decline of many host species, there are currently no described *Bd*-epizootics. Here, we describe the historical and recent biogeographical spread of *Bd* and assess its risk to amphibians across the continent of Africa. We provide a 165-year view of host-pathogen interactions by (i) employing a *Bd* assay to test 4,623 specimens (collected 1908–2013); (ii) compiling 12,297 published *Bd* records (collected 1852–2017); (iii) comparing the frequency of *Bd*-infected amphibians through time by both country and region; (iv) genotyping *Bd* lineages; (v) histologically identifying evidence of chytridiomycosis, and (vi) using a habitat suitability model to assess future *Bd* risk. We found a pattern of *Bd* emergence beginning largely at the turn of the century. From 1852–1999, we found low *Bd* prevalence (3.2% overall) and limited geographic spread, but after 2000 we documented a sharp increase in prevalence (18.7% overall), wider geographic spread, and multiple *Bd* lineages that may be responsible for emergence in different regions. We found that *Bd*

¹ Previously published as Ghose, S. L., Yap, T. A., Byrne, A. Q., Sulaeman, H., Rosenblum, E. B., Chan-Alvarado, A., Chaukulkar, S., Greenbaum, E., Koo, M. S., Kouete, M. T., Lutz, K., McAloose, D., Moyer, A. J., Parra, E., Portik, D. M., Rockney, H., Zink, A. G., Blackburn, D. C., and Vredenburg, V. T., 2023. Continent-wide recent emergence of a global pathogen in African amphibians. *Front. Conserv. Sci.* 4:1069490. DOI: 10.3389/fcsc.2023.1069490.

risk to amphibians was highest in much of eastern, central, and western Africa. Our study documents a largely overlooked yet significant increase in a fungal pathogen that could pose a threat to amphibians across an entire continent. We emphasize the need to bridge historical and contemporary datasets to better describe and predict host-pathogen dynamics over larger temporal scales.

Introduction

Amphibians are one of the most vulnerable groups of vertebrates with 41% of all species currently threatened with extinction (Stuart et al., 2004; The IUCN Red List of Threatened Species, 2021; Wake & Vredenburg, 2008). An emerging infectious disease, chytridiomycosis, caused by the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*), has been a major driver of amphibian declines (Skerratt et al., 2007; Wake and Vredenburg, 2008; Fisher et al., 2009). Invasion and spread of the *Bd* pathogen are linked with declines and extinctions of hundreds of amphibian species worldwide and *Bd* has been documented on every continent where amphibians occur (Skerratt et al., 2007; Fisher et al., 2009; Olson et al., 2013; Scheele et al., 2019; Fisher and Garner, 2020). However, the pathogen's specific historical distribution and geographic spread remain unclear, hampering efforts to predict which amphibian species are at greatest risk of disease outbreak (Berger et al., 2016).

While effects of *Bd* on vertebrates are greater than those of any other observed pathogen (Scheele et al., 2019; Fisher and Garner, 2020), there is still much that is not understood. For example, while *Bd*-caused epizootics resulting in amphibian species decline or extirpation have been documented in detail in various parts of the world (Lips et al., 2006; Fisher et al., 2009; Vredenburg et al., 2010; Walker et al., 2010), it is still not known why epizootics are found in

some areas but not others (Berger et al., 2016). Delineating the geographic origin and spread of the pathogen, as well as its evolutionary history and population genetic structure, are necessary to describe the dynamics of this pathogen-host system. Recent studies reveal a complicated geographic and phylogenetic history for *Bd*, including multiple lineages that vary in pathogenicity and may have spread across and between continents over a period of hundreds or even thousands of years. The genetic structure of *Bd* populations suggests that competition and hybridization among and between *Bd* lineages has occurred (Farrer et al., 2011; Schloegel et al., 2012; Bataille et al., 2013; Rosenblum et al., 2013; O’Hanlon et al., 2018). The global panzootic lineage (*Bd*-GPL; Farrer et al., 2011)) is regarded as the most virulent lineage, has the widest geographic distribution, and is associated with epizootic events (Schloegel et al., 2012; O’Hanlon et al., 2018). Although *Bd* is believed to have originated in Asia, it is unclear when, where, or how *Bd*-GPL diverged from older lineages (O’Hanlon et al., 2018). Other lineages thought to be less virulent are associated with areas that generally lack epizootics (Berger et al., 2016; O’Hanlon et al., 2018), including several identified in Asia (*Bd*-ASIA-1, believed to have originated in Korea, which includes *Bd*-CH, originally thought to have originated in Switzerland), South America (*Bd*-ASIA-2/*Bd*-BRAZIL) and southern Africa (*Bd*-CAPE, first described from Cape Province, South Africa) (Farrer et al., 2011; Bataille et al., 2013; Rodriguez et al., 2014; O’Hanlon et al., 2018).

Africa harbors nearly 16% of known living species of amphibians (AmphibiaWeb, 2021) and multiple lineages of *Bd* have been found to infect these hosts in the wild (including *Bd*-CAPE and *Bd*-GPL). There are few relevant *Bd* host studies in Africa and no described *Bd*-epizootics in the region (Berger et al., 2016; O’Hanlon et al., 2018; Doherty-Bone et al., 2020; Zimkus et al., 2020). However, this is likely due to a lower *Bd* sampling effort in Africa relative to other continents rather than a true absence of such events. Several studies have reported a small number

of *Bd*-infected amphibians collected in the early 20th century in Africa (Weldon et al., 2004; Soto-Azat et al., 2010). More recent African amphibian studies have revealed much higher *Bd* infection prevalence that may be associated with host declines (Goldberg et al., 2007; Kielgast et al., 2010; Bell et al., 2011; Gower et al., 2012; Tarrant et al., 2013; Seimon et al., 2015; Hirschfeld et al., 2016; Jongsma et al., 2016). Although these *Bd*-host infection data vary in spatial and temporal resolution, they suggest a pattern of *Bd* emergence that may represent a threat to African amphibian species, warranting a comprehensive analysis encompassing the broadest possible temporal and geographic scales.

We set out to address an important prediction regarding continent-wide infection dynamics of *Bd*: that the emergence of *Bd* in Africa is not limited to isolated outbreaks but instead represents a broad pattern of synchronized emergence across the entire continent. We address this prediction by comparing the historical frequency of *Bd* occurrence (spanning over a century) to the recent frequency of *Bd* occurrence (roughly the last 20 years). These comparisons of the frequency of *Bd* occurrence (revealing epizootic dynamics) are made both within African countries and within broader geographic regions. In addition, we include comparisons of changes in overall *Bd* infection intensity (zoospore counts) for specific countries in central Africa (Cameroon) and eastern Africa (Kenya) that are known to harbor widespread *Bd* in recent years (Hirschfeld et al., 2016; Kielgast et al., 2010). For our analyses, we have gathered 16,920 original and previously published museum- and field-collected records between 1852 and 2017 from a total of 36 countries. Importantly, our survey of Africa is unique in that it includes both positive and negative *Bd* samples as opposed to a more restricted reporting of only the number of *Bd* positives over time (Zimkus et al., 2020).

Additionally, if there is a continent-wide synchronous emergence of *Bd*, there could be regional differences in the magnitude of *Bd* prevalence. Such differences are likely associated with the distributions of the two described *Bd* lineages detected on the African continent (*Bd*-CAPE and *Bd*-GPL), depending on the presence of one or a combination of both in certain areas. *Bd*-CAPE has been detected in Cameroon and was found to be widely present in South Africa, while the *Bd*-GPL lineage has invaded almost every region of the continent (Rosenblum et al., 2013; O’Hanlon et al., 2018; Byrne et al., 2019). While it is speculated that the hypervirulent *Bd*-GPL is the more virulent of these two lineages based on ex situ experiments, less is known about the impacts of *Bd*-CAPE in nature, and there is evidence that *Bd*-CAPE does exhibit high virulence under certain conditions (Doddington et al., 2013; O’Hanlon et al., 2018; Fisher et al., 2021). Competition among invasive *Bd* strains, and the potential for hybridization has been documented on other continents (Jenkinson et al., 2016) and genotyping has revealed lineages in Africa that are intermediary to the more derived *Bd*-GPL complex and the more early diverging *Bd*-CAPE clade (Rosenblum et al., 2013; O’Hanlon et al., 2018; Byrne et al., 2019). These intermediaries are likely to be hybrids of the two described lineages and could be impacting disease dynamics (Farrer et al., 2011; Greenspan et al., 2018).

To understand how the dynamics of *Bd* lineage competition and/or hybridization may lead to future epizootics in some areas and not in others, we utilize our large database to document changes in *Bd* prevalence in regions that have experienced invasion by different lineages. To predict the risk to future populations of amphibians across the continent, we develop a spatially explicit model of *Bd* risk across regions of Africa, based on our current understanding of the habitat suitability of these *Bd* lineages and amphibian species richness. Combined, this work provides

novel predictions for the regions in Africa that are at high risk of *Bd* invasion and is complementary to a recent spatially explicit suitability model for the African continent (Zimkus et al., 2020).

Methods

Ethics Statement

The animal study was reviewed and approved by San Francisco State University Institutional Animal Care and Use Committee (protocol A20-05-SFSU).

Generating and Compiling *Bd* Records Dataset

We sought to characterize historical and recent *Bd* prevalence in African amphibians. To do this we first compiled a dataset including (1) original records we generated (referred to as “our data”) from amphibian skin swabs assayed for *Bd* and (2) published *Bd* records (referred to as “published data”) for African amphibians.

For records we generated (our data), we collected skin swabs from 2,972 amphibian museum specimens collected from 1908–2009 in Cameroon, Ethiopia, Kenya, Lesotho, Tanzania and Uganda, and from 1,651 live field animals collected by coauthors and collaborators from Burundi and Equatorial Guinea (in 2011), and from Cameroon and the Democratic Republic of Congo (in 2011 and 2013) for a combined total of 4,623 samples. We used a standard Taqman *Bd*-qPCR assay to determine the frequency of *Bd* occurrence and *Bd*-infection intensity (Boyle et al., 2004; Hyatt et al., 2007). This assay was designed to detect *Bd*-GPL, and therefore a limitation is that divergent lineages with mutations in the Taqman binding site may not amplify (Mutnale et al., 2018). Other retrospective studies utilized historical specimens (collected during a similar time-frame) and using the same *Bd*-qPCR assay, found evidence for *Bd*-infection even in their oldest

specimens (Cheng et al., 2011; Rodriguez et al., 2014; Talley et al., 2015). This technique was shown to generate a recovery rate of 82% from museum specimens (Cheng et al., 2011), and has robust power of detection, especially with large datasets. We focused historical sampling on areas with putative *Bd*-related declines [Lesotho (Minter et al., 2004), Ethiopia (Gower et al., 2012; Gower et al., 2013), Tanzania (Channing et al., 2006; Weldon et al., 2020)], areas with many available samples (Uganda and Kenya), and those with the most extensive recent sampling [Cameroon (Blackburn et al., 2010; Hirschfeld et al., 2016) and Kenya (Kielgast et al., 2010)]. Museum specimens are housed in the permanent collections of the California Academy of Sciences (CAS), the Museum of Vertebrate Zoology (MVZ) at the University of California, Berkeley, and Harvard University's Museum of Comparative Zoology (MCZ). Field sampling locations (live animals) were selected opportunistically based on field trips of coauthors and collaborators. As our goal was to assess *Bd* prevalence across all amphibians, we sampled amphibians from all taxonomic classes that were available in collections or captured at field sites (these included Anura and Gymnophiona).

Gloves were worn during swab sampling and changed between animals to prevent cross contamination. In addition, archived museum specimens were rinsed with 70% ethanol before swabbing to reduce cross contamination between animals housed in the same jar. We used standard skin swabbing procedures (which include 30 strokes of ventral surfaces) for *Bd* qPCR assays on live animals and museum specimens using *Bd*-specific ITS1 primers (Boyle et al., 2004; Hyatt et al., 2007; Cheng et al., 2011). Swabs were dried and stored in 1.5 ml microcentrifuge tubes at 4°C until DNA extraction using Prepman Ultra DNA Extraction Kits following the manufacturer's protocol (using 40 µL Prepman Ultra per sample). Standard real-time PCR quantification techniques were used: one replicate per sample was run with standard controls of 0, 0.1, 1, 10, and

100 zoospore equivalents (ZE) (Boyle et al., 2004; Hyatt et al., 2007; Cheng et al., 2011). *Bd* standards were created using *Bd* isolate CJB7 collected in 2009 from host *Rana muscosa* during an epizootic at Sixty Lake Basin in the southern Sierra Nevada, California (Vredenburg et al., 2010), identified as part of the highly virulent *Bd* Global Panzootic Lineage (*Bd*-GPL1; James et al., 2015; Piovia-Scott et al., 2014; Rosenblum et al., 2013). We multiplied the qPCR genomic equivalents (GE) by a PCR dilution factor of 80 in order to quantify the number of zoospore equivalents on each swab (Z_{swab} ; Briggs, Knapp and Vredenburg, 2010; Vredenburg *et al.*, 2010). Any sample with a $Z_{\text{swab}} > 0$ was considered *Bd*-infected or *Bd*-positive. Because some animals had very high Z_{swab} values, we report infection intensity on a base-10 logarithmic scale, represented with units of logZE.

For our compilation of published data, we obtained records from *Bd* surveys conducted in continental Africa (excluding Madagascar but including the islands of the Gulf of Guinea) from scientific literature and from the *Bd*-maps Legacy Project (Olson et al., 2021; dataset available at: <https://geome-db.org/workbench/project-overview?projectId=300>). We included records from Anura, Caudata, and Gymnophiona from all life-stages with location data (*i.e.*, country, latitude and longitude when available), collection year, and *Bd* infection status (*i.e.*, presence or absence), and excluded food markets and the pet trade. While many published records used methods for swab sampling and *Bd* qPCR described above, we also included records using different sample materials (including skin scrapes/brush samples, toe clips, skin/mouthpart tissue, *Bd* isolates) and/or *Bd* screening methods (including conventional PCR, histology, microscopic visualization, *Bd* genome sequencing or genotyping). These published data include 12,297 *Bd* records (collected 1852–2017). When combined with our data (museum and field records we generated; N=4,623),

we include a total of 16,920 *Bd* records from 36 African countries collected between 1852 to 2017 for this study. For more information on the dataset, see the Supplementary Material.

***Bd* Emergence**

To characterize the temporal pattern of *Bd* throughout Africa and estimate the timing of *Bd* emergence, we examined the frequency of *Bd* occurrence over time for all of Africa, for individual countries, and for regions (as defined by the United Nations). For frequency of *Bd* occurrence across all of Africa, we analyzed 16,857 records from our compiled dataset (summarized in Table 1-1). These included our data (all new records generated for this study; N=4,623) and 12,234 records from published data (we excluded 63 of the 12,297 published records in analyses of frequency of *Bd* occurrence because the associated studies did not report both *Bd*-negative and *Bd*-positive samples for *Bd* detection in amphibian hosts; these 63 *Bd*-positive records were included, however, in our *Bd* risk analysis described below that only required *Bd*-positive samples with latitude and longitude data). To examine temporal changes in *Bd* occurrence throughout Africa, we binned records by decade (excluding an additional 171 records from published data for which collection year did not fall into a single decade; N=16,686; see *Supplementary Material*; Table S1-1) and calculated the frequency of *Bd* occurrence by dividing the number of *Bd*-positive records by the total number of animals tested. We calculated a 95% binomial confidence interval (95% CI) for each decade using the R function `binom.test` (R Core Team, 2022; Phillips and Puschendorf, 2013; De León et al., 2017). We assessed our power to detect a prevalence <11%, a conservative estimate of *Bd* endemism based on a previous study that spanned over a 100 year period (Talley et al., 2015), by calculating the probability of detecting zero positives for each decade (defined as “Pr(no *Bd*)”). To do so, we used the R function `dbinom` (R Core Team, 2022; Doraj-Raj, 2014),

which is based on the binomial distribution; we used 11% as the “true” probability that each sample was positive and the total number of samples evaluated in a particular time period as the number of trials.

Next, we examined the frequency of *Bd* occurrence and *Bd*-infection intensity by decade in countries for which we had the most comprehensive data: Cameroon and Kenya. For these countries, we analyzed a subset of records for which we had data on *Bd* infection intensity in addition to infection status (this included our data generated for this study for Cameroon and Kenya, data from Hirschfeld et al. (2016) and Zimkus et al. (2020) for Cameroon, and data from Kielgast et al. (2010) for Kenya; *see Supplementary Material*; Table S1-2; Table S1-3). In addition to calculating frequency of *Bd* occurrence with 95% CI and Pr(no *Bd*) (as described above), mean and maximum *Bd*-infection intensity for *Bd*-positive samples were calculated and visualized by decade. We emphasize, however, that estimates of infection intensity from museum specimens are unreliable due to DNA degradation that occurs over time, so our historical infection intensities may be inaccurate estimates.

Finally, we defined a time of emergence based on the increase in frequency of *Bd* occurrence we observed across decades for all of Africa, Cameroon, and Kenya (year = 2000). We binned *Bd* records by whether they were pre-emergence (“historical,” which we define as “pre-2000”) and post-emergence (“recent,” defined as “2000-present”) and calculated frequency of *Bd* occurrence with 95% CI and Pr(no *Bd*) (as described above) by country and region. To assess whether data across Africa support our proposed timing of emergence statistically, we tested whether the frequency of samples with *Bd*-infection was dependent on time period for countries and regions that had both pre-emergence and post-emergence records (Chi-square tests using the `chisq.test` function in R; R Core Team, 2022). For cases where we rejected the null ($p < 0.05$), we

inferred the direction of change based on the values for frequency of *Bd* occurrence in historical and recent time periods. Statistical analyses were conducted in R (version 4.0.4) in RStudio (version 1.4.1106) using ‘pwr,’ ‘binom,’ and “Hmisc” packages (R Core Team, 2022; Harrell, 2012; Doraj-Raj, 2014; RStudio Team, 2016; Champely, 2018).

***Bd* Genotyping Assay**

To determine which lineages of *Bd* were present, we genotyped *Bd* from a subset of our swab samples taken from 32 frogs collected in Cameroon in 2013 (N=25) and Burundi in 2011 (N=7) using a custom genotyping assay (Table S1-4) (Byrne et al., 2017). Briefly, this genotyping assay uses the Fluidigm Access Array platform to perform microfluidic multiplex PCR on 192 target loci of which 188 are on the *Bd* nuclear genome, 3 are on the *Bd* mitochondrial genome, and one is designed to target the internal transcribed spacer (ITS) region of the related pathogenic fungus *Batrachochytrium salamandrivorans*. Each target locus is 150–200 base pairs (bp) long. First, extracted DNA from swab samples was cleaned and concentrated from 30µl to 10µl using an isopropanol precipitation protocol. All samples were preamplified in two separate PCR reactions, each containing 96 primer pairs at a final concentration of 500nM. For each preamplification PCR reaction, we used the FastStart High Fidelity PCR System (Roche) at the following concentrations: 1x FastStart High Fidelity Reaction Buffer with MgCl₂, 4.5mM MgCl₂, 5% DMSO, 200µM PCR Grade Nucleotide Mix, 0.1 U/µl FastStart High Fidelity Enzyme Blend. We added 1µl of DNA to each preamplification reaction and used the following thermocycling profile: 95°C for 10min, 15 cycles of 95°C for 15sec and 60°C for 4min. Preamplified products were treated with 4µl ExoSAP-it (Affymetrix Inc.) and incubated for 15min at 30°C, then 15min at 80°C. Treated products were diluted 1:5 in PCR-grade water. The diluted products from each of

the two preamplification reactions were combined in equal proportions and used for downstream amplification and sequencing.

Each preamplified sample was loaded into the Fluidigm Access Array IFC (Fluidigm, Inc.) for amplification. All samples were barcoded and pooled for sequencing on ¼ of an Illumina MiSeq lane using the 300bp paired-end kits at the University of Idaho IBEST Genomics Resources Core. We pre-processed all sequencing data as described in Byrne et al. (2017) and generated consensus sequences for all variants present for each sample at each locus. We filtered reads by selecting sequence variants that were present in at least five reads and represented at least 5% of the total number of reads for that sample/locus using dbcamplicons (<https://github.com/msettles/dbcAmplicons>).

We constructed a phylogeny to explore the relationship of the *Bd* sequences collected for this study (N=32) to those from previously-published *Bd* isolates (N=31; Rosenblum et al., 2013; O’Hanlon et al., 2018)). The isolates from Rosenblum et al. (2013) (N=28) were sequenced and processed exactly as described for the swab samples. The consensus amplicon sequences for the isolates from O’Hanlon et al. (2018) (N=3) were calculated from raw sequencing reads. Briefly, we downloaded raw reads from NCBI’s Sequence Read Archive (accession numbers SRS2757071, SRS2757072, SRS2757205) and cleaned the reads using seqyclean v 1.9.9 (<https://github.com/ibest/seqyclean>). We aligned the paired reads to the reference genome JEL423 (Broad Institute v. 17-Jan-2007) using BWA MEM (Li, 2013). Aligned reads were sorted using picard v.2.9.0 (<http://broadinstitute.github.io/picard>) and variants were called using samtools mpileup v.1.6 and bcftools call v.1.6 (Li, 2011). We produced a consensus genome for each isolate using the bcftools consensus. After producing a consensus genome for each of the three isolates, we used BLAST v.2.7.1 to match our reference amplicon sequences to the amplicon sequences in

each genome. We then extracted the best-scoring BLAST match for each isolate to produce a single FASTA containing a consensus sequence for each target locus.

The isolates represent three of the major *Bd* clades: *Bd*-GPL (Global Panzootic Lineage), *Bd*-Brazil/*Bd*-ASIA-2, and *Bd*-CAPE (Farrer et al., 2011; O’Hanlon et al., 2018). First, we trimmed our consensus sequence dataset to eliminate loci that had more than 50% missing data. The remaining dataset contained 186 loci (183 nuclear, 3 mitochondrial). To ensure proper alignment, we filled in missing sequences with a string of Ns (length = maximum sequence length of that locus) using custom R scripts. Next, we individually aligned all loci using the MUSCLE package v.3.18.0 (Edgar, 2004) in R (v.3.4.3) (R Core Team, 2022) and concatenated all aligned loci for each sample for a total length of 25,617bp. We visually checked the concatenated alignment for errors in Geneious v.10.2.3 (Kearse et al., 2012) and used the RAxML plugin (Stamatakis, 2014) in Geneious to search for the best scoring maximum likelihood tree using rapid bootstrapping (1000 replicates).

***Bd* Risk Model**

We integrated abiotic and biotic factors to predict the areas in Africa with the greatest risk of *Bd* establishment, following previous studies (Murray et al., 2011; Liu et al., 2013; Yap et al., 2018). We first created a presence-only habitat suitability model (HSM) driven by climate and land use factors using Maxent v.3.4.1 (Phillips et al., 2017). We used 1,914 georeferenced *Bd* occurrence records for continental Africa and the islands in the Gulf of Guinea (Bioko, São Tomé, Príncipe); north of the Sahara, recent data were only available from Morocco. Records included all *Bd*-positives with latitude and longitude from our data and from published data. To reduce sampling bias, we applied a 10 arc-minute spatial filter by randomly choosing one *Bd* occurrence

site from every 10 arc-minute ($\sim 12 \text{ km}^2$) area (Boria et al., 2014) using R packages ‘dismo’ (Hijmans et al., 2013) and ‘maptools’ (Bivand and Lewin-Koh, 2014). This resulted in 202 *Bd*-positive sites; 195 sites had environmental data and were used for model training. We further minimized sampling bias by restricting background sampling areas to a minimum convex hull polygon around large clusters of occurrence points (Phillips et al., 2009; Rödder et al., 2009; Thorne et al., 2012; Merow et al., 2013; Phillips and Elith, 2013; Mainali et al., 2015; Yap et al., 2018).

Similar to Yap et al. (2018), we used 19 bioclimatic variables from the Worldclim database (<http://www.worldclim.org/bioclim>) (Hijmans et al., 2005) and the global human footprint (HF) (Venter et al., 2016) as predictor variables. To reduce the chances of overfitting the model due to multicollinearity, we calculated Spearman’s rank correlations (r) among all the variables to determine which variables were highly correlated with each other (James et al., 2015). We removed one variable from each pair that had an $r^2 > 0.7$. We used the resulting subset of 11 environmental variables to create the *Bd* HSM: mean diurnal temperature range (Bio2), isothermality (Bio3), maximum temperature of the warmest month (Bio5), minimum temperature of the coldest month (Bio6), mean temperature of driest quarter (Bio9), precipitation of the wettest month (Bio13), precipitation of the driest month (Bio14), precipitation seasonality (Bio15), precipitation of the warmest quarter (Bio18), precipitation of the coldest quarter (Bio19), and human footprint (HF). These variables are biologically relevant to *Bd* and performed well in previous *Bd* HSMs (Ron, 2005; Rödder et al., 2009; Murray et al., 2011; Liu et al., 2013; James et al., 2015; Yap et al., 2018). We ran 20 replicates using cross-validation: occurrence data were divided into 20 equal-sized folds, or groups, and for each replicate 19 folds were used for model

training and one fold was used for model testing, and the results were then averaged by Maxent to produce a final occurrence probability (Phillips et al., 2017).

In addition to abiotic factors, biotic factors such as host availability are critical for disease maintenance and spread (Lloyd-Smith et al., 2005). Although host abundance data would be ideal, such data at the community level for a continental assessment is not available for Africa. However, several studies have shown species richness can be an important factor in *Bd* spread (Rödder et al., 2009; Liu et al., 2013; Boria et al., 2014; Yap et al., 2016, 2018; Hydeman et al., 2017). Thus, in lieu of abundance data, we incorporated species richness to refine our predictions of *Bd* risk. We obtained amphibian richness data from AmphibiaWeb (amphibiaweb.org; AmphibiaWeb, 2021), which included range maps from the IUCN Red List as well as expert-based range maps supplied by AmphibiaWeb to calculate richness.

We calculated the product of the *Bd* HSM and amphibian richness to determine *Bd* risk (*i.e.*, areas of overlap between suitable *Bd* habitat and potential hosts) (Yap et al., 2015, 2016, 2018). We categorized *Bd* risk from low to high using the Jenks optimization method, which identifies natural clusters, or classes, of data and reduces variance within the classes while maximizing the differences between classes (Jenks, 1967). This method is more informative than the binary threshold options provided by Maxent when predicting species range expansions (Creley et al., 2019).

Results

***Bd* Emergence**

Our *Bd*-assay data for 4,623 amphibian specimens (collected 1908–2013) compiled with 12,234 *Bd* records from published data (collected 1852–2017) over a 165-year period show a

pattern of rapid *Bd* emergence beginning largely at the turn of the century (Figure 1-1A). From 1852–1999, we found low overall *Bd* prevalence (3.2%; 2.7–3.7% CI) and limited geographic spread, but after the year 2000 (from 2000–2017), we found a sharp increase in continent-wide prevalence (18.7%; 18.1–19.4% CI) and wider geographic spread. When we examined *Bd* prevalence by decade, we found that it remained below 5% for every decade until the 2000s when it increased to 17.2% (16.6–18.0% CI; Figure 1-1B; Table S1-1). In the second decade of the new century (2010s), overall frequency continued to increase to 21.6% (20.4–22.9% CI). In several countries where we had more complete data, the pattern was more pronounced. For example, in Cameroon, we found only one *Bd* positive in samples we collected up to 1999, but in the next decade, the 2000s, the frequency of *Bd* occurrence rose to 11.0% (8.9–13.3% CI), followed in the 2010s, by an increase to 36.2% (33.0–39.5% CI; Figure 1-1C; Table S1-2). In Cameroon, the average *Bd* infection intensity also increased over time and in the most recent samples, maximum values (2000–2009, 5.5 logZE; 2010–present, 6.4 logZE) surpassed a threshold infection intensity associated with chytridiomycosis-caused mortality (4.0 logZE; Vredenburg’s “10,000 zoospore rule”; Vredenburg et al., 2010; Kinney et al., 2011). In Kenya, frequency of *Bd* occurrence was zero or very low in the first four decades of data (the highest frequency in these early decades was in the 1970’s at 5.2%, 2.5–9.3% CI; Figure 1-1D; Table S1-3) and increased approximately six-fold in the 2000s (31.5%; 28.4–34.7% CI; Figure 1-1D; Table S1-3). The average *Bd* infection intensity in Kenya also increased during this same time period, with maximum *Bd*-infection intensity surpassing the mortality threshold in the 2000s by several orders of magnitude (7.9 logZE; Figure 1-1D, Table S1-3).

In our test of whether frequency of samples with *Bd*-infection was dependent on time period (historical vs recent), we found that the overall frequency of *Bd* occurrence across Africa

in the historical time period (pre-2000; 3.2%, 2.7–3.8% CI), was significantly different from the recent time period (2000–2017; 18.7%, 18.1–19.4% CI; Chi-sq., $p < 0.001$; Table 1-1). We also found that the frequency of *Bd* occurrence was significantly different regionally (see regions; Figure 1-1A) across central, eastern, and southern Africa, between historical ($< 7\%$) and recent ($> 17\%$) time periods (Chi-sq., $p < 0.001$, $p < 0.001$, $p < 0.001$, respectively; Table 1-1). One country in central Africa, Equatorial Guinea, showed a significant decrease in the frequency of *Bd* occurrence between historical and recent time periods (all samples from Bioko Island, Equatorial Guinea; Chi-sq., $p = 0.02$), though all the *Bd*-positives from the historical time-period were collected close to the year 2000 (in 1998). Burundi, in eastern Africa, had the highest frequency of *Bd* infections after 2000 (73.7%, 66.3–80.2% CI) with no *Bd* positives detected historically; however, a Chi-square p-value could not be accurately estimated for this country because of the small historical sample size ($N = 13$). We did not detect a recent surge of *Bd* infections in the country of South Africa (25.7%, 17.8–34.9% CI, historic vs. 23.3%, 21.1–25.6% CI, recent; Chi-sq, $p = 0.65$), though we did in the region overall (6.6%, 5.0–8.5% CI, historic vs. 24.2%, 22.0–26.4% CI, recent; Chi-sq, $p < 0.001$). There were no historical data for northern Africa, which only included data from Morocco, and the recent frequency of *Bd* occurrence there was low (2.0%, 0.9–4.0% CI). In western Africa, although the frequency of *Bd* occurrence also showed an increase from 0% historically to $> 20\%$ recently, low historical sample size ($N = 10$) prevented accurate estimation of a Chi-square p-value. *Bd* was almost entirely absent from western Africa (Table 1-1) except in Nigeria, where the recent frequency of *Bd* occurrence was 44.5% (40.8–48.2% CI).

***Bd* Lineage Genotyping**

We found multiple lineages of *Bd* in Burundi and Cameroon (Figure 1-2A, B; Table S1-4; (Byrne et al., 2019)). In Cameroon, all genotyped samples formed a well-supported clade that includes the previously-published *Bd*-CAPE isolate CCB1 (Rosenblum et al., 2013), indicating that amphibians in Cameroon are widely infected by the *Bd*-CAPE lineage (Figure 1-2B). In contrast, *Bd* samples collected in Burundi were more variable, with some samples grouping with previously-published *Bd*-GPL isolates (CAS250667, CAS250764, CAS250826, CAS250829) while others were not clearly members of either the *Bd*-GPL or *Bd*-CAPE lineages (CAS250662, CAS250742, CAS250744) (Figure 1-2B). For one of these ambiguous samples, CAS250742, the average number of alleles found at each locus across all amplified loci was 2, which was higher than for all other sequenced samples (Figure S1-1). Bootstrap support in this group of samples is low, thus no firm conclusions can be made from data for these three samples. However, the ambiguous Burundi samples are distinct from both from *Bd*-CAPE samples as well as other previously published *Bd*-CAPE/*Bd*-GPL hybrids (Figure 1-2B; O’Hanlon et al., 2018)).

***Bd* Risk Model**

The *Bd* risk model predicts moderate to high *Bd* vulnerability throughout much of western, central, and eastern Africa, including within the Ethiopian Plateau (Figure 1-3). Some portions of Mediterranean northern Africa and the southeastern tip of Africa are also predicted to have some *Bd* risk. Our model predicts the highest *Bd* risk in the tropical rainforests of western and central Africa and the montane forests surrounding the Great Rift Valley, while less vulnerable areas include the arid Sahara Desert throughout most of northern Africa and the semi-arid Kalahari Desert in the western region of southern Africa.

Discussion

The 165 years of amphibian sample collections show a distinct signal of *Bd* emergence and geographic spread across the African continent that began in the first decade of the 21st century. In general, in the 20th century (1852-1999), we found few, geographically scattered *Bd*-positives, showing no signal of epizootic spread (Figure 1-1A, B; earliest *Bd* infected host was 1933 in Cameroon; Soto-Azat et al., 2010)). However, beginning in the 2000–2009 sample period (Figure 1-1A, B) we see a large increase in prevalence and evidence of widespread infection across the continent, which are signals consistent with epizootics. Similarly, in Central America and western North America, studies found few and scattered *Bd*-infected specimens historically in areas that decades later experienced rapid *Bd* expansion, epizootics, and host population collapse (Vredenburg et al., 2010, 2019; Cheng et al., 2011; Sette et al., 2015, 2020; Yap et al., 2016, 2018; Adams et al., 2017; De León et al., 2017). In Africa, we saw only one major exception to this general pattern of emergence: in South Africa, infections were widespread even in our oldest samples (Table 1-1). Thus, generally we find strong evidence indicating a recent surge (beginning in 2000) in the frequency of *Bd* occurrence in amphibian specimens collected across a large portion of the continent (Figure 1-1A, B; Table 1-1, Table S1-1). Disturbingly, we found this broad pattern of *Bd* emergence in areas of Africa where many amphibian species are either suspected or known to have collapsed (Channing et al., 2006; Gower et al., 2012, 2013; Hirschfeld et al., 2016; Weldon et al., 2020). Considering that *Bd* invasion was followed by *Bd*-epizootics resulting in extinctions of amphibian populations in western North America (Briggs et al., 2010; Vredenburg et al., 2010), Central America (Berger et al., 1998; Lips et al., 2006), South America (Catenazzi et al., 2011; Carvalho et al., 2017), Europe (Walker et al., 2010; Bates et al., 2018), and Australia (Laurance et

al., 1996; Berger et al., 1998), amphibian declines in Africa that coincide with widespread expansions of *Bd* may also be linked.

Recent *Bd* emergence was most pronounced in eastern and central Africa where many species are threatened amid reports of declines and die-offs. In fact, most reports place the declines and extinctions occurring near or after the year 2000 (e.g., in Tanzania, Ethiopia, and Cameroon; Channing et al., 2006; Gower et al., 2012, 2013; Hirschfeld et al., 2016; Weldon et al., 2020), which coincides with our *Bd* emergence data. The historical and recent *Bd* occurrence data from Cameroon and Kenya are the most complete. In these countries, we see a large and significant increase in the number and proportion of *Bd* infected hosts after 2000 (Figure 1-1C, D; Table S1-2, S1-3). Studies have shown that high levels of infection (i.e., *Bd* infection intensity or *Bd* load) are associated with severe chytridiomycosis, death, and population collapse (e.g., *mortality threshold*, Vredenburg’s “10,000 zoospore rule”; Vredenburg et al., 2010; Kinney et al., 2011); and, low *Bd* infection intensity is associated with host survival and coexistence with *Bd* suggestive of enzootic dynamics (Briggs et al., 2010; Knapp et al., 2016; Catenazzi et al., 2017). In both Cameroon and Kenya, we detected a recent increase in the average *Bd*-infection intensity of hosts. We note, however, that *Bd* infection intensities from historical *Bd* occurrences may be unreliable (see *Methods*). Regardless, the maximum values for infection intensity that we documented from live animals in the most recent decade for Cameroon and Kenya surpass the *mortality threshold* (Figure 1-1C, D; Table S1-2, S1-3). Thus, we conclude that our evidence of recent and rapid emergence of *Bd* and presence of highly infected animals signal that *Bd* epizootics may be occurring in these regions. By contrast, in South Africa, we found no evidence of a recent *Bd* emergence (Table 1-1). There have been a few cases of observed mortality and population bottlenecks associated with *Bd* in some regions of South Africa, however no widespread declines

or extinctions have been linked to *Bd* despite comprehensive population survey data (Doherty-Bone et al., 2020; Griffiths et al., 2018; Hopkins and Channing, 2003; Tarrant et al., 2013). This suggests that either *Bd* may be endemic to South Africa, that there may be *Bd* genetic lineages with lower virulence there, or that sampling remains incomplete.

Global studies on *Bd* genomics suggest that *Bd* has a complex history with many lineages that appear to have varied effects on amphibian populations (Rosenblum et al., 2013; Fisher and Garner, 2020). Generally, lineages identified as endemic to a single part of the world are thought to have a long evolutionary history of co-occurrence with hosts, and thus have evolved lower virulence than invasive (or novel) lineages that cause epizootics and host die offs. In some places, endemic and invasive lineages are hypothesized to be co-occurring and hybridizing to form new lineage variants (O’Hanlon et al., 2018), and the effects of these hybrid lineages on hosts is unknown. *Bd*-epizootics can also be triggered by enigmatic abiotic or biotic factors that have not been deeply explored (Fisher and Garner, 2020; Fisher et al., 2021). Previous work has reported multiple genetic lineages of *Bd* in Africa (O’Hanlon et al., 2018; Byrne et al., 2019), suggesting the potential for complex hybridization and epizootic dynamics.

In Cameroon, we found evidence of *Bd*-CAPE, a lineage generally considered to be endemic to Africa and less virulent than *Bd*-GPL; however, our emergence data and results of histology (*see Supplementary Material*) suggest this lineage may in fact be virulent in Africa. All genotyped samples from Cameroon clustered genetically with *Bd*-CAPE (Figure 1-2B), which supports previous work (O’Hanlon et al., 2018). Histological examination of animals from Cameroon show evidence of chytridiomycosis (*see Supplementary Material*; Table S1-5; Figure S1-2), and we found *Bd* prevalence in the area has increased and spread geographically. The reported amphibian declines from Cameroon (Hirschfeld et al., 2016) coincide with the recent

increase in frequency of *Bd* occurrence that we report here. We concur with previous work that states that pathogen lineage alone cannot fully predict disease outcomes since lineages that appear less pathogenic in native hosts may cause negative impacts on new or naïve host populations, especially when abiotic factors promote infection and disease (O’Hanlon et al., 2018; Fisher et al., 2021). We suggest this may be the case with *Bd*-CAPE in parts of Africa. It is possible that *Bd*-CAPE was present in Cameroon historically and has only recently increased in frequency due to shifts in some environmental, human, or other unknown factor, but it is also possible that the recent increase in prevalence represents a new invasion and spread of *Bd*-CAPE in Cameroon, perhaps from elsewhere on the continent. *Bd*-CAPE has been widely detected in South Africa (O’Hanlon et al., 2018; Byrne et al., 2019) and may be endemic there. In South Africa, we found a relatively constant proportional occurrence of *Bd* in hosts through a long time-period, and while there have been cases of *Bd*-associated mortality there, these could be associated with different *Bd* lineages (*Bd*-GPL is also present) or result from cofactors that are not yet known. Previous work proposed that *Bd*-CAPE spread from South Africa to Europe, where it has thus far been associated with host declines (Doddington et al., 2013; O’Hanlon et al., 2018). Thus, we propose that *Bd*-CAPE may have recently spread within continental Africa and may also be associated with *Bd*-epizootics in some locations where conditions are suitable. Studies have reported recent evidence of mass amphibian die-offs in Cameroon (Blackburn et al., 2010; Hirschfeld et al., 2016) and Lesotho (Minter et al., 2004), areas where more detailed studies are needed to discern whether *Bd*-CAPE is associated with *Bd*-epizootics.

Our genomic data also identified samples as *Bd*-GPL (Global Panzootic Lineage) in samples from Burundi. The *Bd*-GPL lineage is considered the most virulent *Bd* lineage (Farrer et al., 2011; Rosenblum et al., 2013; O’Hanlon et al., 2018; Byrne et al., 2019). Consistent with this,

histological examination of specimens from Burundi showed signs of chytridiomycosis (*see Supplementary Material*; Table S1-5). In fact, our samples from Burundi had the highest proportion of *Bd* infected hosts in our recent time frame (73.7%; 66.3–80.2% CI), and this could suggest *Bd*-epizootics are occurring there. Furthermore, we found no historical *Bd* positive data in Burundi (Table 1-1) or in adjacent Tanzania (samples collected 1926–2003). In 2003, the Tanzanian Kihansi spray toad (*Nectophrynoides asperginis*) went extinct in the wild purportedly due to a *Bd* epizootic (Weldon et al., 2020). While there was no statistical support that frequency of *Bd* occurrence increased in the recent timeframe in Burundi and Tanzania (due to low availability of historical samples), we did find that frequency of occurrence was significantly higher statistically after 2000 in eastern Africa overall (Table 1-1). Therefore, the recent presence of *Bd*-GPL detected after a dramatic increase in *Bd* occurrence in eastern Africa along with histological evidence of chytridiomycosis, may indicate that *Bd*-GPL driven epizootics are occurring there.

Hybridization between distinct *Bd* lineages has been proposed as a way that virulent lineages have emerged, and these hybrid lineages may be more virulent than either parent lineage (Farrer et al., 2011; Greenspan et al., 2018). Three of our genotyped samples from Burundi did not clearly cluster with previously characterized *Bd* lineages on the phylogenetic tree, but rather were positioned between *Bd*-GPL and *Bd*-CAPE (Figure 1-2B). We see three possibilities for the identity of *Bd* lineages from these samples: (i) *Bd*-GPL and *Bd*-CAPE are able to hybridize, and while our ambiguous samples did not cluster with known hybrids, they could be novel hybrids of these two lineages (Figure 1-2B; O’Hanlon et al., 2018)); (ii) these samples could each contain multiple lineages of *Bd*, as evidenced by a higher average number of alleles found in one of these samples than any other (Figure S1-1), and this supports the possibility that more than one lineage

was present in that sample; or (iii) *Bd* detected in these samples could represent novel genetic variation that has not previously been characterized. Further work on ambiguous lineages in Burundi could resolve this issue and help in our general understanding of this complex pathogen. Further studies may identify lethal and sublethal infections (potentially from different *Bd* lineages) that could help us understand factors that could affect pathogen success (and how it may vary across lineages) such as differences in pathogen dispersal ability, reproductive ability, and vulnerability to predators. Additionally, we emphasize that future studies should prioritize genotyping *Bd* throughout Africa. The genotyping work presented here is limited, and further *Bd* genotyping is needed to better characterize the distribution of lineages across the continent.

Habitat suitability models combined with potential host availability provide an opportunity to understand the potential distribution of *Bd* risk over large geographic areas (Murray et al., 2011; Liu et al., 2013; Yap et al., 2018). The results of our HSM generally align with previous potential distribution models (Penner et al., 2013; Zimkus et al., 2020); however, two key distinctions in our model are that (1) we incorporated potential host availability to determine risk of disease spread (Yap et al., 2015, 2016), and (2) we included the global human footprint (Venter et al., 2016). Although host abundance data at the community and continental scale would be ideal to identify host availability, such data are not available. Therefore, as a proxy, we used species richness, which has been shown to be an important factor in *Bd* spread (Rödder et al., 2009; Liu et al., 2013; Boria et al., 2014; Yap et al., 2016, 2018; Hydeman et al., 2017). We included the global human footprint (Venter et al., 2016) in our model because the presence of humans or human activities can play a role in disease spread (Liu et al., 2013; James et al., 2015). We also took additional steps to reduce sampling bias and minimize the chances of overfitting due to multicollinearity of the abiotic variables. Thus, our model is complementary to previous potential distribution models (Penner et

al., 2013; Zimkus et al., 2020). Our model identifies geographic areas with moderate to high risk of *Bd* spread as well as further highlighting African regions with high *Bd* suitability and supporting the assertion that countries in these regions are potential hotspots for the negative impacts of *Bd* (Figure 1-3).

In central Africa, the HSM predicted high *Bd* risk in Angola, Cameroon, Democratic Republic of Congo, Equatorial Guinea, Gabon, and Republic of Congo. The observed *Bd* dynamics in Cameroon and Democratic Republic of Congo, coupled with population declines in Cameroon (Hirschfeld et al., 2016) suggest a recent emergence, yet more information and monitoring is also needed in Angola, Equatorial Guinea, Gabon, and Republic of Congo to determine their *Bd* status (Table 1-1). We found a high proportion of *Bd* positives from Bioko Island (Equatorial Guinea) from 1998, and *Bd*-GPL has also been detected there (O’Hanlon et al., 2018; Byrne et al., 2019; Zimkus et al., 2020), but the lack of population survey data across a longer timescale prevents a proper understanding of *Bd*-host dynamics there.

In eastern Africa, the HSM model predicted high *Bd* risk in Kenya, Malawi, Mozambique, Tanzania, Zambia, and Zimbabwe. Mozambique and Zimbabwe border South Africa, where *Bd*-Cape may be endemic and there is evidence suggesting spread to Lesotho could be associated with amphibian declines (Minter et al., 2004). Tanzania experienced a documented *Bd*-driven species extinction in the wild in 2003 (Weldon et al., 2020). Additionally, Tanzania borders Kenya, where we found evidence of *Bd* emergence and high *Bd* risk, and Burundi, where we report high *Bd* prevalence and ambiguous *Bd* lineages (Seimon *et al.*, 2015). Because eastern Africa is identified as having widespread high *Bd* risk and multiple locations have high recent *Bd* prevalence, *Bd*-host dynamics in these countries should be closely monitored and studied.

In western Africa, our HSM model predicts high *Bd* risk in Ghana, Côte d'Ivoire, Liberia, Guinea, and Sierra Leone. However, there are no records of *Bd* positives there, and it has been hypothesized that the Dahomey Gap (a non-forested area between western Nigeria and eastern Ghana) may act as a natural barrier creating a safe haven from chytridiomycosis for amphibians (Penner et al., 2013). However, it is worrisome that in nearby Nigeria (a country where we identified moderate *Bd* risk) the frequency of *Bd* occurrence is very high (44.5%; 40.1–48.2% CI), and our model predicts highly suitable habitat just west of the Dahomey Gap, similar to previous studies (Penner et al., 2013; Zimkus et al., 2020). Also, other studies show that once *Bd* invades an area, it spreads over large distances and into remote areas rapidly (Lips et al., 2006; Berger et al., 2016; Yap et al., 2016; Vredenburg et al., 2019; Fisher and Garner, 2020). We suggest that future research should focus on *Bd*-host relationships in western Africa, including monitoring disease presence and spread to determine whether species are threatened by future *Bd* invasion and surveillance of amphibians in Nigeria to determine whether population declines have occurred.

In northern Africa, our HSM model predicted moderate risk in Morocco and in northern Algeria, Tunisia, and Egypt. However, this risk may be underestimated as our analysis did not include recent studies showing an expansion of *Bd* positives in Morocco (El Cadi et al., 2019; Thumsová et al., 2022). Despite these newer records for Morocco, data for northern Africa are limited; in fact, we are not aware of any published records for other countries in the region. Thus, northern Africa represents another understudied region where monitoring of amphibian populations and *Bd* are crucial.

New studies of *Bd*-host dynamics and interactions between *Bd* lineages in African amphibians could greatly advance our knowledge of chytridiomycosis. By bringing together new and published data spanning 165 years, our study demonstrates recent increases in the frequency

of *Bd* occurrence in much of Africa; yet, we also find areas where frequency of *Bd* occurrence has not changed over time. In some areas, increases in frequency of *Bd* occurrence are coincident with amphibian declines (*e.g.*, Cameroon, Tanzania, and Ethiopia; Hirschfeld et al., 2016; Channing et al., 2006; Weldon et al., 2020; Gower et al., 2012, 2013), whereas in regions where *Bd* has not changed in frequency over long periods of time, evidence of widespread declines has not been detected (*e.g.*, South Africa; Hopkins and Channing, 2003; Tarrant et al., 2013). Combined, these results indicate that Africa may harbor regions of *Bd* endemism (where host species may not be susceptible to epizootics) as well as regions where *Bd* is invading (posing large risks to host species). We find that multiple genetic lineages of *Bd* occur in Africa and suggest that (1) lineages generally considered to be low in virulence may actually be responsible for epizootic dynamics in some areas (*e.g.*, *Bd*-CAPE), and that (2) co-occurring lineages may be hybridizing. Our results provide an opportunity to further investigate virulence of *Bd* lineages in nature and to determine how *Bd* hybridization may affect virulence, pathogenicity, and health outcomes in hosts. Finally, our initial data and model predictions help expand our study to enable risk prediction in areas where no *Bd* data are presently available. Our study highlights the importance of determining the temporal context of host-pathogen dynamics and emphasizes the need to bridge historical and contemporary datasets to better describe host-pathogen dynamics over larger temporal scales and predict future scenarios.

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Data Availability Statement

All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials and are available on Zenodo (DOI: https://zenodo.org/record/5514129#.Y_Th5XbP02w). Georeferenced *Bd* records generated by the authors for this study are freely available through the Amphibian Disease Project hosted on the Genomic Observatories MetaDatabase (DOI: <https://n2t.net/ark:/21547/Dvp2>). Genotyping data

was deposited in the National Center for Biotechnology Information Sequence Read Archive (Bioproject PRJNA555719).

Author Contributions

VTV and DCB conceived and designed the study. DCB, EG, MTK, and DMP collected field samples. SLG and DCB collected museum samples. SLG and AQB performed laboratory work. DM performed histology. SLG, TAY, HS, ACA, SC, KL, AJM, EP, MSK and HR compiled and organized data from the literature. SLG, TAY, AQB, EBR, VTV, and HS contributed to the analysis of the data. SLG, VTV, TAY, AQB, HS, DM, and AGZ wrote the manuscript. All authors contributed to manuscript revision, read, and approved the final version submitted for publication.

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Tables and Figures

Table 1-1 Compiled historical and recent frequency of *Batrachochytrium dendrobatidis* (*Bd*) occurrence by African country and region. N=16,857; Results are grouped by time period (historical: 1852–1999; recent: 2000–2017); percent *Bd*-infected is frequency of *Bd* occurrence with 95% confidence intervals based on a binomial distribution; Pr(no *Bd*) is the probability of finding no *Bd*-positives in each decade (based on a binomial distribution) if frequency of *Bd* occurrence were 11% (Talley et al., 2015); For countries that had historical and recent data, we used Chi-square tests to determine if frequency of *Bd* occurrence was significantly associated with time period based on p-values (NA indicates missing historical or recent data; NA* indicates that a Chi-square p-value could not be approximated (expected values were less than 5); *Bd* survey data can be found in the associated references (“this study” indicates our data (records we generated for this study)); ¹Central Africa includes grouped data from Cameroon, Democratic Republic of the Congo, Equatorial Guinea, and Gabon, and from Congo and Democratic Republic of the Congo (Soto-Azat et al., 2010); ²Eastern Africa includes grouped data from Rwanda and Uganda (Soto-Azat et al., 2010); ³Southern Africa includes grouped data from South Africa, Lesotho, and Swaziland (Weldon et al., 2004); ⁴ Multiple Regions includes grouped data from Angola, Democratic Republic of the Congo, Tanzania and Zambia, from Cameroon, Equatorial Guinea, Gabon, Ghana, Guinea-Bissau, Liberia, Nigeria and Sierra Leone, from Cameroon, Equatorial Guinea, Gabon and Nigeria, from Democratic Republic of the Congo, Eritrea, Kenya, Lesotho, Malawi, Mozambique, Namibia, South Africa, Sudan, Tanzania, Uganda and Zimbabwe, from Democratic Republic of the Congo and Uganda, from Democratic Republic of the Congo and Zambia, from Ethiopia and Sudan, and from Malawi, Mozambique, Nigeria, Tanzania and Zambia (Soto-Azat et al., 2010).

Region/Country	Historical (1852–1999)			Recent (2000–2017)			Chi-sq. p-value	References
	Percent <i>Bd</i> Infected (95% CI)	No. <i>Bd</i> - Positive/Total Tested	Pr(no <i>Bd</i>)	Percent <i>Bd</i> Infected (95% CI)	No. <i>Bd</i> - Positive/Total Tested	Pr(no <i>Bd</i>)		
Central Africa ¹	7.04 (5.4, 8.99)	59 / 838	<0.001	16.97 (16.02, 17.95)	1003 / 5910	<0.001	<0.001	
Cameroon	0.62 (0.07, 2.21)	2 / 325	<0.001	16.72 (15.50, 18.00)	588 / 3516	<0.001	<0.001	Baláz, Kopecký, & Gvoždík, 2012; Blackburn et al., 2010; Doherty-Bone et al., 2013; Gower, Doherty-Bone, et al., 2013; Hirschfeld et al., 2016; Miller et al., 2018; Soto-Azat et al., 2010; Zimkus et al., 2020; This study
Central African Republic				0.00 (0.00, 70.76)	0 / 3	0.7	NA	“Bd-maps,” 2014
Democratic Republic of the Congo	0.65 (0.02, 3.56)	1 / 154	<0.001	19.82 (17.40, 22.42)	199 / 1004	<0.001	<0.001	“Bd-maps,” 2014; Greenbaum, Kusamba, Aristote, & Reed, 2008; Greenbaum, Meece, Reed, & Kusamba, 2014, 2015; Seimon et al., 2015; Soto-Azat et al., 2010; This study
Equatorial Guinea	17.89 (13.81, 22.60)	56 / 313	<0.001	0.00 (0.00, 11.22)	0 / 31	0.03	0.02	Hydeman et al., 2017; This study
Gabon	0.00 (0.00, 84.19)	0 / 2	0.79	17.09 (14.59, 19.82)	142 / 831	<0.001	NA*	“Bd-maps,” 2014; Bell et al., 2011; Gratwicke et al., 2011; Hydeman et al., 2017; Jongsma & Blackburn, 2016; Soto-Azat et al., 2010
Republic of Congo				6.67 (1.85, 16.20)	4 / 60	<0.001	NA	Jongsma & Blackburn, 2016; Zimkus et al., 2020

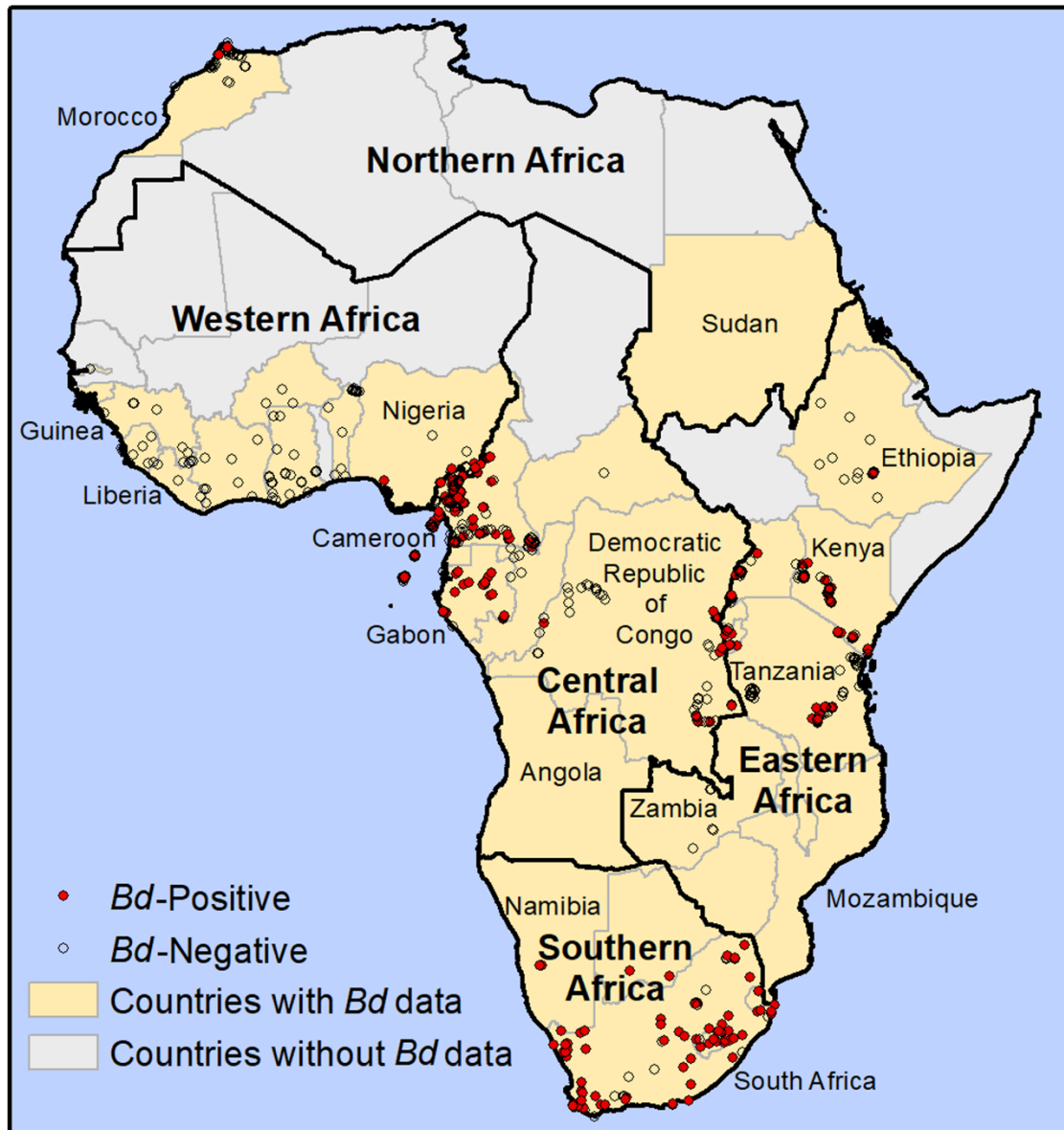
São Tomé and Príncipe	0.00 (0.00, 84.19)	0 / 2	0.79	15.05 (11.93, 18.63)	70 / 465	<0.001	NA*	Hydeman, Bell, Drewes, & Zamudio, 2013; Hydeman et al., 2017
Eastern Africa ²	1.31 (0.90, 1.84)	32 / 2443	<0.001	20.74 (19.30, 22.24)	618 / 2980	<0.001	<0.001	
Burundi	0.00 (0.00, 24.71)	0 / 13	0.22	73.65 (66.29, 80.16)	123 / 167	<0.001	NA*	Seimon et al., 2015; This study
Ethiopia	3.85 (0.10, 19.64)	1 / 26	0.05	42.50 (33.53, 51.85)	51 / 120	<0.001	<0.001	Gower et al., 2012; Soto-Azat et al., 2010; This study
Kenya	1.86 (1.00, 3.16)	13 / 698	<0.001	31.48 (28.38, 34.69)	271 / 861	<0.001	<0.001	Kielgast et al., 2010; Ouellet, Mikaelian, Pauli, Rodrique, & Green, 2005; Soto-Azat et al., 2010; This study
Malawi	100 (2.50, 100.00)	1 / 1	0.89	18.42 (10.45, 28.97)	14 / 76	<0.001	NA*	Conradie, Harvey, Kotzé, Dalton, & Cunningham, 2011; Soto-Azat et al., 2010
Mozambique				0.77 (0.02, 4.21)	1 / 130	<0.001	NA	Conradie, Bittencourt-silva, Loader, Dalton, & Tolley, 2016
Rwanda	0.00 (0.00, 23.16)	0 / 14	0.2	17.12 (12.41, 22.73)	38 / 222	<0.001	NA*	Seimon et al., 2015
Tanzania	0.00 (0.00, 6.16)	0 / 58	<0.01	5.38 (4.05, 6.97)	53 / 986	<0.001	NA*	“Bd-maps,” 2014; Gower, Doherty-Bone, et al., 2013; Moyer & Weldon, 2006; Zancolli, Storfer, & Rödel, 2013; This study
Uganda	1.05 (0.61, 1.67)	17 / 1621	<0.001	19.14 (15.15, 23.66)	67 / 350	<0.001	<0.001	Goldberg et al., 2007; Seimon et al., 2015; Soto-Azat et al., 2010; This study
Zambia				0.00 (0.00, 5.28)	0 / 68	<0.001	NA	“Bd-maps,” 2014
Northern Africa				2.03 (0.88, 3.96)	8 / 394	<0.001	NA	

Morocco				2.03 (0.88, 3.96)	8 / 394	<0.001	NA	“Bd-maps,” 2014; El Mouden et al., 2011
Southern Africa ³	6.56 (4.98, 8.46)	55 / 838	<0.001	24.16 (22.00, 26.42)	358 / 1482	<0.001	<0.001	
Botswana	100.00 (15.81, 100.00)	2 / 2	0.79				NA	“Bd-maps,” 2014; Weldon, 2005
Lesotho	15.38 (4.36, 34.87)	4 / 26	0.05	72.73 (39.03, 93.98)	8 / 11	0.28	NA*	“Bd-maps,” 2014; Weldon, 2005; This study
Namibia				60.00 (36.05, 80.88)	12 / 20	0.1	NA	Zimkus et al., 2020
South Africa	25.69 (17.80, 34.94)	28 / 109	<0.001	23.29 (21.14, 25.56)	338 / 1451	<0.001	0.65	“Bd-maps,” 2014; Conradie, Weldon, Smith, & Du Preez, 2011; Griffiths et al., 2018; Hopkins & Channing, 2003; Soto-Azat et al., 2010; Tarrant et al., 2013; Weldon, 2005
Swaziland	50.00 (6.76, 93.24)	2 / 4	0.63				NA	“Bd-maps,” 2014; Weldon, 2005
Western Africa	0.00 (0.00, 30.85)	0 / 10	0.31	20.69 (18.71, 22.79)	323 / 1561	<0.001	NA*	
Benin				0.00 (0.00, 2.64)	0 / 138	<0.001	NA	“Bd-maps,” 2014; Penner et al., 2013
Burkina Faso				0.00 (0.00, 52.18)	0 / 5	0.56	NA	“Bd-maps,” 2014; Penner et al., 2013
Cote D'Ivoire	0.00 (0.00, 97.50)	0 / 1	0.89	0.00 (0.00, 5.78)	0 / 62	<0.001	NA*	“Bd-maps,” 2014; Penner et al., 2013
Ghana				0.00 (0.00, 1.23)	0 / 297	<0.001	NA	“Bd-maps,” 2014; Penner et al., 2013

Guinea				0.00 (0.00, 1.22)	0 / 300	<0.001	NA	“Bd-maps,” 2014; Penner et al., 2013
Liberia				0.00 (0.00, 20.59)	0 / 16	0.15	NA	“Bd-maps,” 2014; Penner et al., 2013
Nigeria	0.00 (0.00, 52.18)	0 / 5	0.56	44.49 (40.84, 48.19)	323 / 726	<0.001	NA*	Imasuen et al., 2011; Ouellet et al., 2005; Reeder, Cheng, Vredenburg, & Blackburn, 2011; Zimkus et al., 2020
Sierra Leone	0.00 (0.00, 60.24)	0 / 4	0.63	0.00 (0.00, 21.80)	0 / 15	0.17	NA*	“Bd-maps,” 2014; Penner et al., 2013; Soto-Azat et al., 2010
The Gambia				0.00 (0.00, 84.19)	0 / 2	0.79	NA	“Bd-maps,” 2014
Multiple Regions ⁴	0.00 (0.00, 0.92)	0 / 401	<0.001				NA	
TOTAL AFRICA	3.22 (2.73, 3.78)	146 / 4530	<0.001	18.74 (18.05, 19.44)	2310 / 12327	<0.001	<0.001	

Figure 1-1. Distribution and frequency of *Batrachochytrium dendrobatidis* (*Bd*) occurrence in Africa 1852-2017. (A) Georeferenced *Bd* survey data for Africa (N=11,472; includes records generated for this study and previously published records for which we had latitude and longitude); red dots indicate *Bd*-positive records (N=1,914); open circles indicate *Bd*-negative records (N=9,558); yellow fill indicates countries with *Bd* data (including georeferenced and non-georeferenced locations); black outlines indicate regions, as defined by the United Nations; (B) frequency of *Bd* occurrence for all of Africa by decade; N=16,686 (171 samples included in Table 1-1 not included here because collection years did not fall into a single decade); (C) frequency of *Bd* occurrence and infection intensity results from Cameroon; N=1,940 (includes our data records generated for this study, records from Hirschfeld et al. (2016) and from Zimkus et al. (2020)); (D) frequency of *Bd* occurrence and infection intensity results from Kenya; N=1,503 (records through 1999 generated for this study; 2006 records from Kielgast et al. (2010)); (B–D) grey bars represent sample size (N); blue line represents frequency of occurrence (percent infected) and blue bars represent the corresponding 95% binomial confidence intervals (95% CI); (C–D) green line represents the average infection intensity (the logarithm of zoospore equivalents or logZE) and green bars represent the corresponding standard error; green diamonds represent maximum logZE for each time period; green hashed horizontal line indicates a logZE value of 4, the threshold for deadly infection intensity (Vredenburg *et al.*, 2010; Kinney *et al.*, 2011).

A



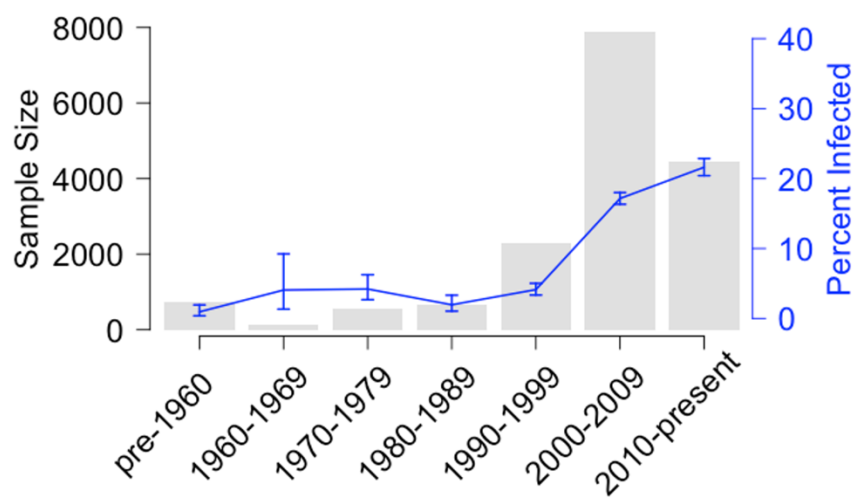
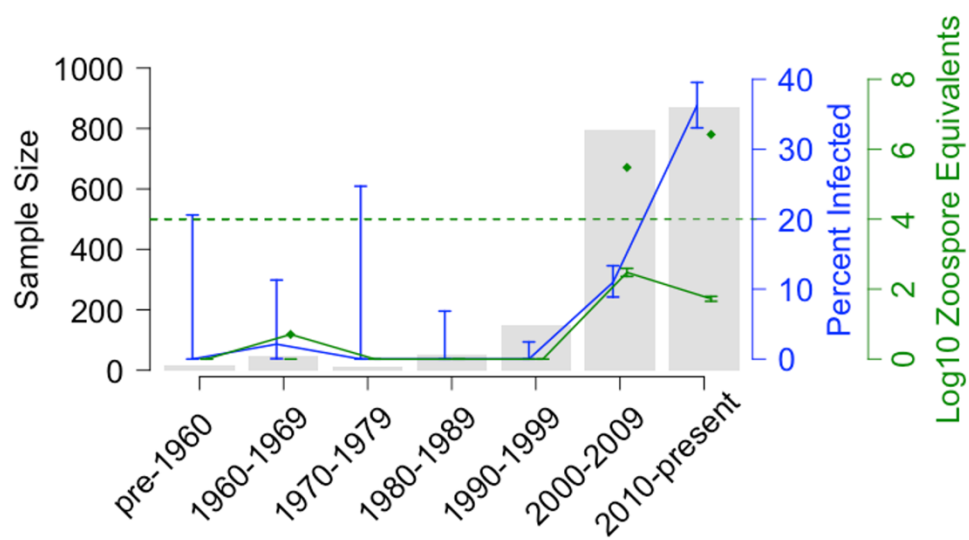
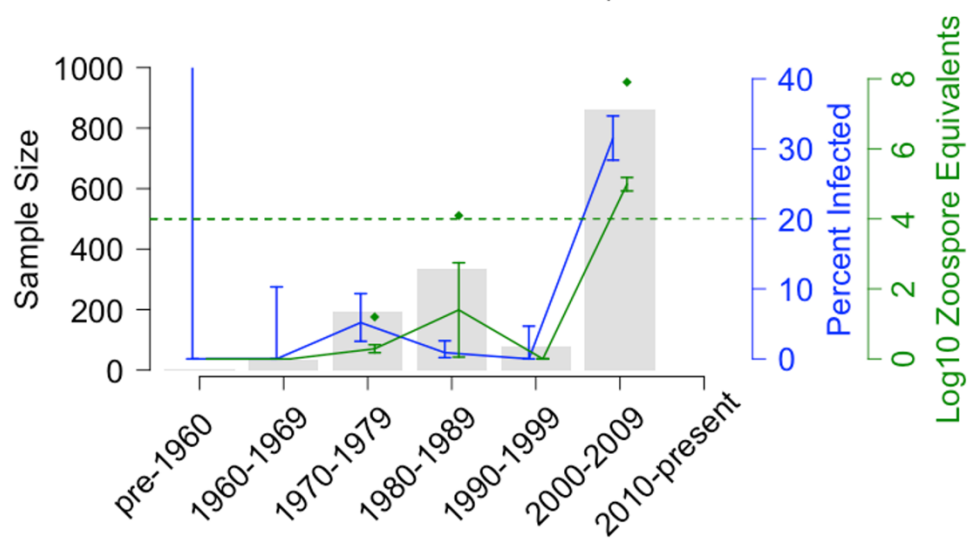
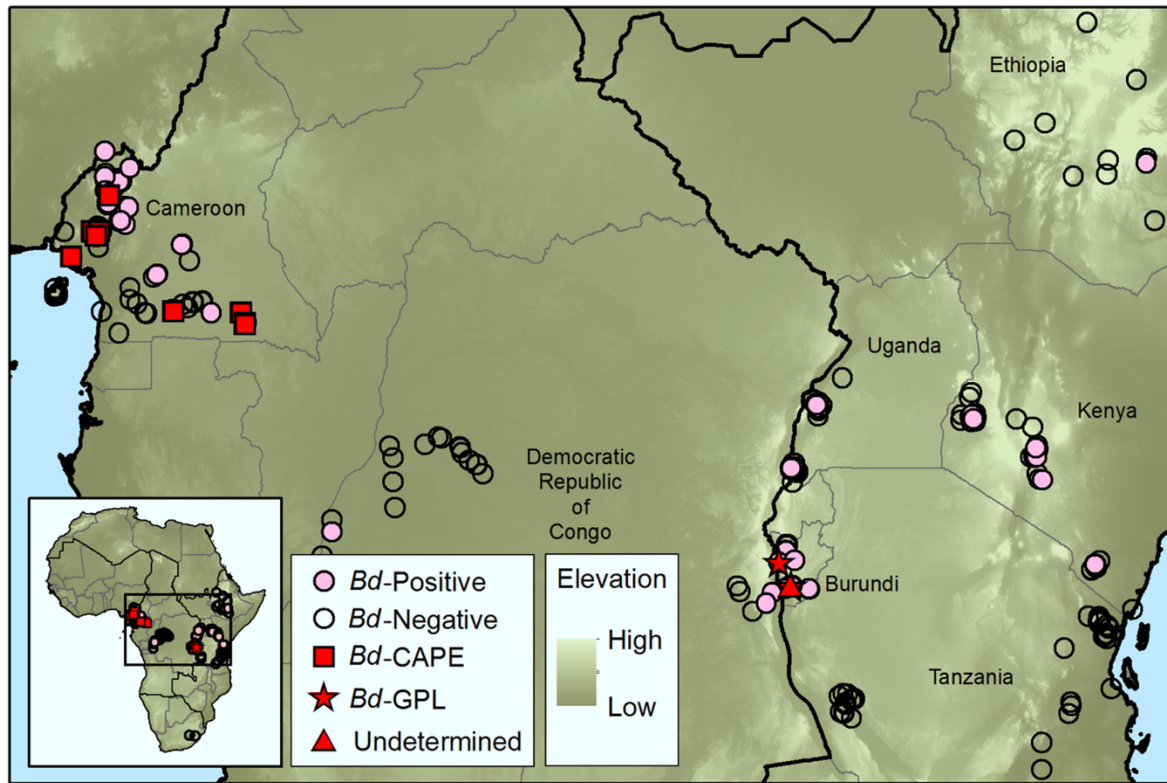
B**C****D**

Figure 1-2. Genotyping of *Batrachochytrium dendrobatidis* (*Bd*) lineages from samples processed in this study. (A) Locations and infection status of georeferenced samples processed by the authors of this study (N=4,349) showing *Bd*-positive (pink circles) and *Bd*-negative (open circles) locations; locations of *Bd* samples genotyped in this study (N=32) are indicated by red squares (*Bd*-GPL), red stars (*Bd*-Cape), and red triangles (Undetermined lineage); **(B)** best-scoring unrooted maximum likelihood tree for 61 *Bd* samples (32 sequenced for this study, 31 previously-published *Bd* isolates). Tree created from concatenated, aligned consensus sequences from 186 *Bd* loci (25,617 bp). Nodes with less than 10% bootstrap support have been collapsed and the size of the black node markers are scaled based on bootstrap support. Geographic origin for each previously published isolate is indicated with a colored dot. Known *Bd* lineages are inside grey circles and samples genotyped for this study are in yellow circles. See Table S1-4 for sample information associated with the newly genotyped swabs.

A



B

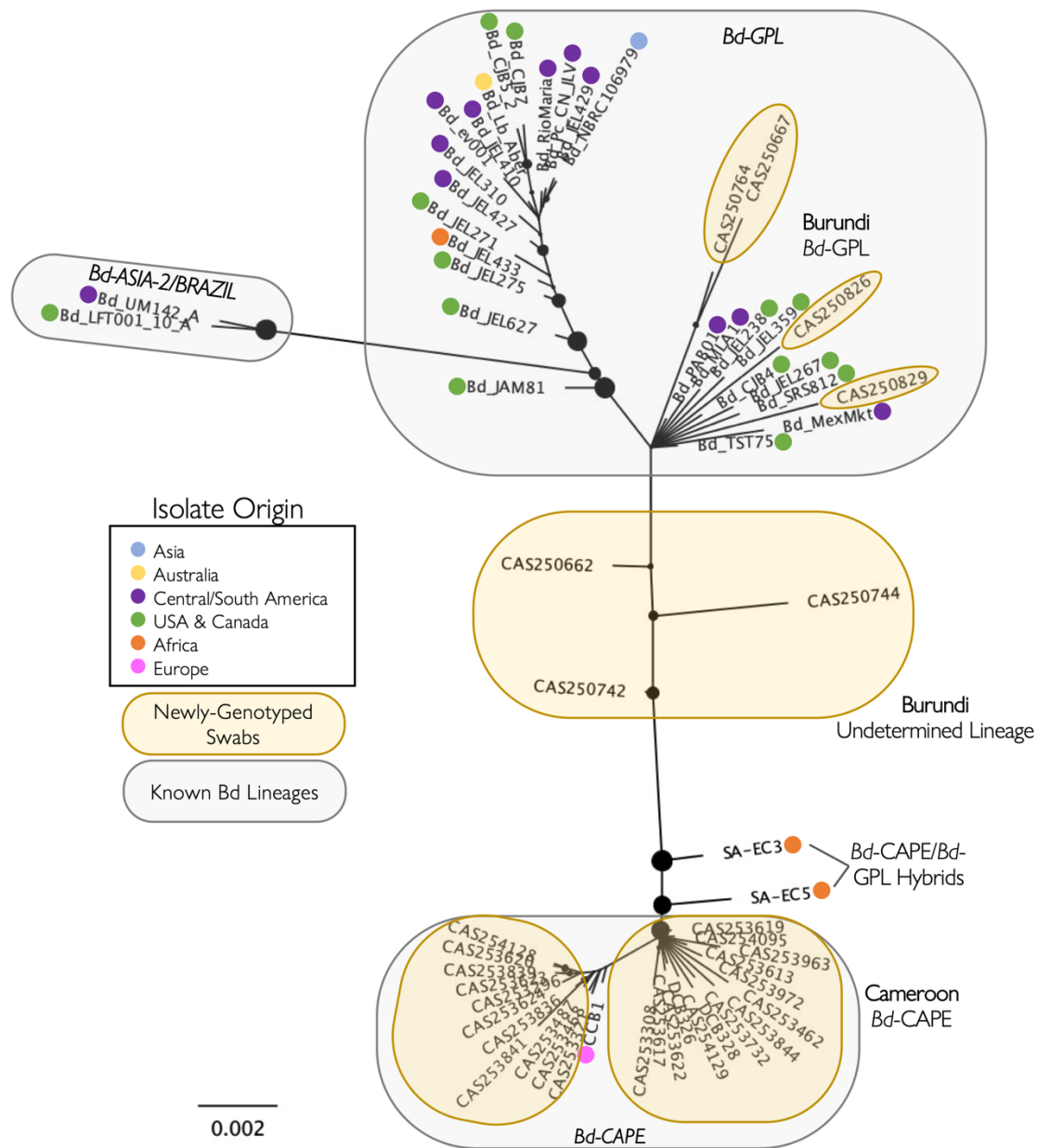
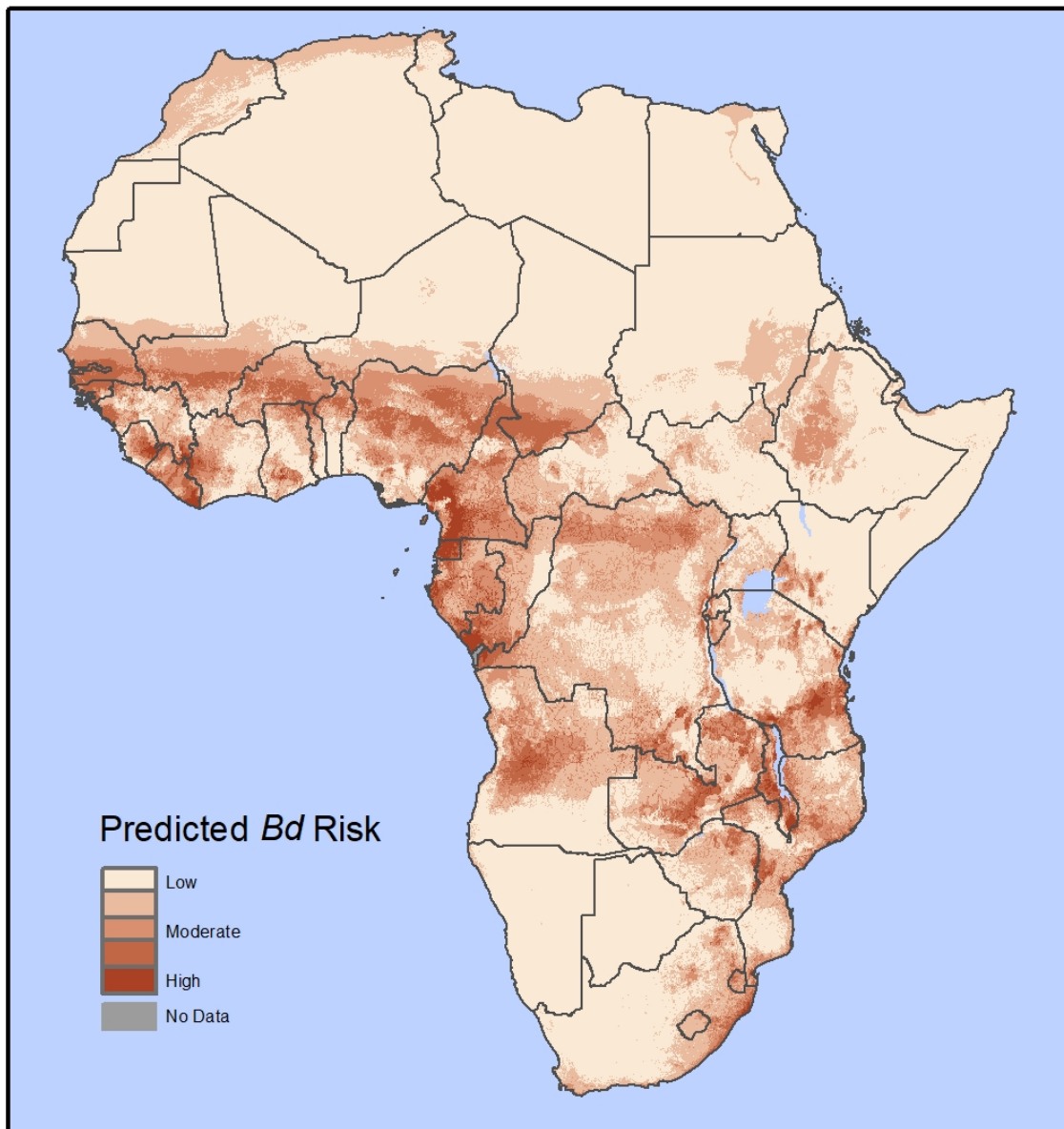


Figure 1-3. Predicted *Bd* disease risk in Africa from our habitat suitability model (HSM);
Based on 1,914 georeferenced *Bd* occurrence records with 202 unique *Bd*-positive localities in the African mainland and islands in the Gulf of Guinea.



Supplementary Material

Supplemental Methods: Notes on Dataset

To detect *Bd* occurrence and intensity in African amphibians, we collected swab samples from 2,972 museum preserved amphibian specimens as well as 1,076 live animals in the field (4,048 samples total). We also include data for an additional 575 field-collected samples from Cameroon (Hirschfeld *et al.*, 2016) collected and processed using the same methods, making the total number of records 4,623 in our study.

We also gathered data from the literature amounting to 12,297 *Bd* records. When compiling the data, we found that most records published in Doherty-Bone *et al.* (2008) were also included in Hirschfeld *et al.* (2016), and we therefore did not include Doherty-Bone *et al.* (2008) so as not to duplicate those records. Of the 12,297 records, 63 were from studies that did not report both *Bd*-positive and *Bd*-negative samples, and therefore were not included in our calculations of the frequency of *Bd* occurrence. Those 63 records were used in our *Bd* risk model as *Bd* positive localities.

When binning our data by decade, records associated with a range of years across multiple decades were included in the decade with most overlap as long as dates extended no more than 3 years into another decade. Records that extended more than 3 years into a consecutive decade were excluded from our analysis of *Bd* occurrence by decade.

For analysis of *Bd* infection intensity in Cameroon and Kenya by decade, we included data from three previous publications in addition to our generated data (Cameroon: Hirschfeld *et al.*, 2016; Zimkus *et al.*, 2020; Kenya: Kielgast *et al.*, 2010). These studies reported the same methods for qPCR that we used, and so we calculated the infection intensity for those samples as we did

for our samples (multiplying reported GE by a dilution factor of 80 and log-transforming the resultant $ZE + 1$). For Kenya, data for the 2000s were all collected in 2006 and previously published in Kielgast *et al.* (2010). Their infection intensity data was available in a summary table that reported the average GE from each of 31 *Bd* positive populations. Therefore, after calculating our log transformed ZE from those average GE values, we took the weighted mean of their reported means using the R function `wtd.mean`, and calculated the variance of the distribution of their reported means using `wtd.var`. Standard deviation and standard error were calculated from the variance across populations (N=31 *Bd* positive sites) (see error bars in Figure 1-1D for 2000–2009). The SE error bars graphed around the mean infection intensity value may therefore be smaller than if we had access to raw data.

For our analysis of pre- and post- emergence data (emergence defined as the year 2000; Table 1-1), we used a Chi-square test of independence of *Bd* detection (positive vs. negative) and time period (pre-2000 vs. 2000-present) to determine whether there was a relationship between these two categorical variables. For countries and regions that had both pre- and post- emergence data, a significant Chi-square p-value for these tests would indicate that knowing the frequency of *Bd* detection would help to predict whether samples came from the pre- or post- emergence time period and vice versa.

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Table S1-1. Summary of all *Bd* data for Africa by decade. N=16,686 (171 samples included in Table 1-1 were not included here because collection years did not fall into a single decade); Lower and upper CI (95% confidence interval) are based on the binomial distribution; Pr(no *Bd*) is the probability of finding no *Bd*-positive samples in each decade (based on a binomial distribution) if frequency of *Bd* occurrence were actually 11% (Talley et al., 2015). The data tabulated here are graphed in Figure 1-1B.

Decade	No. <i>Bd</i> Positives	No. Examined	% Infected	Lower CI	Upper CI	Pr(no <i>Bd</i>)
Pre-1960	7	739	0.95	0.38	1.94	<0.001
1960–1969	5	123	4.07	1.33	9.23	<0.001
1970–1979	23	546	4.21	2.69	6.25	<0.001
1980–1989	13	664	1.96	1.05	3.32	<0.001
1990–1999	94	2277	4.13	3.35	5.03	<0.001
2000–2009	1355	7901	17.15	16.32	18.00	<0.001
2010–present	959	4436	21.62	20.41	22.86	<0.001

Table S1-2. Summary of Cameroonian *Bd* data by decade. N= 1,940; No. *Bd* Pos. is the number of samples testing positive for infection; Total No. is the total number of samples tested; % infected is based on the ratio of positives to the total; Lower and upper CI (95% confidence interval) are based on the binomial distribution; Pr(no *Bd*) is the probability of finding no *Bd* positive samples in each decade (based on a binomial distribution) if frequency of *Bd* occurrence were actually 11% (Talley et al., 2015); $\bar{x}$ LogZE is the mean of log transformed zoospore equivalents; SD LogZE is the standard deviation of log transformed zoospore equivalents; SE LogZE is the standard error of log transformed zoospore equivalents; Max. LogZE is the maximum value of log transformed zoospore equivalents in the decade; The data tabulated here are graphed in Figure 1-1C.

Decade	No. <i>Bd</i> Pos.	Total No.	% Infected	Lower CI	Upper CI	Pr(no <i>Bd</i>)	$\bar{x}$LogZE	SD LogZE	SE LogZE	Max. LogZE
Pre-1960	0	16	0.00	0.00	20.59	0.15	0.00	0.00	0.00	NA
1960–1969	1	47	2.13	0.05	11.29	<0.01	0.70	0.00	0.00	0.70
1970–1979	0	13	0.00	0.00	24.71	0.22	0.00	0.00	0.00	NA
1980–1989	0	52	0.00	0.00	6.85	<0.01	0.00	0.00	0.00	NA
1990–1999	0	149	0.00	0.00	2.45	<0.001	0.00	0.00	0.00	NA
2000–2009	87	794	10.96	8.87	13.34	<0.001	2.48	1.07	0.11	5.48
2010–present	315	869	36.25	33.05	39.54	<0.001	1.72	1.24	0.07	6.42

Table S1-3. Summary of Kenyan *Bd* data by decade. N= 1,503; No. *Bd* Pos. is the number of samples testing positive for infection; Total No. is the total number of samples tested; % infected is based on the ratio of positives to the total; Lower and upper CI (95% confidence interval) are based on the binomial distribution; Pr(no *Bd*) is the probability of finding no *Bd* positive samples in each decade (based on a binomial distribution) if frequency of *Bd* occurrence were actually 11% (Talley et al., 2015); $\bar{x}$ LogZE is the mean of log transformed zoospore equivalents; SD LogZE is the standard deviation of log transformed zoospore equivalents; SE LogZE is the standard error of log transformed zoospore equivalents; Max. LogZE is the maximum value of log transformed zoospore equivalents in the decade; The data tabulated here are graphed in Figure 1-1D.

Decade	No. <i>Bd</i> Pos.	Total No.	% Infected	Lower CI	Upper CI	Pr(no <i>Bd</i>)	$\bar{x}$LogZE	SD LogZE	SE LogZE	Max. LogZE
Pre-1960	0	2	0.00	0.00	84.19	0.79	0.00	0.00	0.00	NA
1960–1969	0	34	0.00	0.00	10.28	0.02	0.00	0.00	0.00	NA
1970–1979	10	193	5.18	2.51	9.32	<0.001	0.29	0.36	0.11	1.20
1980–1989	3	336	0.89	0.18	2.59	<0.001	1.40	2.33	1.35	4.09
1990–1999	0	77	0.00	0.00	4.68	<0.001	0.00	0.00	0.00	NA
2000–2009	271	861	31.48	28.38	34.69	<0.001	4.99	1.07	0.19	7.90
2010–present	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

Table S1-4. Histology results for samples analyzed in this study. N=14; NHL = no histologic lesions.

Museum ID	Field ID	LogZE	Genus	species	Col. Year	Country	decimal Long	decimal Lat	Morphological Diagnosis
CAS25081 7	DCB 183	2.21	<i>Leptopelis</i>	<i>bocagii</i>	2011	Burundi	29.474300	-4.013680	NHL
CAS25081 8	DCB 184	2.10	<i>Leptopelis</i>	<i>bocagii</i>	2011	Burundi	29.474300	-4.013680	NHL
CAS25082 0	DCB 186	4.37	<i>Leptopelis</i>	<i>bocagii</i>	2011	Burundi	29.474300	-4.013680	Epidermal hyperplasia and hyperkeratosis, moderate with moderate numbers of intraepithelial sporangia consistent with <i>Bd</i>
CAS25082 1	DCB 187	4.01	<i>Leptopelis</i>	<i>bocagii</i>	2011	Burundi	29.474300	-4.013680	Epidermal hyperplasia and hyperkeratosis, moderate with moderate numbers of intraepithelial sporangia consistent with <i>Bd</i>
CAS25001 1	BJE 3216	4.62	<i>Arthroleptis</i>	<i>poecilonotus</i>	2011	Cameroon	10.759983	6.548617	Epidermal hyperplasia and hyperkeratosis, severe with numerous intraepithelial sporangia consistent with <i>Bd</i>
CAS25003 9	BJE 3274	0.00	<i>Arthroleptis</i>	<i>poecilonotus</i>	2011	Cameroon	10.035133	6.335950	NHL
CAS25330 8	DCB 266	4.13	<i>Hylarana</i>	<i>albolabris</i>	2013	Cameroon	13.904100	2.900570	NHL
CAS25331 5	DCB 273	0.27	<i>Hylarana</i>	<i>albolabris</i>	2013	Cameroon	13.049200	2.900960	NHL

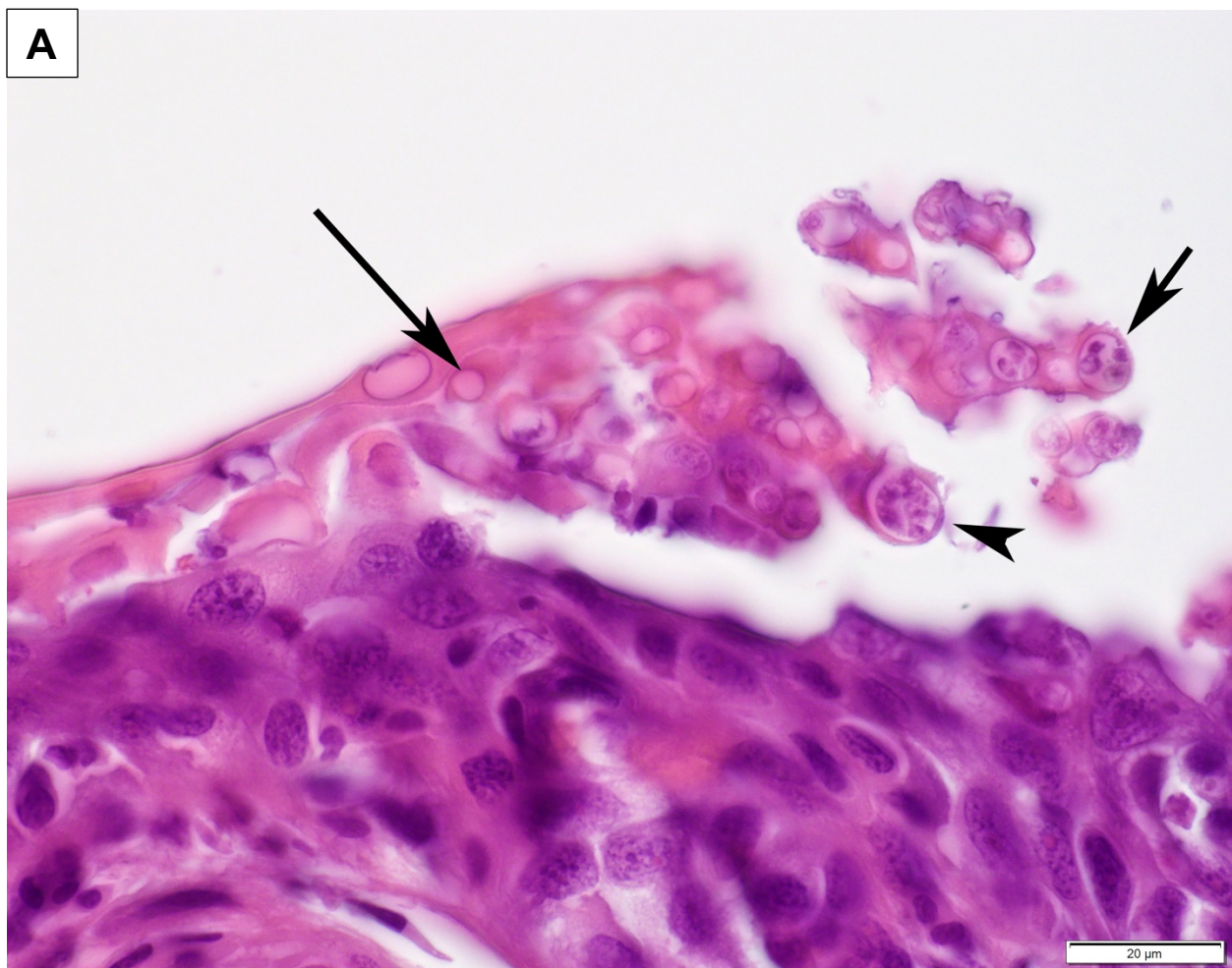
CAS25361 7	DCB 609	4.06	<i>Hyperolius</i>	<i>riggenbachi</i>	2013	Cameroon	10.158530	5.855733	Epidermal hyperplasia, mild to moderate
CAS25361 6	DCB 608	2.10	<i>Hyperolius</i>	<i>riggenbachi</i>	2013	Cameroon	10.158530	5.855733	Epidermal hyperplasia, mild to moderate
CAS25373 2	DMP 703	4.46	<i>Leptopelis</i>	<i>aubryi</i>	2013	Cameroon	9.066090	4.326760	NHL
CAS25372 8	DMP 699	0.00	<i>Leptopelis</i>	<i>aubryi</i>	2013	Cameroon	9.066090	4.326760	NHL
CAS25370 4	NA	1.65	<i>Xenopus</i>	<i>longipes</i>		Cameroon			NHL
CAS25370 0	NA	0.00	<i>Xenopus</i>	<i>longipes</i>		Cameroon			NHL

Table S1-5. Genotyped *Bd* swab samples in this study. N=32; No. Loci is the number of loci that amplified.

Museum ID	Field ID	Log ZE	No. Loci	Genus	species	Col. Year	Country	decimal Lon	decimal Lat
CAS250829	DCB195	2.34	156	<i>Leptopelis</i>	<i>bocagii</i>	2011	Burundi	29.27305	-3.34964
CAS250742	DCB119	3.73	180	<i>Hyperolius</i>	<i>kivuensis</i>	2011	Burundi	29.62737	-3.94678
CAS250826	DCB192	2.21	133	<i>Ptychadena</i>	cf. <i>mascareniensis</i>	2011	Burundi	29.27311	-3.34304
CAS250764	DCB125	2.22	133	<i>Xenopus</i>	sp.	2011	Burundi	29.6284	-3.94108
CAS250744	DCB121	2.39	96	<i>Hyperolius</i>	<i>viridiflavus</i>	2011	Burundi	29.62737	-3.94678
CAS250662	DCB39	2.28	162	<i>Hyperolius</i>	<i>viridiflavus</i>	2011	Burundi	29.6147	-3.9534
CAS250667	DCB44	2.22	98	<i>Hyperolius</i>	<i>viridiflavus</i>	2011	Burundi	29.6147	-3.9534
CAS253622	DCB614	3.25	181	<i>Hyperolius</i>	<i>adametzi</i>	2013	Cameroon	10.15853	5.855733
CAS253623	DCB615	3.17	176	<i>Hyperolius</i>	<i>adametzi</i>	2013	Cameroon	10.15853	5.855733
CAS253624	DCB616	3.45	178	<i>Hyperolius</i>	<i>adametzi</i>	2013	Cameroon	10.15853	5.855733
CAS253308	DCB266	4.13	101	<i>Hylarana</i>	<i>albolabris</i>	2013	Cameroon	13.9041	2.90057
CAS253732	DMP703	4.46	180	<i>Leptopelis</i>	<i>aubryi</i>	2013	Cameroon	9.06609	4.32676
CAS253972	DMP956	3.35	180	<i>Leptopelis</i>	<i>aubryi</i>	2013	Cameroon	9.77183	4.84975
CAS254095	DMP1038	3.68	169	<i>Leptopelis</i>	<i>boulengeri</i>	2013	Cameroon	9.69563	4.94781
CAS254128	DMP1076	3.43	182	<i>Leptopelis</i>	<i>calcaratus</i>	2013	Cameroon	9.6686	4.9606

CAS2541 29	DMP 1077	3.35	179	<i>Leptopelis</i>	<i>calcaratus</i>	2013	Cameroon	9.6686	4.9606
CAS2534 62	DCB 438	2.35	93	<i>Afrivalus</i>	<i>fulvovittatus</i>	2013	Cameroon	11.97633	2.93994
CAS2534 68	DCB 444	2.38	172	<i>Afrivalus</i>	<i>fulvovittatus</i>	2013	Cameroon	11.97633	2.93994
CAS2533 63	DCB 326	2.55	162	<i>Sclerophrys</i>	<i>gracilipes</i>	2013	Cameroon	14.0234	2.6106
CAS2533 65	DCB 328	3.34	177	<i>Sclerophrys</i>	<i>gracilipes</i>	2013	Cameroon	14.0234	2.6106
CAS2538 41	DMP 781	2.20	67	<i>Afrivalus</i>	<i>lacteus</i>	2013	Cameroon	9.865061	4.959439
CAS2538 44	DMP 784	3.32	96	<i>Afrivalus</i>	<i>lacteus</i>	2013	Cameroon	9.865061	4.959439
CAS2536 13	DCB 605	3.44	180	<i>Leptopelis</i>	<i>nordequatorialis</i>	2013	Cameroon	10.15853	5.855733
CAS2539 63	DMP 903	2.27	102	<i>Afrivalus</i>	<i>paradorsalis</i>	2013	Cameroon	9.77183	4.84975
CAS2532 96	DCB 254	3.37	177	<i>Arthroleptis</i>	<i>poecilonotus</i>	2013	Cameroon	13.90334	2.90072
CAS2533 21	DCB 283	2.20	153	<i>Arthroleptis</i>	<i>poecilonotus</i>	2013	Cameroon	14.02324	2.63101
CAS2534 87	DCB 463	2.66	167	<i>Arthroleptis</i>	<i>poecilonotus</i>	2013	Cameroon	11.97633	2.93994
CAS2536 17	DCB 609	4.06	182	<i>Hyperolius</i>	<i>riggenbachi</i>	2013	Cameroon	10.15853	5.855733
CAS2536 19	DCB 611	2.44	138	<i>Hyperolius</i>	<i>riggenbachi</i>	2013	Cameroon	10.15853	5.855733
CAS2536 20	DCB 612	3.30	180	<i>Hyperolius</i>	<i>riggenbachi</i>	2013	Cameroon	10.15853	5.855733
CAS2538 36	DMP 769	2.37	144	<i>Leptopelis</i>	<i>rufus</i>	2013	Cameroon	9.865061	4.959439
CAS2538 39	DMP 772	3.53	174	<i>Leptopelis</i>	<i>rufus</i>	2013	Cameroon	9.865061	4.959439

Figure S1-1. Images of histological samples. Samples were stained with hematoxylin and eosin and visualized at 1000X magnification; **(A)** *Bd*-infected skin from a specimen of *Arthroleptis poecilonotus* collected in Cameroon (CAS250011); The epidermis and stratum corneum are severely thickened, hyperplastic, and hyperkeratotic. Numerous organisms consistent with *Bd* at various stages of maturation are present in the keratinized epithelium; These include empty thalli (long arrow), zoosporangia filled with multiple zoospores (short arrow), and a zoosporangium with a discharge papilla (arrowhead); **(B)** Normal skin from a specimen of *Xenopus longipes* collected in Cameroon (CAS253704); The epidermis and stratum corneum (keratinized layer) are uniformly thin.



B

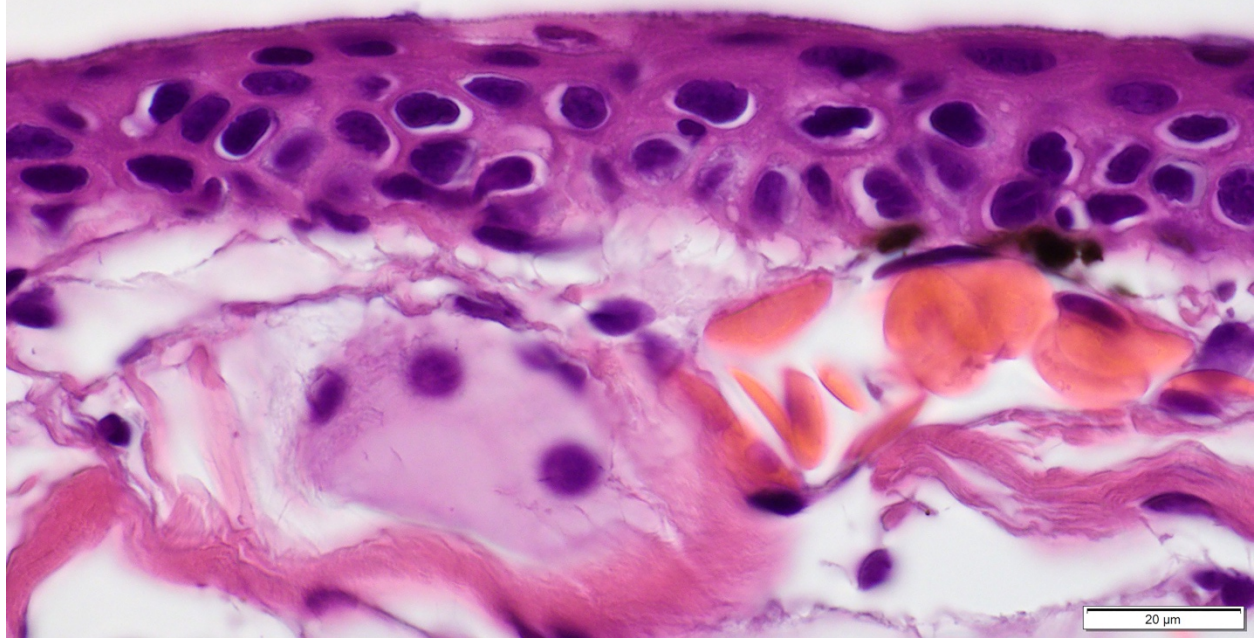
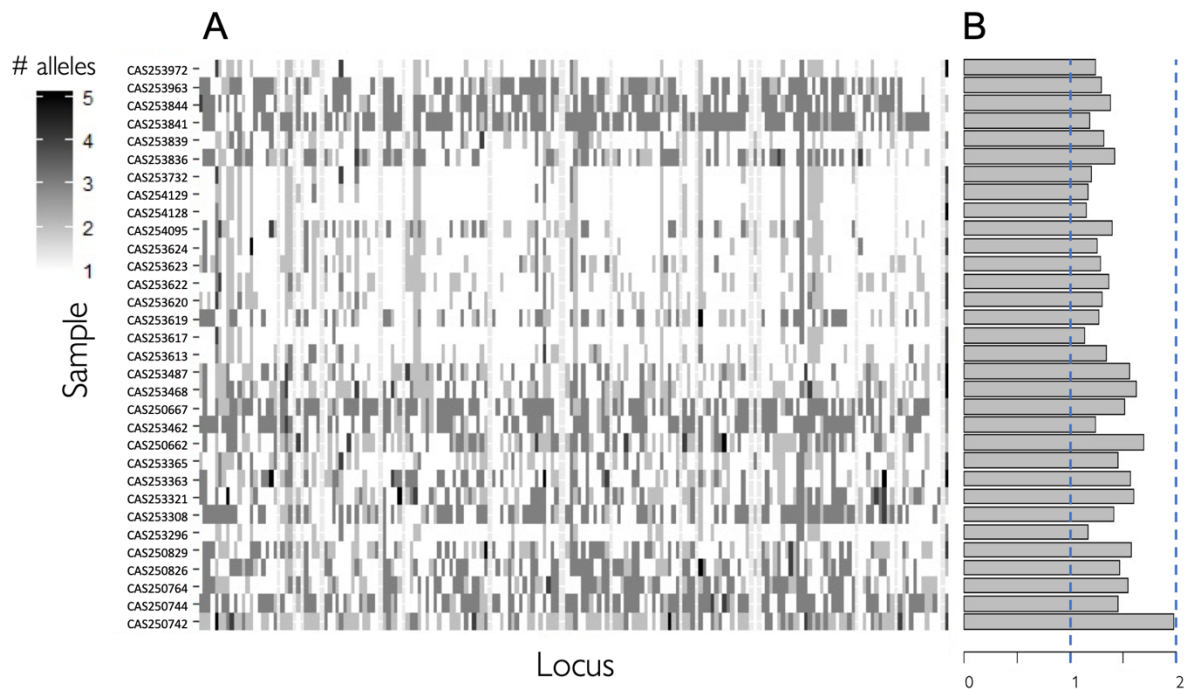


Figure S1-2. Unique alleles from newly genotyped *Bd* swab samples. (A) Heat-map showing the number of unique alleles for each swab sample (N=32) at each locus (N=192); (B) average number of alleles for each sample across all amplified loci.



Chapter Two:

Skin microbiomes of captive Sierra Nevada yellow-legged frogs (*Rana sierrae*) vary among body regions and individuals

Abstract

Studies of the microbiome provide insights relevant to the health and conservation of host organisms. In amphibians, the skin microbiome can defend against pathogens, including *Batrachochytrium dendrobatidis* (*Bd*). Intra-species variation in disease susceptibility and intra-individual variation in infection distribution across the skin, therefore, may relate to differences in skin microbiomes. However, characterization of microbiome variation within and among amphibian individuals is needed. We utilized 16S rRNA gene amplicon sequencing to compare microbiomes of ten body regions from nine captive *R. sierrae* individuals and their tank environments. While frogs harbored distinct microbial communities compared to their tank environments, tank identity was associated with more variation in frog microbiomes than individual frog identity. We detected differences between microbiomes of body regions where *Bd* infection would be expected compared to regions that infrequently experience infection. Notably, the bacterial families Burkholderiaceae (phylum Proteobacteria) and Rubritaleaceae (phylum Verrucomicrobia) were dominant on frog skin, and the relative abundances of undescribed members of these families were important to describing differences among and within individuals. Two undescribed Burkholderiaceae taxa were found to be putatively *Bd*-inhibitory, and both showed higher relative abundance on body regions where *Bd* infection is often localized. These findings highlight the importance of considering intrapopulation and intraindividual

heterogeneities, which could provide insights relevant to predicting localized interactions with pathogens.

Introduction

Communities of microbiota associated with multicellular organisms, also known as the microbiome, can play a significant role in the health and disease of their hosts (Cho and Blaser, 2012; Lee and Hase, 2014; Oever and Netea, 2014; Robinson et al., 2010; Zilber-Rosenberg and Rosenberg, 2008). Skin microbiome composition and structure can influence host health by contributing to immune defenses and maintaining skin homeostasis (Sanford and Gallo, 2013). In amphibians, the skin-associated microbiome serves as a primary defense mechanism against invading pathogens (Walke and Belden, 2016).

The fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*) poses a significant threat to amphibians globally, causing the disease chytridiomycosis in hundreds of species, which has led to dramatic declines and species extinctions (Fisher et al., 2009; Fisher and Garner, 2020; Scheele et al., 2019; Skerratt et al., 2007). *Bd* infects keratinized epidermal cells, disrupting host osmoregulation and electrolyte balance, and often leading to mortality (Berger et al., 1999, 1998; Voyles et al., 2009). Interestingly, susceptibility to *Bd* varies widely among amphibian species, populations, and individuals (Jiménez and Sommer, 2017; Rosenblum et al., 2010). While this variation is influenced by multiple factors, including host genetics and environmental conditions, the skin-associated microbiome may also play a crucial role in determining susceptibility (Becker et al., 2015a).

Studies have shown that several amphibian skin microbes can inhibit *Bd*, and that the structure of the skin microbiome can predict the severity of infection and disease outcomes (Bates

et al., 2018; Becker et al., 2015a, 2015b; Harris et al., 2009a; Jani et al., 2017; Woodhams et al., 2015, 2007b). These findings have spurred interest in probiotics for amphibians, although results have been mixed (Becker et al., 2009; Harris et al., 2009a, 2009b; Kueneman et al., 2016a; Becker et al., 2021, 2015a, 2011; Knapp et al., 2022; Woodhams et al., 2020, 2012). Probiotic effectiveness often depends on the ability of beneficial bacteria to persist on the skin, which is influenced by the existing microbial community (Becker et al., 2015a; Knapp et al., 2022; Woodhams et al., 2012). This underscores the need for a deeper understanding of skin microbiome complexity and dynamics (Becker et al., 2011; Costello et al., 2012; Garner et al., 2016).

Captivity is known to alter the amphibian skin microbiome, with several studies finding differences in microbiome structure and diversity between captive and wild individuals across many amphibian species, likely due to environmental and dietary differences (Antwis et al., 2014; Becker et al., 2014; Kueneman et al., 2022; Loudon et al., 2014; Sabino-Pinto et al., 2016). These captivity-induced shifts in the microbiome could impact the success of reintroduction programs, warranting closer attention to the microbiome in captivity prior to release (Redford et al., 2012). Additionally, variability in the microbiome within populations or within individuals could affect health outcomes post-release and contribute to differences in *Bd* susceptibility and infection intensities observed within populations (Ellison et al., 2019; Jani and Briggs, 2014; Jiménez and Sommer, 2017; Rosenblum et al., 2010). Few studies have examined individual variability or within-individual variability in amphibian microbiomes, although such variations have been documented in humans and other animals (Asangba et al., 2022; Bouslimani et al., 2015; Grice et al., 2009; Krog et al., 2022; Shibagaki et al., 2017; Sugden et al., 2021). One study that examined individual variation in amphibian microbiomes over time found that skin microbiomes can vary between wild-captured individuals within the same population (Ellison et al., 2021). Furthermore,

heterogeneity in microbiome structure among body regions has been detected in certain amphibian species (Bataille et al., 2016; Sabino-Pinto et al., 2016; Sanchez et al., 2017), suggesting that different skin regions may harbor distinct microbial communities.

Bd infections in frogs are primarily limited to the ventral skin surfaces and toes (Berger et al., 2005, 1998; North and Alford, 2008; Pessier et al., 1999). This pattern of infection may indicate a difference in the microbial communities and niche space available in certain regions of the body, warranting an examination of the microbiomes of different body regions to understand potential regional defenses against *Bd*. Evidence indicates that frog skin selects for specific microbes from the environment (Bates et al., 2018; Loudon et al., 2016; Walke et al., 2014), but whether there is selection for different microbes in body regions preferentially infected by *Bd* has not been examined.

The Sierra Nevada yellow-legged frog (*Rana sierrae*) has experienced dramatic population declines due to invasive fish and disease (Vredenburg et al., 2010, 2007). Restoration efforts for this species often involve head-starting, where frogs are reared to adulthood in captivity before being reintroduced into the wild. However, to our knowledge there has not been research on whether microbiomes of captive-reared *R. sierrae* may affect their success post-release. Additionally, no previous studies have investigated differences in microbiomes within and among captive *R. sierrae* individuals.

In this study, we utilized high-throughput sequencing of bacterial 16S rRNA gene amplicons to characterize the skin microbiome of captive adult *R. sierrae* reared at the San Francisco Zoo. We sought to address the following questions: (1) Are captive *R. sierrae* skin microbiomes distinct from their tank environment microbiome? (2) How much variation is there among microbiomes of captive *R. sierrae* individuals? (3) How much variation is there among

microbiomes of different body regions within captive *R. sierrae* individuals? and (4) Are there consistent differences in the skin microbiome that correspond to body regions preferentially infected by *Bd*? Based on previous evidence, we hypothesized that we would detect differences in microbiome structure between frogs and their tank environments (Bataille et al., 2016; Walke et al., 2014), among individuals (Ellison et al., 2021), and among body regions (Bataille et al., 2016; Sabino-Pinto et al., 2016; Sanchez et al., 2017). Further, we hypothesized that certain microbes would be differentially abundant between body regions that tend to harbor *Bd* infections (ventral surfaces and feet) and those where infection is often absent (dorsal surfaces like the back). Our fine-scale analysis of the skin microbiome identifies characteristics that vary within and among individuals and their tank environments, providing insights relevant to amphibian health and conservation.

Methods

Ethics Statement

Sampling of *Rana sierrae* individuals housed at the San Francisco Zoo was conducted with approval from the UC Davis IACUC (Protocol #18732) and the San Francisco Zoo Research Review Committee.

Frog Population and Handling

Frogs used in this study came from a source population of *R. sierrae* in the Sierra Nevada Mountains. More specifically, this source site is located in the Desolation Wilderness (El Dorado County, California), in the Lake Tahoe basin, at about 2500 m elevation. Frogs were collected as egg masses in the summer of 2014 by Roland Knapp and the Mountain Lakes Research Group

(Sierra Nevada Aquatic Research Laboratory, Mammoth Lakes, CA) and transported to the San Francisco Zoo, where they hatched approximately one week later and were reared to adulthood over the following year by Jessie Bushell, Director of Conservation at the SF Zoo. Once frogs reached adulthood (snout–vent length (SVL) ≥ 40 mm) individuals were tagged with 8 mm unique passive integrated transponder (PIT) tags, which allow for differentiation among individuals. At the Zoo, adult frogs were housed in tanks (groups of 8-13 individuals) filled with tap water purified using biological filters to remove toxic nitrogenous compounds and supplemented with Kent Marine R/O Right, a formulation of dissolved solids and electrolytes used to restore natural water chemistry to water that has been distilled, deionized, or purified by reverse osmosis.

Sample Collection

We collected samples for this study at the San Francisco Zoo from frogs and their tank environments. We wore nitrile gloves during sample collection from frogs and surfaces in tanks using sterile synthetic fine tip dry swabs (Medical Wire & Equipment, MW113). For frog sampling, we rinsed each individual with 60 mL of sterile water prior to swabbing (Culp et al., 2007; Lauer et al., 2007). From each frog, we collected a separate swab from each body region for microbiome analysis (“microbiome swabs”) and an additional swab for assessing *Bd* presence and infection intensity (“*Bd* swabs”). To do this, we first collected one swab per body region by taking 10 strokes of the region as a way to standardize sample material from body regions of various sizes. Body regions included the back, outer hindlimbs, snout, vocal sack, ventral abdomen, inner forelimbs, forefeet, inner hindlimbs, hindfeet, and cloaca (Figure 2-1). Next we collected one *Bd* swab per individual by taking 30 strokes of ventral surfaces (10 strokes of ventral abdomen, 10 strokes of inner hindlimbs, and 5 strokes on each hindfoot; “Chytrid Swabbing Protocol,” 2009).

For each frog we also recorded the sex, tank identity, and individual identity (recording both the PIT tag number and the “Tahoe ID” assigned to each frog by the Zoo). We collected swabs of surfaces in tanks including rock perches (above the water surface; two samples per tank), underwater rocks (rock perch submerged in water; one sample per tank), and tank walls (above the water surface; three samples per tank) by taking 40 strokes across each surface. Tank water was sampled by filling a 60 mL syringe with tank water, passing the water through a 0.22 μ m Sterivex filter (Millipore), and repeating this process four times (total water filtered = 240 mL per sample; two filter samples collected per tank) (Ellison et al., 2019). We also recorded air and water temperatures. All samples were kept on dry ice during collection and transferred to a -80 °C freezer for storage on the same day.

In this study, we analyze samples collected from nine frogs (n=90 microbiome swabs and n=9 *Bd* swabs) and their tank environments (n=18 microbiome swabs; n=6 water filters) on 7/28/2015. These frogs were not distributed among tanks in a balanced manner: six frogs were co-housed in one tank, two frogs were co-housed in a second tank, and one frog was housed in a third tank. Therefore, several comparisons of microbiomes among individuals were confounded with between-tank comparisons. In our microbiome analyses described below, we estimated the relative importance of individual variation and tank variation to several microbial community metrics. To do this, we calculated the percent of pairwise comparisons of co-housed individuals (*i.e.*, within tanks) that were significantly different, and compared to the percent of pairwise comparisons of individuals from distinct tanks (*i.e.*, between tanks) that were significantly different (*see Methods: Microbial sequence analysis and visualization*).

Molecular Methods

Bd swab samples

DNA was extracted from *Bd* swab samples using the PrepMan Ultra Sample Preparation Reagent following the manufacturer's protocol (40 μ L used per sample). DNA extracts were stored at -20 °C or -80 °C. To confirm that frogs reared at the Zoo were not infected with *Bd*, we used a standard quantitative PCR (qPCR) assay with *Bd*-specific primers for ITS1 (Boyle et al., 2004; Hyatt et al., 2007). qPCR assays of DNA extracts were completed in Roland Knapp's laboratory at the Sierra Nevada Aquatic Research Laboratory (Mammoth Lakes, California, USA). Plasmid *Bd* standards were used in assays, which are based on single ITS1 PCR amplicons (Joseph and Knapp, 2018; Longo et al., 2013).

Microbiome samples

We extracted DNA from microbiome swabs and water filter samples using MoBio PowerSoil DNA Isolation Kits, using a modified protocol for low-biomass samples discussed with the manufacturer. Swabs or filters were swirled in the PowerBead tubes and left inside these tubes. Modifications to the manufacturer's standard protocol included the following: (1) after adding Solution C1 and vortexing to mix, tubes were incubated at 65 °C for 10 minutes; (2) tubes were then secured in a bead beater set to "homogenize," and bead-beated for a total of 3 minutes (90 seconds on, 60 seconds rest, followed by 90 seconds on); (3) all centrifugation steps throughout were done for 1 minute at 13,000 x g unless otherwise noted below; (4) we combined steps for Solutions C2 and C3 by adding 100 μ L of each at once prior to 5 minute incubation on ice, (5) after the C2/C3 step, we transferred 700 μ L of lysate to a clean collection tube and added 700 μ L of Solution C4 and 600 μ L of 100% ethanol before loading on the spin filters; (6) before washing

the filter with solution C5, we inserted a step to wash with 650 μ L of 100% ethanol; (7) After washing with solution C5, we dried the spin column by centrifuging for 2 minutes at 13,000 x g; (8) We added 60 μ L of Solution C6 (heated to 60 °C) to the filter membrane, and we allowed this solution to sit on the filter for 5 minutes before centrifuging into a storage tube. Following DNA extraction, we quantified DNA concentration using a Qubit (Invitrogen, Carlsbad, CA, United States) and the dsDNA High Sensitivity Kit, and stored extracts at -80 °C.

Sequence Generation

Sequencing libraries were prepared following the protocol “16S Metagenomic Sequencing Library Preparation” (Part # 15044223 Rev. B, Illumina, Inc., San Diego, CA, USA) with some modifications. Briefly, we PCR amplified the hypervariable V3-V4 region of the bacterial 16S rRNA gene using 341F and 805R primers (Klindworth et al., 2013) with overhang adaptors (forward primer with overhang = 5'

TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG; reverse primer with overhang = 5'

GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC)

from each sample in triplicate, using 4 μ L DNA extract per reaction. We pooled PCR products from each sample (75 μ L pool) and purified them using magnetic beads (Axygen AxyPrep Mag PCR Clean-Up Kit) using 60 μ L beads per pool (for a 0.8X ratio of beads to PCR product), following the steps in the above Illumina protocol for “PCR Clean-Up,” and eluting in 25 μ L of 10 mM Tris pH 8.5. Next, we attached dual indices and Illumina sequencing adaptors in a second round of PCR described in the Illumina protocol (“Index PCR”). We then purified and normalized 25 μ L of each index PCR product using SequalPrep Normalization Plate Kits (Invitrogen),

following the manufacturer's protocol with an extension of the binding step incubation to 2-6 hours. We then pooled 10 uL of purified, normalized, and indexed PCR product per sample, and used the Zymo Clean and Concentrator Kit to increase the DNA concentration of the pool following the manufacturer's protocol (using a ratio of 5:1 of DNA Binding Buffer:PCR product, and final elution using 200uL DNA Elution Buffer). We quantified DNA in the final pool using a Qubit (Invitrogen), and sent the pooled libraries to the UC Davis Genome Center DNA Technology Core for sequencing on an Illumina MiSeq (Illumina, Inc., San Diego, CA, USA) with v3 chemistry in 2×300 bp run mode. We sequenced 20 negative control samples in addition to our true samples. These included six controls for swab sample collection (three dry swabs and three swabs rinsed with sterile water, processed in the same way as true samples), eight blank DNA extraction kit controls (two to three preparations from each of three PowerSoil kits for which no sample was added to the PowerBead tube, but otherwise processed in the same way as true samples), and six PCR controls (for which no sample DNA was added to the first PCR step, and subsequent processing was the same as for true samples).

Sequence Processing

We used a modified version of a custom script designed by G. Jospin (https://github.com/gjospin/scripts/blob/master/Demul_trim_prep.pl) to demultiplex the sequence data. Primers were removed using cutadapt v. 3.5 (Martin, 2011) with Python v. 3.9.10 (Van Rossum and Drake, 2009), discarding reads for which primers were not present. We processed the resulting sequences using the DADA2 v.1.24.0 (Callahan et al., 2016) workflow in R v. 4.2.1 with RStudio v. 2022.07.0-548 (Posit Team, 2022; R Core Team, 2022). We trimmed forward and reverse reads at 250 base pairs, truncated at the first quality score of 2, and removed them if the

expected errors were greater than 4 (this removed 32.8% of sequences). We then merged reads and inferred amplicon sequence variants (ASVs; 7.9% of sequences did not pass through these steps). Next, we identified 1.8% of merged reads to be chimeric and removed them. After chimera removal, samples had a mean read depth of 23,832 with a range of 294 to 98,808 reads. We assigned taxonomy to genus level using the Ribosomal Database Project (RDP) naive Bayesian classifier algorithm and the SILVA high quality ribosomal RNA database v. 132, and species level assignments were made based on exact matching of ASVs to reference strains in the SILVA database (Quast et al., 2012; Wang et al., 2007; Yilmaz et al., 2014). We then assigned unique names to ASVs, beginning with “SV” (sequence variant) followed by a number (*e.g.*, SV1, SV2, etc.). We then removed ASVs based on taxonomic classification that were (1) non-bacterial at the domain level (including Eukaryota, Archaea, and those unclassified to domain), (2) chloroplasts, and (3) mitochondria, which resulted in 2373 unique ASVs in the dataset.

We used Decontam v. 1.16.0 to identify putative contaminants, implementing the prevalence method with a probability threshold of 0.5 (which identifies sequences that have a higher prevalence in negative controls than in true samples) and setting the batch argument so that contaminants were identified independently within groups of samples associated with specific negative controls (Davis et al., 2018). We identified contaminants separately for each of the following four control sample groups, using batch as indicated: (1) dry swabs (n=3) using batch by the sample material so as to identify contaminants in swab samples and not filters, (2) swabs rinsed with sterile water (n=3) using batch by whether sampling involved rinsing with sterile water so as to identify contaminants from frogs that were rinsed prior to swabbing, (3) extraction kit blanks (n=8) using batch by the PowerSoil kit used so as to identify contaminants from each kit separately, and (4) PCR negative controls (n=6) without specifying batch so as to identify

contaminants associated with PCR across all samples. We then compiled a list of putative contaminants identified using each control group (102 unique ASVs) and removed them, leaving 2271 unique ASVs in the dataset.

There has been an ongoing debate in the literature regarding the validity of using rarefaction as a sample normalization technique for microbiome data (Cameron et al., 2021; Gloor et al., 2017; McKnight et al., 2019; McMurdie and Holmes, 2014; Weiss et al., 2017). We chose to implement this method for many analyses because we were interested in community level comparisons that can become distorted using other normalization methods (McKnight et al., 2019). Additionally, rarefaction was shown to be more effective than other methods at controlling effects of sample library size when sample depths are very uneven (Schloss, 2023; Weiss et al., 2017), which is the case for our dataset. Therefore, for all subsequent sequence analyses with the exception of the DESeq2 differential abundance testing (see below), samples were rarefied at an even sampling depth of 2727, which was the minimum depth of a true sample. All negative control samples had fewer than 2727 reads, and were therefore discarded during rarefaction.

After rarefying the dataset, we aligned remaining sequences using DECIPHER v. 2.22.0 (Wright, 2016) and built a maximum likelihood tree with a GTR + $\Gamma(4)$ + I model using phangorn v. 2.8.1 (Schliep et al., 2017; Schliep, 2011) on the UC Davis Bioinformatics Core High Performance Computing Cluster in R v. 4.1.0 (R Core Team, 2022). We midpoint rooted the tree using phangorn v. 2.9.0 (Schliep et al., 2017; Schliep, 2011).

The resulting dataset analyzed for this study included 1861 unique ASVs across 114 true samples (10 samples from each of nine frog individuals and 24 tank environment samples). We note that we also sequenced samples from a tenth frog individual, however we do not include that individual in our analyses for this study because it was treated with the anti-fungal drug

itraconazole approximately 11 weeks prior to sample collection and none of the other nine individuals sampled underwent that treatment (Tahoe097; n=10 samples removed in order to minimize variation due to factors outside the scope of our study).

Microbial Sequence Analysis and Visualization

Alpha diversity analysis

To assess within-sample microbial community diversity (*i.e.* alpha diversity), we used the `estimate_richness` function in `phyloseq` v. 1.40.0 (McMurdie and Holmes, 2013) to calculate alpha diversity metrics including observed richness and Shannon diversity. Observed richness is measured as the number of ASVs present, while Shannon diversity takes into account both the number of ASVs and their frequencies (*i.e.*, evenness). We estimated these community diversity metrics for all samples in the dataset to compare diversity between frog and environment samples, as well as for a subset of samples in the dataset (frog samples only) to compare diversity between and within frog individuals. We conducted Shapiro-Wilk normality tests for all groups of alpha diversity estimates that we sought to compare, and found that estimates for at least one group in each comparison were not normally distributed, warranting use of nonparametric statistical tests. We used Kruskal-Wallis rank sum tests to test for significant differences in alpha diversity values between metadata groupings of the samples (including frog vs. environmental sample type, frog individual, and frog body region) utilizing the `kruskal.test` function in base R v. 4.2.1 (R Core Team, 2022). For significant Kruskal-Wallis results, we implemented *post hoc* Dunn tests using the `dunnTest` function in `FSA` v. 0.9.3 (Ogle et al., 2022) in order to identify which groups were significantly different, correcting resultant p-values using the Benjamini-Hochberg procedure to control the false discovery rate (FDR) with multiple comparisons. Because individual identity was

confounded with tank identity for many *post hoc* comparisons of frog individuals, we determined the total number of pairwise comparisons of individuals co-housed in the same tanks, and calculated the percent of those comparisons that were significantly different. We also determined the total number of pairwise comparisons of individuals that were housed in distinct tanks, and calculated the percent of those comparisons that were significantly different.

Beta diversity analysis

To assess community structure we compared between-sample diversity (*i.e.*, beta diversity) using three ecological distance metrics: unweighted Unifrac, weighted Unifrac, and Bray-Curtis dissimilarities. Unweighted UniFrac distance is calculated from the community phylogenetic tree as the unique fraction of branch length within a sample community that is not shared with other communities sampled (Lozupone and Knight, 2005). This metric, therefore, can be thought of as measuring distances based on community membership (presence/absence). When UniFrac is weighted, it takes into account the relative abundances (*i.e.*, evenness) of branch lengths in addition to membership, giving more weight to dominant organisms than rare ones (Lozupone et al., 2007). Bray-Curtis also takes into account species richness and relative abundances, but this metric is not informed by phylogeny. We used the ordinate function in phyloseq to calculate these distances for different subsets of the data, and visualized ordinations using principal coordinate analysis (PCoA).

To test for significant differences in community structure between metadata groupings of the samples, we ran permutational multivariate analysis of variance (PERMANOVA) tests using the *adonis* function in *vegan* v. 2.6.2 with 9999 permutations (Anderson, 2001; Oksanen et al., 2022). For each ecological distance metric, we ran a PERMANOVA on the whole dataset (frog

samples and environmental samples) to test for significant differences in microbiome structure between frogs and environmental sample types, and then ran a PERMANOVA on the frog samples alone to test for significant differences between frog individuals (“Tahoe ID”) and between frog body regions (“Sample Type”), as well as testing for significant effects of other metadata factors like frog sex (*i.e.*, males versus females). Next, for factors that rejected the null hypothesis in these PERMANOVA tests ($p < 0.05$), we performed *post hoc* pairwise PERMANOVA tests to identify which levels within factors differed significantly, using the `adonis.pair` function in `EcolUtils` v. 0.1 with 9999 permutations (Salazar, 2022). We corrected p-values for multiple comparisons using the Benjamini-Hochberg procedure. We also calculated mean dispersion for factors included in PERMANOVA tests and tested for significant differences in dispersion using the `betadisper` and `permutest.betadisper` functions in `vegan`. For significant comparisons ($p < 0.05$), we then implemented *post hoc* Tukey Honest Significant Differences tests to identify which levels within factors differed significantly in their group dispersion (using the `TukeyHSD` function in base R, which corrects p-values for the family wise error rate in multiple comparisons).

For *post hoc* pairwise comparisons of frog individuals, we determined the total number of pairwise comparisons of individuals co-housed in the same tanks, and calculated the percent of those comparisons that were significantly different. We also calculated the percent of pairwise comparisons of individuals from distinct tanks that were significantly different. We compared these percentages to assess the relative importance of tank effects and individual effects.

Analysis of prevalence and relative abundance of taxa

We first calculated the proportion of taxa shared between frog samples and environmental samples. We merged samples in the rarified dataset by “SampleCategory,” so that read counts were

collapsed into either frog or environment, and removed any ASVs that had zero counts across frog and environmental samples. Next we calculated the proportion of ASVs that were present in both frog and environment samples (“shared”). We also calculated the proportion of bacterial families that were shared between frog and environmental samples. To do this we collapsed ASVs from the same families using the `tax_glom` function in `phyloseq` (specifying `NArm=FALSE` to include unclassified taxa) prior to merging samples by “SampleCategory.”

We visualized and compared the relative abundance of taxa for the whole dataset (frog samples and environmental samples) and for a subset of the dataset (frog samples only). To do this, we first transformed the rarefied sample counts to relative abundances. To examine relative abundance of bacterial families across the whole dataset, we merged ASVs from the same families using the `tax_glom` function in `phyloseq` (specifying `NArm=FALSE` to include unclassified taxa). For visualization purposes, we filtered families to retain only those with mean relative abundance greater than 0.6%. We grouped the filtered families by a metadata factor to compare frog samples to the four environmental sample types (“EnvironType_FrogCategory”) and by taxonomic ranks desired for visualization, and then plotted mean relative abundance with standard error bars faceted by levels of the metadata grouping factor. For visualizations of the relative abundance of taxa within frog samples, we focused on two dominant ASVs (“SV1” and “SV2”). We used the `prune_taxa` function in `phyloseq` to retain only these ASVs, grouped data by metadata factors (frog individual or frog body region) and taxonomic rank for visualization, and plotted the mean relative abundances of these two ASVs with standard error bars by each metadata grouping factor (frog individual and frog body region).

To determine whether the relative abundance of bacterial families or ASVs differed significantly between metadata groupings, we implemented nonparametric Kruskal-Wallis rank

sum tests followed by *post hoc* Dunn tests when results of Kruskal-Wallis tests were significant ($p < 0.05$). We corrected p-values from Dunn tests using the Benjamini-Hochberg procedure. For *post hoc* comparisons of frog individuals, we compared the percent of pairwise comparisons within tanks that were significant to the percent of pairwise comparisons between tanks that were significant.

For frog samples, we determined whether the relative abundances of our two ASVs of interest (“SV1” and “SV2”) were significantly different from each other within sample metadata groupings (*i.e.*, within each individual or within each body region). To do this, we used nonparametric Wilcoxon signed rank exact tests with the Benjamini-Hochberg procedure for p-value correction.

DESeq2 differential relative abundance testing

We used DESeq2 v. 1.36.0 in R on filtered but un-rarified merged read counts to determine which ASVs showed significant $\log_2$ fold differences between frog body regions (Love et al., 2014). ASVs with significant $\log_2$ fold differences in DESeq2 normalized read counts between body regions were considered differentially abundant. Based on previous findings that *Bd* infection is more prominent on ventral surfaces and toes than on the dorsal back surface of many anurans (Berger et al., 2005, 1998; North and Alford, 2008; Pessier et al., 1999), we chose to compare frog back samples to (1) frog abdomen samples, (2) frog inner hindlimb samples, (3) frog hindfeet samples, and (4) frog forefeet samples, to examine whether bacterial community members were differentially associated with these body regions. First, we subsetting the data to only include frog samples, and used the `phyloseq_to_deseq2` function to format the raw read count data for DESeq2. Our ASV counts table was sparse with only two ASVs present across all samples, therefore we

calculated geometric means across samples for each ASV by ignoring samples with zero counts so that geometric means and resulting estimated size factors would not be based on only those two ASVs. Then, we estimated size factors for each sample based on the geometric means (applying the `estimateSizeFactors` function to the calculated geometric means). We filtered out low abundance ASVs with 10 or fewer reads total across frog samples (this filtered out 1984 unique ASVs, leaving 287 unique ASVs across the frog samples). We then ran the `DESeq` function on the dataset for each contrast of interest (back ↔ abdomen, back ↔ inner hindlimbs, back ↔ hindfeet, and back ↔ forefeet), identifying ASVs that showed significant $\log_2$ fold differences (*i.e.*, that were differentially abundant; Benjamini-Hochberg corrected p-values <0.05). For ASVs that were identified as differentially abundant, we calculated the mean relative abundance across body regions from the rarefied dataset and implemented Kruskal-Wallis rank sum tests to determine if the mean relative abundance differed significantly between body regions.

Bd-inhibitory predictions for select taxa

We predicted putative *Bd*-inhibitory function of microbial community members of interest. Predictions were based on a database of full length 16S rRNA gene sequences from bacteria that were isolated and assayed for their effects on growth of *Batrachochytrium* pathogens (Woodhams et al., 2015). This database, which is regularly updated, has been used in several previous studies to predict anti-*Bd* function from amplicon data (Bletz et al., 2017; Chen et al., 2022; Jiménez et al., 2022; Kueneman et al., 2022, 2016a; Muletz Wolz et al., 2018). We used the strict inhibitory subset of the database (AmphiBac_InhibitoryStrict_2023.2; accessed from <https://github.com/AmphiBac/AmphiBac-Database/>) which included sequences from 2,056 inhibitory taxa. We used NCBI nucleotide BLAST to make a multiple sequence alignment with

our ASVs as query sequences and the inhibitory database as subject sequences (Camacho et al., 2009). We checked that query coverage was 100%, and then documented cases where the percent identity was $\geq 99\%$ (*i.e.*, cases where our query ASVs shared $\geq 99\%$ sequence similarity with an inhibitory database sequence).

Results

***Bd* Assay**

No amplification of *Bd* ITS1 was detected during qPCR of frog *Bd* swab samples, indicating that *Bd* was not present on frogs sampled in this study.

Alpha Diversity Analysis

Within-sample diversity was significantly different between frog samples and tank environment sample types (including rock perch, tank wall, tank water, and underwater rock samples) based on both the observed richness (*i.e.*, the number of ASVs in the rarefied dataset; Kruskal-Wallis, chi-squared = 55.62, df = 4, $p < 0.001$; Figure 2-2A) and Shannon diversity, which we refer to as abundance-weighted diversity (Kruskal-Wallis, chi-squared=56.77, df = 4, $p < 0.001$; Figure 2-2B). These tests revealed that frog samples had significantly lower within-sample diversity than tank water, tank wall, rock perch, and underwater rock samples for both observed richness and abundance-weighted diversity (Dunn tests, $p < 0.05$; Table S2-1). None of the environment sample types differed significantly from each other for either diversity metric (Dunn tests, $p > 0.05$; Table S2-1).

For frog samples, we found that both rarefied observed richness and abundance-weighted diversity differed significantly by frog individual (Observed: Kruskal-Wallis, chi-squared=40.84,

df = 8, $p < 0.001$; Shannon: Kruskal-Wallis, $\chi^2 = 29.95$, df = 8, $p < 0.001$; Figure 2-2C, D). *Post hoc* Dunn tests were used to determine which individuals showed significant differences in alpha diversity. For observed richness we identified 12 significant differences out of 36 pairwise comparisons of our 9 individuals (Dunn test, $p < 0.05$; 33% of comparisons were significant; Table S2-2). While 25% of within-tank pairwise comparisons of individuals were significantly different, 40% of between-tank pairwise comparisons of individuals were significantly different. The *post hoc* Dunn test for abundance-weighted diversity (*i.e.*, Shannon diversity) identified 11 significant differences out of 36 pairwise comparisons of individuals (Dunn test, $p < 0.05$; 30.5% of comparisons were significant; Table S2-2). 31.3% of within-tank pairwise comparisons were significantly different and 30% of between-tank pairwise comparisons were significantly different. These results show that there is variability in the number of ASVs and their evenness among frog individuals and among tanks, but that a greater percentage of differences in observed richness were between tanks than among individuals within tanks.

We found that abundance-weighted diversity differed significantly by frog body region, (Kruskal-Wallis, $\chi^2 = 21.54$, df = 9, $p = 0.010$; Figure 2-2F), but that observed richness did not (Kruskal-Wallis, $\chi^2 = 8.67$, df = 9, $p = 0.47$; Figure 2-2E). This suggests that while all frog body regions harbor a similar number of microbial community members, some regions harbored communities with higher evenness. We applied *post hoc* Dunn tests to determine which body regions were significantly different in terms of abundance-weighted alpha diversity. We identified three significant differences out of 45 possible pairwise comparisons of body regions (Dunn test, $p < 0.05$; Table S2-3), showing that the forefeet had higher abundance-weighted diversity than the abdomen, back, and hind-feet.

Beta Diversity Analysis

Microbial community structure was significantly different between frog samples and tank environment sample types (including tank water, tank wall, rock perch, and underwater rock samples) based on three ecological distance metrics: weighted and unweighted Unifrac and Bray-Curtis dissimilarity (PERMANOVA, $p < 0.001$; Figure 2-3, A–C, Table S2-4). This shows that frog-associated microbial communities were distinct from environment-associated communities when considering only community membership (*i.e.*, differences in presence/absence of organisms measured by unweighted Unifrac) or when also considering relative abundances of community members (measured by weighted Unifrac and Bray-Curtis). Further, differences in microbial community structure were observed between frog and environmental samples whether phylogeny was considered (UniFrac distances) or not (Bray-Curtis dissimilarity). We found that differences between microbial assemblages on frogs and those from environmental samples explained the highest amount of variation in weighted Unifrac (69%), followed by Bray-Curtis (45%), and finally unweighted Unifrac (29%) (Table S2-4). These results suggest that differences in the relative abundances of community members were more important to describing variation between frogs and their environment than presence/absence of community members alone, and that differences were explained to a greater extent by phylogeny than by taxonomy alone. *Post hoc* pairwise PERMANOVA tests revealed that all groups (frog, tank water, tank wall, rock perch, and underwater rock) were significantly different from each other based on all three metrics for community structure (PERMANOVA, $p < 0.05$; Table S2-5).

PERMANOVA tests are sensitive to differences in group dispersion (*i.e.*, within-group variance) and thus significant effects detected by these tests could indicate differences in the average location of groups in ordination space (*i.e.*, group centroids), differences in group

dispersion, or some combination of the two (Anderson, 2001). If PERMANOVA tests indicate a significant effect of a factor and group dispersions for that factor do not differ significantly, then we know that centroid location differs for these groups. However, if the group dispersions are significantly different, then our tests are unable to distinguish whether differences are due to dispersion alone or some combination of dispersion and centroid location. We found group dispersion between frogs and environment sample types did not differ significantly for unweighted Unifrac (betadisper permutest, $p=0.16$; Table S2-6), indicating that differences based on phylogenetically informed community membership were due to differences in mean centroids. There were significant differences in group dispersion, however, for weighted Unifrac and Bray-Curtis (betadisper permutest, $p<0.001$; Table S2-6). For weighted Unifrac, pairwise comparisons of group dispersion (for frog, rock perch, tank wall, tank water, and underwater rock sample groupings) revealed that five out of ten comparisons were significantly different (TukeyHSD, $p<0.05$; Table S2-7), which included two out of the four comparisons with frog samples (frog ↔ rock perch and frog ↔ tank wall group dispersions differed significantly, TukeyHSD, $p<0.05$; frog ↔ tank water and frog ↔ underwater rock group dispersions did not differ, TukeyHSD, $p=0.25$ and $p=0.99$, respectively). For Bray-Curtis dissimilarity, three out of ten pairwise comparisons of group dispersion were significantly different (TukeyHSD, $p<0.05$; Table S2-7), and these represented three of the four comparisons with frog samples (frog ↔ rock perch, frog ↔ tank wall, and frog- tank water group dispersions differed significantly, TukeyHSD, $p<0.05$; frog ↔ underwater rock group dispersions did not differ, TukeyHSD, $p=0.17$). Therefore, in many cases, differences in microbiome structure between frogs and their environment based on relative abundances of community members may have been driven by differences in group variance alone or in combination with differences in group centroids. Ordination visualizations (Figure 2-3, A–

C) showed that frog samples clustered separately from environmental sample types for all three measures of community structure, which is evidence that in cases where differences in dispersion were detected between frog and environment samples, both the group dispersions and centroids may have been distinct. We also note that these ordinations comparing frog and environmental samples (Figure 2-3, A–C) show a characteristic “horseshoe effect,” which could be a consequence of saturation of the distance metrics (Morton et al., 2017). Since distance metrics cannot discriminate between samples that do not share ASVs, this saturation can occur when the dataset has sparse counts of ASVs across samples creating a “band table” (Morton et al., 2017). This effect does not alter our interpretation of the ordinations, and it actually provides further evidence of the high dissimilarity between environment and frog samples.

Our PERMANOVA model to explain variation in community structure within frog samples revealed that the factor with largest effect was individual identity (“TahoeID”; PERMANOVA, $p < 0.001$; Table S2-8). This factor explained the highest amount of variation in weighted Unifrac distances (41%), followed by Bray-Curtis (37%) and lastly by unweighted Unifrac (22%). Even after accounting for the effects of individual identity, we also found a significant effect of the body region sampled (“SampleType”; PERMANOVA, $p < 0.05$; Table S2-8). Body region explained the highest amount of variation in Bray-Curtis (32%), followed by weighted Unifrac (28%), and explained the least amount of variation in unweighted Unifrac distances (11%). Shuffling the order of factors (TahoeID and SampleType) in the PERMANOVA model did not alter these results, and the interaction term for these factors was not significant and was dropped from the model. We also tested whether frog sex (*i.e.*, males versus females) explained variation in the microbiome. However, when this factor was included after individual identity in sequential PERMANOVA models, it was not significant; thus sex was also dropped

from the model. Overall, the results of the omnibus model suggest that frog individual (which includes tank variation) and body region explain more variation in community structure when relative abundances of microbes are incorporated than when community membership is considered alone, which may indicate that shifts in abundant community members are more important to explaining differences than shifts in rare community members. Further, while differences between frog individuals are better explained by phylogenetic distance based metrics, differences between body regions are better explained by metrics uninformed by phylogeny.

To determine how many frog individuals were driving variation in community structure explained by this factor (*i.e.*, “TahoelD”), we conducted *post hoc* pairwise PERMANOVA tests. We also compared the relative importance of individual identity to tank identity in pairwise comparisons, as individual identity was confounded with tank identity for many comparisons. Although frog individual identity explained the smallest amount of variation in unweighted Unifrac distances overall compared to other metrics, that variation was driven by the highest number of significantly different individuals. Out of 36 possible combinations of frog individuals, 26 pairwise comparisons were significantly different for unweighted Unifrac (pairwise PERMANOVAs, $p < 0.05$; 72.2% of comparisons were significant; Table S2-9; Figure 2-3D). 68.8% of pairwise comparisons of co-housed individuals (*i.e.*, within-tank) were significantly different, compared with 75% of pairwise comparisons of individuals between distinct tanks. For weighted Unifrac, 19 of the 36 pairwise comparisons of individuals were significantly different (pairwise PERMANOVAs, $p < 0.05$; 52.8% of comparisons were significant; Table S2-9; Figure 2-3E). This included 25% of pairwise comparisons of co-housed individuals and 75% of pairwise comparisons of individuals between tanks. Finally, 18 of the 36 pairwise comparisons of individuals were significantly different in terms of Bray-Curtis dissimilarity (PERMANOVA,

$P < 0.05$; 50% of comparisons were significant; Table S2-9; Figure 2-3, D–F). This included 31.3% of pairwise comparisons of co-housed individuals within tanks and 65% of pairwise comparisons of individuals between tanks. These results suggest that differences in all three metrics of community structure were primarily associated with tank identity rather than individual identity, however the association between community structure and tank was stronger for abundance weighted metrics than for the unweighted community structure metric (*i.e.*, the percentage of differences in within-tank and between-tank comparisons for unweighted Unifrac were most similar).

Next, we used pairwise PERMANOVA tests to determine which frog body regions drove the variation in community structure explained by this factor (*i.e.*, “SampleType”). For unweighted Unifrac, none of the 45 pairwise comparisons of body regions were significantly different after correcting p-values for multiple comparisons (PERMANOVA, $p > 0.05$; Table S2-10; Figure 2-3G). For weighted Unifrac, nine pairwise comparisons of body regions were significantly different (PERMANOVA, $p < 0.05$; 20% of comparisons were significant; Table S2-10; Figure 2-3H), and these included seven significant differences between the back and other body regions, and two significant differences between the snout and other body regions (hindfeet and cloaca were significantly different from both the back and the snout; the abdomen, forefeet, inner forelimbs, inner hindlimbs, and outer hindlimbs were all significantly different from the back). For Bray Curtis, 13 pairwise comparisons of body regions were significantly different (PERMANOVA, $p < 0.05$; 28.9% of comparisons were significant; Table S2-10; Figure 2-3I), including the same seven significant differences with the back and two significant differences with the snout that were identified for weighted Unifrac, as well as four additional significant differences with the snout only identified for Bray-Curtis (the snout also differed from the abdomen, forefeet, inner hindlimbs

and outer hindlimbs for Bray-Curtis). The vocal sack was the only region that never differed significantly from other body regions in terms of community structure.

We also looked for differences in group dispersion for frog samples by individual and by body region for the three distance metrics. While unweighted Unifrac group dispersion differed significantly by individual in the omnibus test (betadisper permutest, $p=0.04$; Table S2-11), there were no significant differences identified in *post hoc* pairwise comparisons of group dispersions after correcting p-values for multiple comparisons (TukeyHSD, $p>0.05$; Table S2-12). Additionally, we found no significant differences in group dispersion by individual for weighted Unifrac and Bray-Curtis (betadisper permutest, $p>0.05$; Table S2-11). Group dispersion by body region did not differ significantly for any of the distance metrics (betadisper permutest, $p>0.05$; Table S2-13). These results indicate that significant results from pairwise PERMANOVA tests comparing frog individuals and body regions represent significant differences in group centroids for community structure and not differences in dispersion between individuals or body regions.

Analysis of Prevalence and Relative Abundance of Taxa

To investigate the similarity in presence of taxa between frog samples and environmental samples, we calculated the proportion of taxa shared between frog and environment. We found that 15.4% of ASVs were shared between frog and environmental samples (212 out of 1378 ASVs were shared). We note that the proportion of shared ASVs will most likely be reduced compared to the proportion of shared OTUs, the unit of comparison in many previous studies (Kueneman et al 2013; Bates et al 2018; Walke et al 2014), because OTU calling involves collapsing sequence variants by a threshold of similarity (usually 97%), while ASVs are not clustered. We also looked

at the proportion of families shared between frog and environmental samples, which was 38.4% (91 out of 237 bacterial families were shared).

To better understand the composition of microbiomes defining different types of samples, we visualized taxonomic families that had mean relative abundance greater than 0.06% across all samples (an arbitrary threshold selected for optimized visualization). This further revealed that the distribution of taxa was distinct between frog samples and the four types of environmental samples (rock perch, tank wall, tank water, and underwater rock; Figure 2-4). Frog-associated communities were dominated by the families Burkholderiaceae (phylum Proteobacteria; mean relative abundance of $48.0 \pm 2.6\%$ across frogs), Rubritaleaceae (phylum Verrucomicrobia; mean relative abundance of $39.5 \pm 2.5\%$ across frogs), and to a lesser extent the families Pseudomonadaceae and Alcanivoracaceae (both in phylum Proteobacteria; mean relative abundance of $7.3 \pm 1.2\%$ and $2.7 \pm 0.2\%$, respectively). The environment-associated communities were, for the most part, made up of lower relative abundances (mean relative abundance $<17\%$) of an increased number of families representing several phyla in addition to the Proteobacteria and Verrucomicrobia, including the Actinobacteria, Bacteroidetes, Deinococcus-Thermus, and Firmicutes (Figure 2-4).

Next, we examined whether there were significant differences in the relative abundance of taxa among and within samples. Relative abundance of the family Burkholderiaceae was significantly different between frogs and environment sample types (Kruskal-Wallis, chi-squared = 357.31, df = 4, $p < 0.001$; Figure 2-4), with higher relative abundance on frogs than on the four environmental sample types (Dunn test, $p < 0.001$; Table S2-14). We also found that within the Burkholderiaceae, one ASV (“SV2,” which was unclassified at the genus level) was dominant on frogs, making up 98.8% of Burkholderiaceae rarefied read counts across frog samples and 31.42% of Burkholderiaceae rarefied read counts across environmental samples. While SV2 was present

in 100% of samples, its relative abundance was significantly different between frogs and environmental sample types (Kruskal-Wallis, chi-squared = 56.492, df = 4, $p < 0.001$), with significantly higher relative abundance on frogs than in the environmental sample types (Dunn tests, $p < 0.01$; Table S2-15).

The family Rubritaleaceae was also dominated by one ASV present in 100% of samples (“SV1,” which was unclassified at the genus level) that made up 99.9% of Rubritaleaceae rarefied read counts across frog samples and 92.68% of Rubritaleaceae rarefied read counts across environmental samples. While the relative abundance of the family Rubritaleaceae was not significantly different between frogs and environment sample types (Kruskal-Wallis, chi-squared = 3.6618, df = 4, $p = 0.4537$; Figure 2-4), SV1 relative abundance was significantly different between these groups (Kruskal-Wallis, chi-squared = 45.626, df = 4, $p < 0.001$), with higher relative abundance on frogs than in environmental samples (Dunn tests, $p < 0.01$; Table S2-15).

Because frogs were dominated by SV1 (family Rubritaleaceae) and SV2 (family Burkholderiaceae), we examined variation in the relative abundance of these two ASVs across frog individuals (Figure 2-5A) and frog body regions (Figure 2-5B) to ascertain whether variation in these ASVs contributed to differences between and within individuals. The relative abundance of both SV1 and SV2 differed significantly by frog individual (Kruskal Wallis, SV1: chi-squared = 40.828, df = 8, $p < 0.001$, SV2: chi-squared = 27.863, df = 8, $p < 0.001$). *Post hoc* Dunn tests revealed that out of 36 pairwise comparisons of our 9 frog individuals, SV1 relative abundance differed significantly in 13 comparisons (*i.e.*, 36.1% of comparisons were significant; Dunn tests, $p < 0.05$; Table S2-16). Because frogs were not evenly distributed among distinct tank environments, we quantified the proportion of between-tank and within-tank individual comparisons that differed, and found that 25% of between-tank individual comparisons and only

6.25% of within-tank individual comparisons were significantly different in terms of SV1 relative abundance. SV2 relative abundance differed significantly in six of the 36 pairwise comparisons of individuals (*i.e.*, 16.7% of comparisons were significant; Dunn tests, $p < 0.05$; Table S2-16). While 55% of between-tank pairwise comparisons of individuals were significant, only 12.5% of within-tank pairwise comparisons of individuals were significant. Thus, the relative abundances of these ASVs both appeared to be primarily associated with tank identity rather than individual identity.

The relative abundance of both SV1 and SV2 also differed significantly by frog body region (Kruskal-Wallis, SV1: chi-squared = 27.941, $df = 9$, $p < 0.001$, SV2: chi-squared = 35.697, $df = 9$, $p < 0.001$). Out of 45 pairwise comparisons of body regions, SV1 relative abundance differed significantly in nine comparisons. SV1 relative abundance was significantly higher on the back than on seven other body regions (the abdomen, cloaca, forefeet, hindfeet, inner forelimbs, inner hindlimbs, and outer hindlimbs; Dunn tests, $p < 0.05$; Table S2-17), and was significantly higher on the snout than on two other body regions (the cloaca and hindfeet; Dunn tests, $p < 0.05$; Table S2-17). SV2 relative abundance differed significantly in 12 out of 45 pairwise comparisons, with significantly lower relative abundance on the back than on six other body regions (the abdomen, cloaca, hind-feet, inner forelimbs, inner hindlimbs, and outer hindlimbs; Dunn tests, $p < 0.05$; Table S2-17), significantly lower relative abundance on the snout than on five other body regions (the abdomen, cloaca, hind-feet, inner hindlimb, and outer hindlimbs; Dunn tests, $p < 0.05$; Table S2-17), and significantly higher SV2 relative abundance on hindfeet than on the vocal sack (Dunn test, $p = 0.029$; Table S2-17).

We noticed a pattern between the mean relative abundances of SV1 and SV2, where certain individuals and/or body regions had lower SV1 relative abundance and higher SV2 relative abundance while others showed the opposite relationship (Figure 2-5A, B). We were therefore also

interested in whether the relative abundance of SV1 was significantly different from the relative abundance of SV2 within each individual or body region (*i.e.*, whether the relative abundance of one of these ASVs was consistently higher than the other within certain individuals or body regions). Wilcoxon signed rank test results revealed that SV1 and SV2 relative abundances were significantly different within three frog individuals, each of which was housed in a different tank. One individual had significantly higher SV1 relative abundance and two individuals had significantly higher SV2 relative abundance (Wilcoxon, $p < 0.05$; Table S2-18). There were no significant differences between the relative abundances of these ASVs for the other six individuals (Wilcoxon, $p > 0.05$; Table S2-18). By body region, we found that the relative abundance of SV1 was significantly higher than that of SV2 on both the back and the snout (Wilcoxon, $p < 0.05$; Table S2-19), but that the relative abundances of these two ASVs were not significantly different for the other eight body regions examined (Wilcoxon, $p > 0.05$; Table S2-19).

DESeq2 Differential Abundance Testing

To identify ASVs that were differentially abundant between body regions known to experience higher *Bd* infection and pathogenesis (*e.g.*, ventral surfaces and toes) and body regions known to have markedly lower *Bd* infection (*e.g.*, dorsal surfaces, mainly the back) we implemented DESeq2 analysis on raw read count data. We individually compared DESeq2 normalized counts from the abdomen, the inner hindlimbs, the hindfeet, and the forefeet to the back. While there were no ASVs identified to be differentially abundant between the inner hindlimbs, hindfeet, or forefeet and the back, the analysis identified one unique ASV, SV56, that showed a significant $\log_2$ fold difference between the abdomen and the back (Table S2-20). SV56, an undescribed member of the family Burkholderiaceae, had significantly increased normalized

counts on the abdomen compared to the back (estimated $\log_2$ fold difference of 24.18). The mean relative abundance of SV56 in the rarefied dataset, however, was not significantly different among body regions (Kruskal Wallis, chi-squared = 5.5597, df = 9, $p=0.783$; Figure S2-1).

***Bd*-Inhibitory Predictions**

We found that SV2, the dominant undescribed Burkholderiaceae across frog samples, shared 100% sequence identity with a known *Bd*-inhibitory taxon, AmphiBac_1576 (Woodhams et al., 2015). SV56, another undescribed Burkholderiaceae that showed significant $\log_2$ fold higher normalized relative abundance on the *R. sierrae* abdomen than on their back (*see above*), shared 99.53% sequence identity with the same inhibitory taxon (AmphiBac_1576). SV1, the dominant undescribed Rubritaleaceae across frog samples, did not share $\geq 99\%$ sequence similarity with any taxa from the inhibitory database.

Discussion

In this study, we characterized microbiomes of captive *R. sierrae* and their tank environments in order to (1) assess differences between frog skin microbiomes and environmental microbiomes, (2) elucidate the importance of frog individual identity to variation in frog microbiomes, (3) determine whether microbiomes were heterogeneous among frog skin regions, and (4) ascertain whether differences among body regions corresponded to the expected distribution of *Bd* infection across the skin. Our results indicate that while captive frogs harbored distinct microbial communities compared to their local tank environment, more variation in frog microbiomes was associated with distinct tank enclosure than with individual frog identity. In addition, there were detectable differences between microbiomes of body regions preferentially

infected with *Bd* compared to those regions that infrequently experience infection. Further, heightened relative abundances of putatively *Bd*-inhibitory microbes were localized in body regions where we would expect interactions with *Bd* to occur. Together, these results help elucidate the captive microbiome of the endangered Sierra Nevada yellow-legged frog, *Rana sierrae*, and provide a basis for predicting microbiome-pathogen interactions.

Frog skin microbiomes were distinct from their tank environment microbiome and were dominated by fewer organisms

We hypothesized that frog skin microbiomes would be distinct from their surrounding tank environment microbiome, which was supported by our results for within-sample community diversity (*i.e.*, alpha diversity), community structure (*i.e.*, Beta diversity), and presence and relative abundance of microbial community members. We found that tank surfaces and water harbored significantly higher within-sample diversity than frogs (Figure 2-2A, B), which agrees with a previous study of wild *R. sierrae* showing that lake water communities had higher observed richness than frog associated communities (Ellison et al., 2019). However, this finding differed from previous evidence that lake water microbiomes had reduced or equal diversity compared to microbial communities associated with several other species of post-metamorphic amphibians (Bates et al., 2018; Kueneman et al., 2014). This discrepancy could either indicate that (1) lake water collected by Ellison et al. (2019) and tank water collected here were unusually diverse compared to other environments, or that (2) *R. sierrae* may harbor lower diversity microbiomes than other species. Reduced diversity is linked to clinical signs of chytridiomycosis (Becker and Harris, 2010) while higher community richness has been shown to correlate with host persistence after *Bd* invasion (Jani et al., 2017). Therefore, if *R. sierrae* microbiomes harbor lower diversity

and richness than other species, this could relate to their high susceptibility to *Bd* (Vredenburg et al., 2010). Future studies that directly compare the diversity of *R. sierrae* microbiomes to other species are needed.

Low diversity of microbiomes on frogs in this study likely also relates to the fact that we sampled captive frogs. Captive amphibian microbiomes often have reduced diversity compared to their wild counterparts, though there are also cases where amphibians harbor higher microbial diversity in captivity than in the wild (Kueneman et al., 2022). One previous study found that skin microbiomes of captive-reared *R. sierrae* harbored lower richness and diversity than wild counterparts (Jani and Briggs, 2014), providing some support that *R. sierrae* do experience reduced microbiome diversity in the captivity.

We also found that community structure significantly differed between frog-associated and environment-associated microbiomes (Figure 2-3, A–C), which has been previously reported in studies of both captive and wild amphibian populations (Albecker et al., 2019; Bates et al., 2018; Fitzpatrick and Allison, 2014; Jani et al., 2017; Jani and Briggs, 2014; Kueneman et al., 2014; Walke et al., 2014). There are several factors that make amphibian skin a unique and complex environment that could lead to such differences. Mucosal secretions, anti-microbial peptides (AMPs), and other secretions produced by the host regulate microbial presence and abundance on the skin, as do other microbes and the anti-microbial metabolites they produce, all of which vary between and within host species (Lillywhite and Licht, 1975; Myers et al., 2012; Tennessen et al., 2009; Walke et al., 2014; Woodhams et al., 2010, 2007a, 2006a, 2006b). By affecting which microbes can exist and persist on the skin, these interacting skin components act as a filter for microbes from the environment.

Previous studies found variable proportions of taxa shared between amphibian- and environment-associated communities, and usually dominant microorganisms on amphibians were different from those in environmental assemblages (Bates et al., 2018; Kueneman et al., 2014; Walke et al., 2014). Further, abundant microorganisms on amphibians have been shown to be rare in the environment (Bates et al., 2018; Kueneman et al., 2014; Walke et al., 2014). Here, we found that only 15.4% of ASVs were shared between frogs and environmental samples. We also looked at the proportion of shared bacterial families between frog and environmental samples, which was higher at 38.4%. We note that the proportion of taxa shared with their environment may be lower for captive frogs than their wild counterparts, as was shown previously (Bataille et al., 2016).

Additionally, in our study, a major difference in the distribution of taxa between frogs and their tank environments was that the two sequence variants found to be dominant on frogs (SV1 in the family Rubritaleaceae and SV2 in the family Burkholderiaceae) both showed significantly lower relative abundance on tank surfaces and in tank water (Figure 2-4). This supports the idea that high relative abundances of these bacteria were selected for by the frog's skin (Loudon et al., 2016; Walke et al., 2014). A caveat of these statistical comparisons is that the data used is compositional (*i.e.*, relative abundances must sum to 100%). Therefore, care must be taken with the interpretation of differences in relative abundances across samples since they do not represent absolute abundances and are standardized to the rarefied read count. For example, the higher relative abundances of these two taxa on frogs than in their environment could have resulted from higher absolute abundances on frogs, but it also could have resulted from reduced abundances of other taxa on frogs that inflated the relative abundance of these two ASVs. Regardless, it is interesting that microbial relative abundances on *R. sierrae* skin were dominated by only two sequence variants, though dominance by one or few bacterial strains has been reported in previous

studies of both captive and wild amphibians (Bates et al., 2018; Kueneman et al., 2016b, 2014; Loudon et al., 2014).

The Rubritaleaceae are a little studied family of Gram-negative bacteria in the phylum Verrucomicrobia, containing only five described species isolated from marine animals or marine sediment (Kasai et al., 2007; Rosenberg, 2014; Scheuermayer et al., 2006; Yoon et al., 2008, 2007). These species are highly conserved at the 16S rRNA gene, and are therefore not distinguishable by 16S amplicon analysis (Rosenberg, 2014). This may explain why we were unable to assign taxonomy below the family level for the dominant Rubritaleaceae sequence variant on frogs. Described Rubritaleaceae species are non-motile, obligate aerobes that synthesize carotenoid pigments, resulting in red-colored colonies (Rosenberg, 2014). The production of carotenoid pigments by Rubritaleaceae on frog skin may affect the skin-associated microbial community, as previous studies have shown that dietary carotenoid intake by amphibians increased community richness and shifted community structure of frog skin microbiomes (Antwis et al., 2014; Edwards et al., 2017).

The only previous mention of the Rubritaleaceae in amphibian microbiomes was from a study of captive *Rana muscosa*, the sister species to *R. sierrae*, conducted in the same facility at the San Francisco Zoo as our study (Jani et al., 2021). However, the phylum Verrucomicrobia, which includes Rubritaleaceae, has been detected on amphibian skin in several studies based on 16S rRNA gene data (Becker et al., 2014; Belden et al., 2015; Kueneman et al., 2016b, 2014; Longo et al., 2015; Loudon et al., 2014; Sabino-Pinto et al., 2016; Sanchez et al., 2017). Verrucomicrobia is a widely distributed phylum that has been found in various environments including soil, marine and freshwater, and animal intestines (Bergmann et al., 2011; Hugenholtz et al., 1998; Parveen et al., 2013; Passel et al., 2011; van Passel et al., 2011). Although a previous

study found that Verrucomicrobia were more abundant on wild than captive *Atelopus zeteki* (Becker et al., 2014), we hypothesize that the high relative abundance of Rubritaleaceae and Verrucomicrobia observed in the present study may be unique to captivity. This owes to the fact that Verrucomicrobia, though present, were not highly abundant in previous studies of wild *R. sierrae* populations (Jani and Briggs, 2014), even for populations from the same site used to source the San Francisco Zoo population examined here using the same primers for 16S rRNA gene amplification (Ellison et al., 2021, 2019). It is possible that in captivity, these taxa replace other taxa with similar functional abilities on the skin in the wild, but this requires further investigation.

The other dominant amphibian associated sequence variant was a member of the family Burkholderiaceae. This family consists of ecologically, phenotypically, and metabolically diverse Gram-negative bacteria found in soil, water, and in association with plants, animals, and fungi (Coenye, 2014). While some Burkholderiaceae are pathogens to plants and animals including humans (Coenye, 2014), others have been shown to suppress fungal pathogens (Carrión et al., 2018). Our dominant Burkholderiaceae sequence variant exhibited 100% sequence similarity to a bacterial isolate from the *Bd* inhibitory database, suggesting that this bacterium may help suppress *Bd* proliferation on the skin (AmphiBac_1576 / Ranamuscosa-inhibitory_37; Woodhams et al., 2015).

The order Burkholderiales, which includes the family Burkholderiaceae, has been identified as highly abundant on amphibians in several studies (Bataille et al., 2016; Bates et al., 2018; Kueneman et al., 2014), including studies of *R. sierrae* (Ellison et al., 2021, 2019). Previous research on wild *R. sierrae* found Burkholderiaceae on their skin, but it was less abundant compared to another family in the order, Comamonadaceae (Ellison et al., 2019). Notably, several amphibian microbiome studies report that a single Comamonadaceae sequence variant dominated

the community in much the same way as the dominant Burkholderiaceae sequence variant did here (Bates et al., 2018; Kueneman et al., 2016b, 2014). Interestingly, while our taxonomic assignment for this bacterium was to Burkholderiaceae, the *Bd* inhibitory bacterial isolate with identical amplicon sequence was classified as an undescribed Comamonadaceae in the inhibitory database metadata (Woodhams et al., 2015). This discrepancy illustrates how choice of assignment algorithm and/or taxonomic database can impact such classifications. Therefore, the dominant sequence variant we identified as a Burkholderiaceae is likely to be closely related to the dominant Comamonadaceae found in other amphibian studies, or it may even represent the same bacterium. Despite amplicon sequence similarity to a known *Bd*-inhibitor, further work is needed to determine whether the specific taxon identified here exhibits anti-*Bd* function.

Tank identity was more strongly associated with skin microbiomes than frog individual identity

Next, we tested our hypothesis that frog individuals would vary in their microbiomes. However, since frog individuals were not distributed evenly among distinct tank environments, many comparisons of individuals were confounded with tank comparisons. This warranted an evaluation of the relative importance of individual and tank identity to observed variation. Therefore, we compared the proportion of comparisons that were significant within and between tanks for metrics of microbiome diversity, structure, and relative abundance of taxa.

We found that individuals housed in the same tanks did exhibit variation in all metrics. However, a greater percentage of between-tank comparisons were significantly different for all metrics except for abundance-weighted diversity (*i.e.*, Shannon diversity). This indicates that tank identity was more important than individual identity to variation in most metrics. Impacts of

enclosure on animal microbiomes have been shown previously in other systems (Breen et al., 2019; Hildebrand et al., 2013). Further, amphibians have been shown to be impacted by their environment and it has been shown that they select for rare environmental microbes on their skin (Loudon et al., 2014; Walke et al., 2014). Our results are consistent with these previous findings. For example, community structure of the microbiome varied among individuals within and between tanks, however, for all three metrics examined, the percent of significant between-tank comparisons was greater than the percent of significant within-tank comparisons (Figure 2-3, D–F; *see Results*). Further, the percentage of between-tank individual comparisons that showed significant differences in relative abundances of the two dominant taxa on frogs (*i.e.*, the dominant Burkholderiaceae and Rubritaleaceae sequence variants) was roughly four times greater than the percentage of significant within-tank individual comparisons.

While tank identity appeared to be more important than individual to describing variation in many microbiome metrics, we did detect some effect of individual frog identity within tanks. Most published studies have not addressed differences in community composition among individuals, most likely because the majority of studies collected only one sample per individual. Generally, studies have focused on population or species-level variation, however variation between microbiomes of individuals may also be important to predicting disease dynamics. Individual variation could arise from factors such as diet, local habitat, microclimate, age, sex, or host genetics (Jiménez and Sommer, 2017). However, captive *R. sierrae* in our study had the same diet, were housed in the same tank environments, were all the same age, and all originated from egg masses collected from the same population (while different egg masses could be associated with different genotypes, many frogs would have been siblings from the same egg mass). Additionally, we found that frog sex did not explain variation after controlling for differences

between individuals. It is possible that the observed microbiome variation between individuals could be due to variation in efficacy of individual immune responses and in the amount or type of AMPs or other glandular secretions produced by individuals, and these differences require further study.

Considering that our study and Ellison et al (2021) both identify variation among *R. sierrae* individuals, we urge future researchers to collect replicate samples of individuals in order to document and identify sources of this variability, and to test whether individual variation in microbiomes and other components of the frog skin biome are important to within-population differences in *Bd*-driven disease outcomes. Further, future studies of microbiome variation among captive individuals should ensure that frogs are distributed among tank enclosures in a balanced design to more effectively tease apart the impacts of tank environment and individual identity.

Frog body regions showed spatial variation in the microbiome, which corresponded to expected spatial variation in *Bd* infection

Finally, we tested our hypotheses that we would detect differences in the microbiome between body regions of frogs, and that some variation would correspond to body regions preferentially infected by *Bd*. We found evidence supporting these hypotheses in comparisons of within-sample diversity, community structure, and relative abundance of taxa, described below.

Within-sample diversity, for the most part, did not differ between frog body regions; the only exceptions were significantly higher relative abundance-weighted diversity on frog forefeet compared to the abdomen, hindfeet, and back (Figure 2-2E, F). This differs from previous studies of the *Bombina orientalis* microbiome that found ventral surfaces harbored higher richness and diversity than dorsal surfaces (Bataille et al., 2016; Sabino-Pinto et al., 2016) but is consistent with

findings from other amphibian species that showed no such differences (for *Bufo japonicus*, *Cynops pyrrhogaster*, *Odorrana splendida*, and *Rana japonica*) (Sabino-Pinto et al., 2016). Interestingly, there did not appear to be a relationship between the size of each body part and microbiome diversity. The forefeet were the smallest body region sampled, but harbored higher diversity than larger body regions like the back. This suggests that standardizing the number of strokes of each body region was sufficient to control for differences in the area occupied by each body region.

Microbial community structure differed primarily between the back and other body regions. Previous studies also found significant differences in microbiome structure across the skin for two different amphibian species (Bataille et al., 2016; Sabino-Pinto et al., 2016; Sanchez et al., 2017). Though in one study, community structure only differed significantly between dorsal and ventral surfaces in captivity and not in the wild, warranting future investigation of within-individual microbiome heterogeneity of wild *R. sierrae* (Bataille et al., 2016). Here, while we did not detect differences in unweighted community membership among body regions, we found that the back differed significantly from up to seven other body regions based on two metrics of relative abundance weighted community structure (Figure 2-3, G–I). This suggests that dominant community members (*i.e.*, those given more weight in these metrics) may be more important to differences between body regions than are differences in rare organisms.

We also found that the relative abundance of the two dominant skin-associated bacteria (families Rubritaleaceae and Burkholderiaceae), both differed significantly in most comparisons of the back with other body regions (Figure 2-5B). As discussed above, the dominant Burkholderiaceae skin taxon was identified to be putatively *Bd*-inhibitory based on sequence similarity to a known anti-*Bd* bacterium (Woodhams et al., 2015). This Burkholderiaceae ASV

had significantly higher relative abundance on body regions including the abdomen, inner hindlimbs, and hindfeet compared to the back. Further, we found that a different undescribed member of the Burkholderiaceae showed significant $\log_2$ fold higher normalized read counts on the abdomen compared to the back (SV56; Table S2-20), and this taxon was also putatively anti-*Bd* (Woodhams et al., 2015).

Our finding that much of what defines heterogeneity in the skin microbiome are differences between the back and other body regions supports our hypothesis that microbiome variation corresponds to spatial heterogeneity in *Bd* infection across the skin. Studies have shown that *Bd* infection occurs most on ventral surfaces, hindfeet, and toes, and is either absent or minimal (*i.e.*, very few *Bd* sporangia) on dorsal surfaces like the back (Berger et al., 2005, 1998; North and Alford, 2008; Pessier et al., 1999). Further, it has been shown that the back of an amphibian experiences fewer pathological changes due to *Bd* infection than do other body surfaces (Berger et al., 2005). The fact that two putatively anti-*Bd* members of the Burkholderiaceae showed higher relative abundance on ventral surfaces compared to the back suggests that they may directly interact with *Bd* upon infection. However, isolation and functional characterization of these taxa are needed to determine if they would act to inhibit *Bd* growth in practice. Additionally, as discussed above, our data is compositional, and therefore quantitative analyses of taxa are needed to determine whether differences in relative abundances of Burkholderiaceae observed were driven by differences in their absolute abundances or by differences in abundances of other taxa.

The reasons that both *Bd* infection and microbiome structure differ among body regions may relate to differences in skin architecture of the amphibian host. For example, there are usually larger and more numerous granular glands (also referred to as serous glands) on the back compared to ventral surfaces (Berger et al., 2005; Varga et al., 2019). Granular glands secrete bioactive

molecules that assist in host defense, including AMPs (Varga et al., 2019), which could result in lower *Bd* infection load on the back. Such differences in the skin landscape also likely contribute to differences in the skin microbiome that we observed between the back and other body regions (*i.e.*, AMPs and other glandular secretions will impact skin microbiomes). Additionally, it has been shown that bacterially produced compounds can act synergistically with host-produced AMPs to inhibit *Bd* growth in *R. muscosa* (Myers et al., 2012). Thus the combination of skin architecture and bacterial composition are likely directly relevant to the distribution of *Bd* infection across the skin.

Our results suggest that where you collect an amphibian skin swab from (*i.e.*, which body regions) will affect the resultant community observed. However, we emphasize that this may not apply to all types of amphibians. The heterogeneity in skin structure, microbiome structure, and *Bd* localization across the skin are all likely related to the evolved ecology of the amphibian. *R. sierrae* is a semi-aquatic species that spends much of its time basking at the edges of lakes and streams, keeping ventral surfaces and toes more moist than the back. *Bd* zoospores require water to disperse, so differences in moisture across the skin due to an amphibian's lifestyle and ecology may contribute to spatial heterogeneity of *Bd* infection across the skin. For example, in a fully aquatic amphibian species (*Xenopus tropicalis*), no differences were detected in *Bd* infection between dorsal and ventral regions (Parker et al., 2002). Future studies would benefit from comparing differences in the microbiome structure, skin architecture, and spatial heterogeneity in *Bd* infection across amphibians of differing ecologies to determine whether there are consistent patterns and to elucidate the role that ecology has played in the evolution of such differences.

Applications for restoration

R. sierrae are critically endangered, but there are cases where management efforts have led to population recovery (Knapp et al., 2016). Knowledge about the microbiome could help us improve restoration efforts further (Redford et al., 2012). We suggest that microbiome variation between individuals, between distinct local environments, and within individuals could be important to restoration. Differences between individuals and between their local environments should be taken into consideration as they may lead to differences in outcomes after reintroduction and to differences in efficacy of probiotic treatments (*e.g.*, differences in successful colonization of the community using probiotic therapies). Differences across the skin could be exploited to focus on altering microbiomes of ventral surfaces and feet that gain higher *Bd* loads. Additionally, by elucidating microbiome variation between and within individuals, we can better understand and develop models to predict corresponding variation in *Bd* intensity. This natural variation relates to how susceptible frogs will be to high levels of infection (Ellison et al., 2019; Jani and Briggs, 2014), which could also be an indicator of how healthy and resilient frogs are in the face of other pathogens or environmental stressors.

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Figures

Figure 2-1. Diagram of *Rana sierrae* body regions sampled in this study. Ventral surfaces sampled are indicated with purple text and arrows; dorsal surfaces sampled are indicated with orange text and arrows; feet sampled are indicated with blue text and arrows. For limbs and feet, both the right and left were sampled. Forefeet were only sampled on the ventral surface, whereas hindfeet samples were collected from both the ventral and dorsal surfaces.

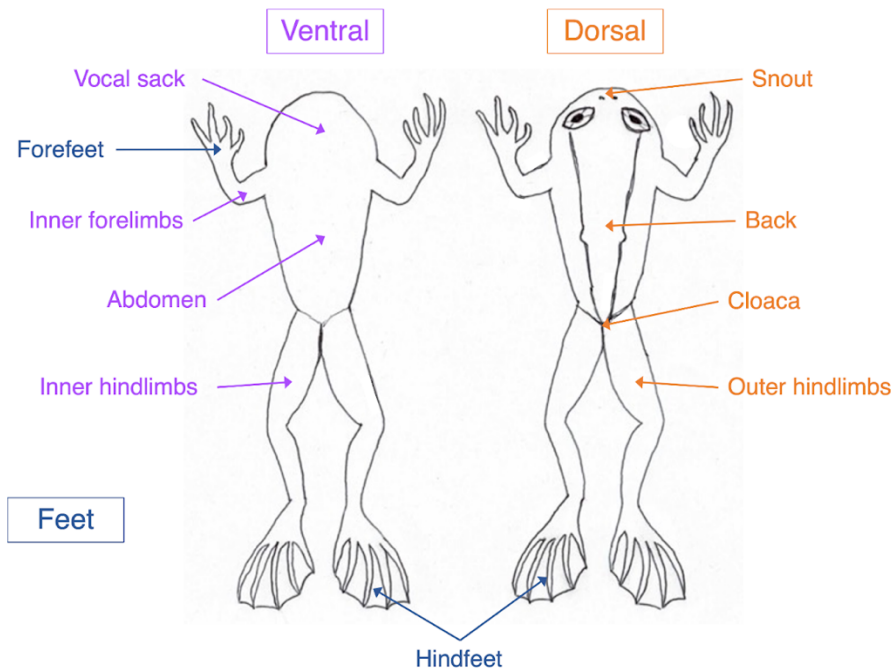


Figure 2-2. Does alpha diversity vary across sample groupings? Within-sample diversity differs between frogs and environment sample types (rock perch, tank wall, tank water, and underwater rock) in terms of **(A)** the observed number of ASVs (“Observed Richness”; Kruskal-Wallis, chi-squared = 55.62, $p < 0.001$) and **(B)** Shannon Diversity (Kruskal-Wallis, chi-squared=56.77, $p < 0.001$); **(A-B)** Boxplots and points are colored by the type of sample substrate; Within-sample diversity differs between frog individuals in terms of **(C)** the observed number of ASVs (“Observed Richness”; Kruskal-Wallis, chi-squared=40.84, $p < 0.001$) and **(D)** Shannon Diversity (Kruskal-Wallis, chi-squared=29.95, $p < 0.001$); **(C-D)** Boxplots and points are colored by frog individual ID; Within-sample diversity does not differ significantly between frog body regions in terms of **(E)** the observed number of ASVs (“Observed Richness”; Kruskal-Wallis, chi-squared=8.67, $p = 0.47$), but does differ significantly in terms of **(F)** Shannon Diversity (Kruskal-Wallis, chi-squared=21.54, $p = 0.010$); **(E-F)** Boxplots and points are colored by frog body region.

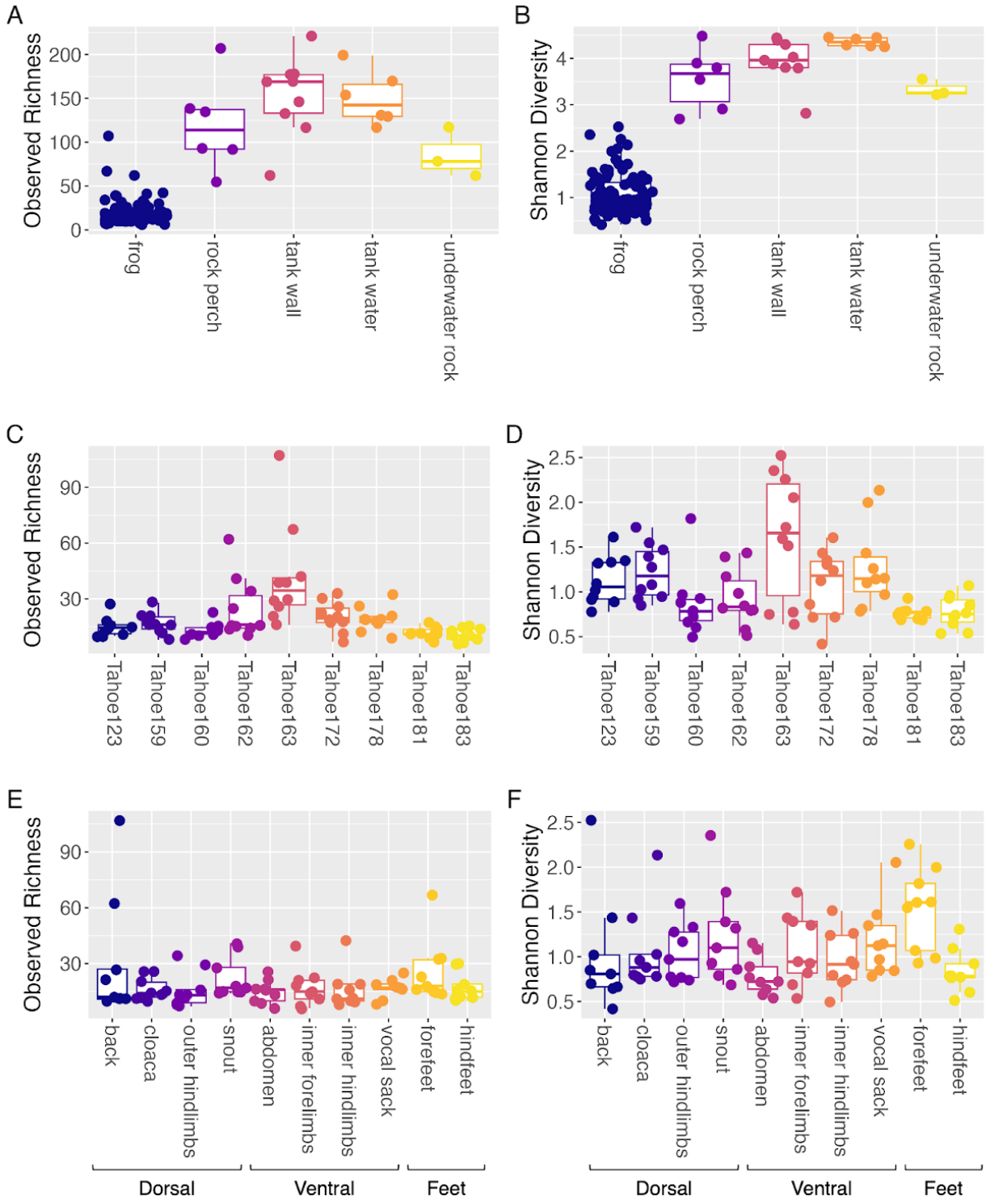


Figure 2-3. Does beta diversity vary across sample groupings? (A-C) Microbial community structure of frogs and environment sample types (rock perch, tank wall, tank water, and underwater rock); points are colored and shaped by frog category and environmental sample types; **(D-F)** Microbial community structure of frog individuals; points are colored and shaped by frog individual (“TahoeID”); **(G-I)** Microbial community structure of frog body regions; points are colored and shaped by frog body region (“SampleType”); **(A, D, G)** PCoA visualizations of unweighted Unifrac distances; **(B, E, H)** PCoA visualizations of weighted Unifrac distances; **(C, F, I)** PCoA visualizations of Bray-Curtis dissimilarities.

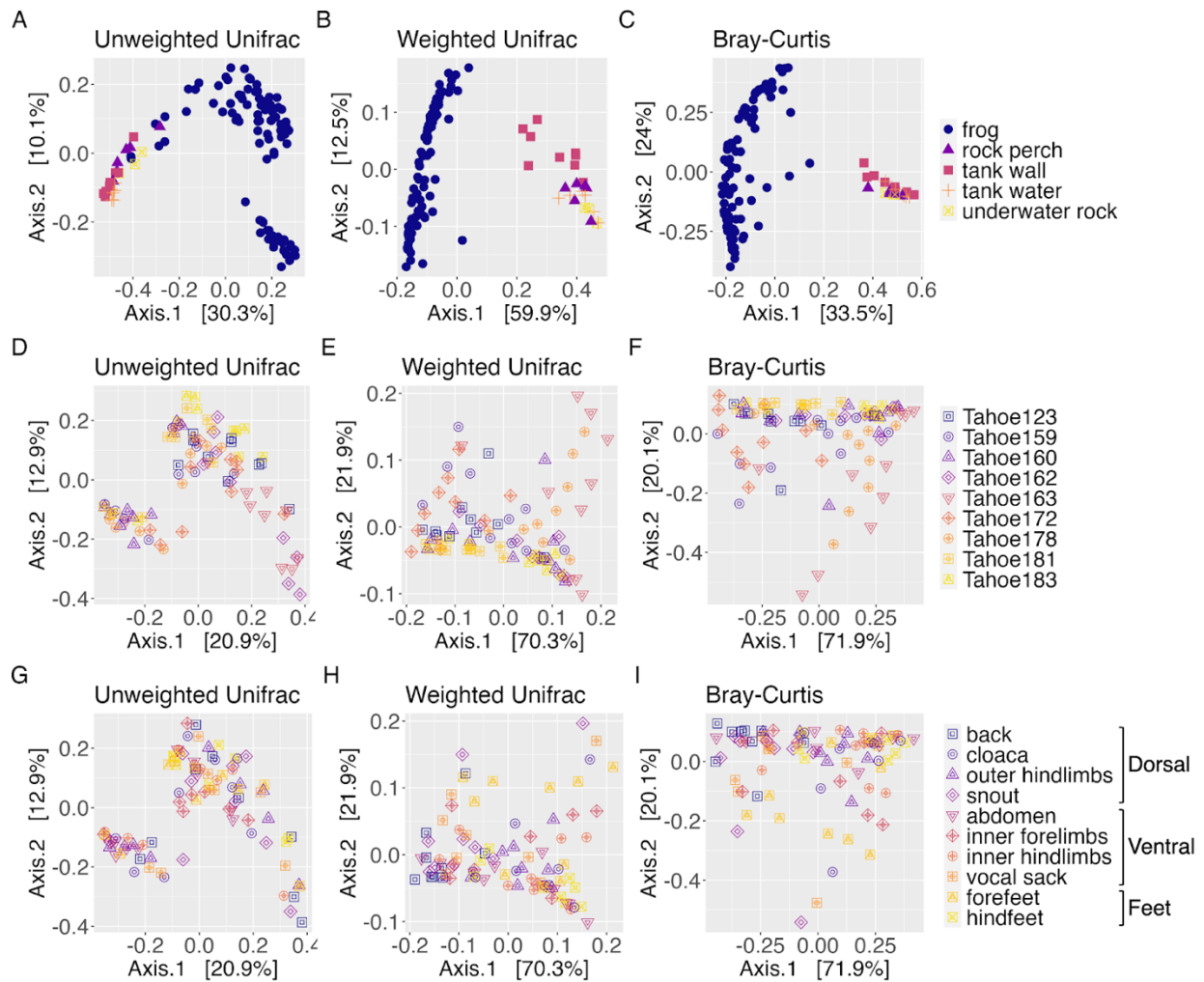


Figure 2-4. How do mean relative abundances of top represented bacterial families vary across samples? Bacterial families with mean relative abundance greater than 0.6% across samples are shown; families are colored and ordered by phylum; plot is faceted by frog, rock perch, tank wall, tank water, and underwater rock samples; error bars represent the standard error around the mean relative abundance.

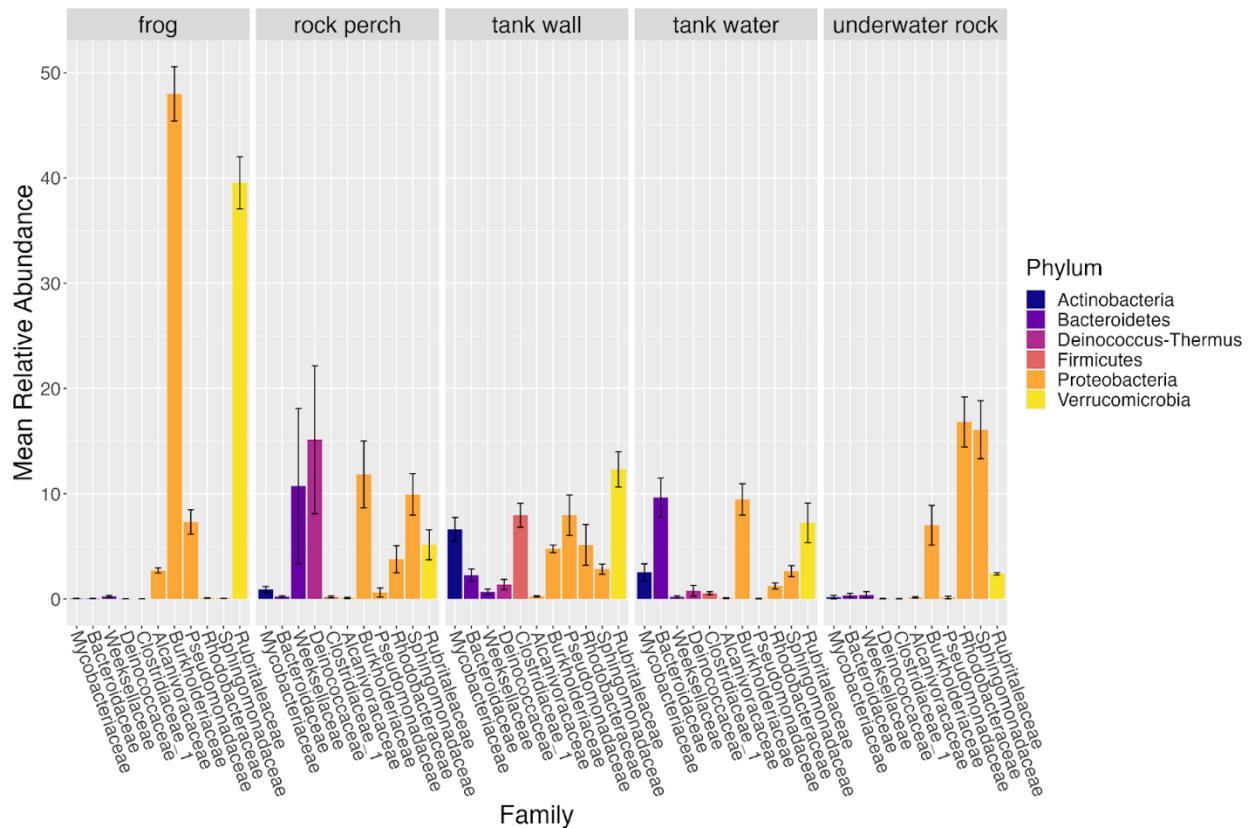
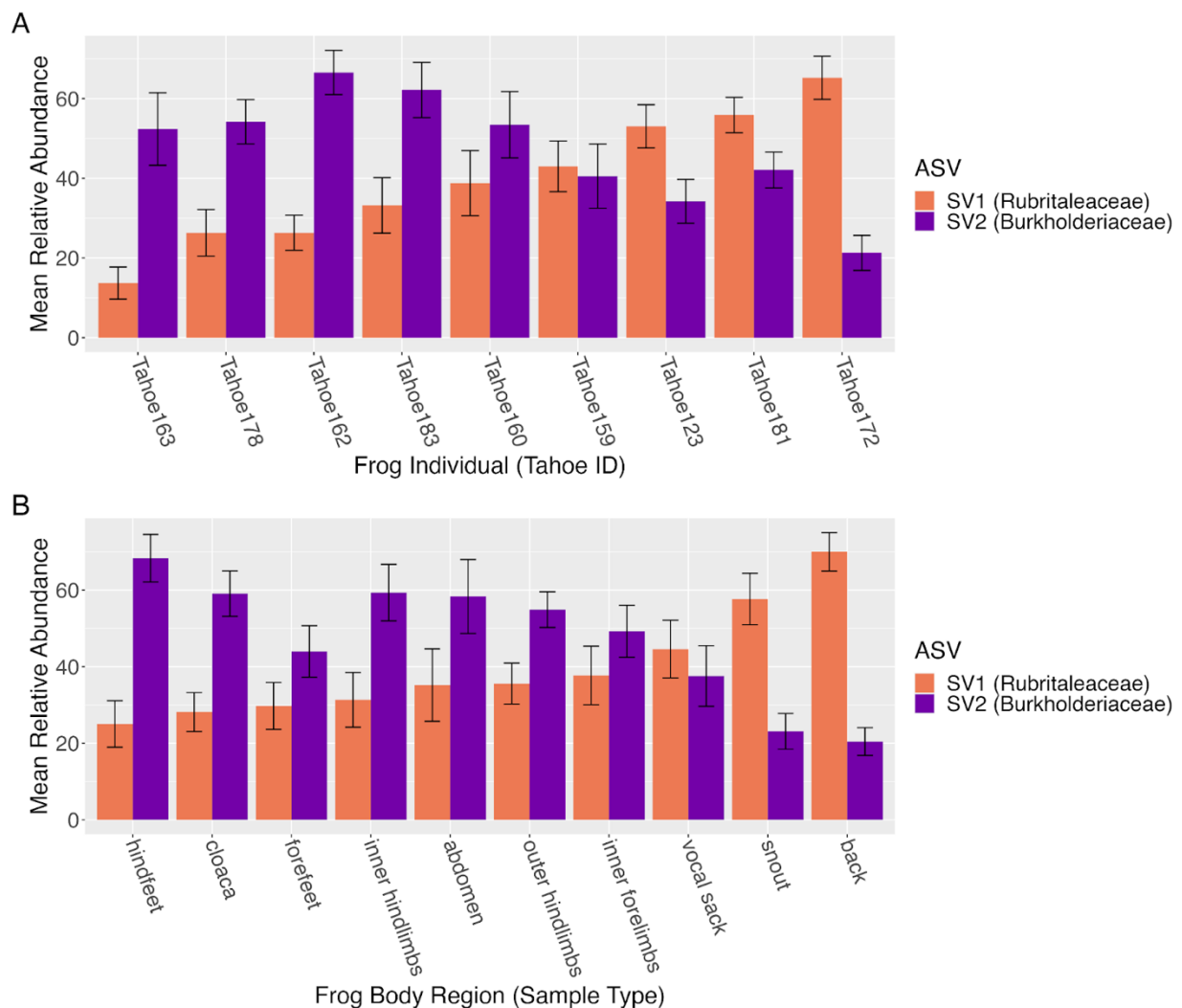


Figure 2-5. How do mean relative abundances of two dominant frog-associated taxa vary across frog samples? Mean relative abundance of two dominant ASVs (SV1, family Rubritaleaceae, and SV2, family Burkholderiaceae) on frogs across **(A)** individuals (*i.e.*, “TahoeID”) and **(B)** body regions (*i.e.*, “SampleType”); relative abundances are calculated from the rarefied dataset; frog individuals and frog body regions are ordered from lowest to highest mean relative abundance of SV1; bars are colored by ASV and associated bacterial family; error bars represent the standard error around the mean relative abundance.



Supplementary Material

Table S2-1. For which pairwise comparisons between frogs and environment types does alpha diversity differ? Following significant results of Kruskal-Wallis tests (Shannon: chi-squared=56.771, df=4, $p<0.001$; Observed: chi-squared=55.624, df=4, $p<0.001$), results of *post hoc* Dunn tests for Shannon diversity and Observed diversity in pairwise comparisons of frog, rock perch, tank wall, tank water, and underwater rock samples are shown; p-values were adjusted using Benjamini-Hochberg procedure to control the false discovery rate (FDR) with multiple comparisons; for significance, “***” = $p<0.001$, “**” = $p<0.01$, “*” = $p<0.05$, “ns” = not significant.

	Comparison	Z	Unadjusted p-value	Adjusted p-value	Significance
Shannon	frog ↔ rock perch	-3.851	0.00011771	0.00039238	***
	frog ↔ tank wall	-4.966	0.00000068	0.00000682	***
	frog ↔ tank water	-4.544	0.00000551	0.00002754	***
	frog ↔ underwater rock	-2.569	0.01020177	0.02550442	*
	rock perch ↔ tank wall	-0.214	0.83080469	0.92311632	ns
	rock perch ↔ tank water	-0.507	0.61246706	1.00000000	ns
	rock perch ↔ underwater rock	0.164	0.86972014	0.86972014	ns
	tank wall ↔ tank water	-0.341	0.73292772	0.91615965	ns
	tank wall ↔ underwater rock	0.343	0.73168590	1.00000000	ns
	tank water ↔ underwater rock	0.578	0.56352445	1.00000000	ns
Observed	frog ↔ rock perch	-3.869	0.00010925	0.00036417	***
	frog ↔ tank wall	-5.143	0.00000027	0.00000271	***
	frog ↔ tank water	-4.216	0.00002485	0.00012425	***
	frog ↔ underwater rock	-2.479	0.01318713	0.03296782	*
	rock perch ↔ tank wall	-0.316	0.75202298	1.00000000	ns
	rock perch ↔ tank water	-0.253	0.79989743	0.99987179	ns
	rock perch ↔ underwater rock	0.250	0.80275295	0.89194772	ns
	tank wall ↔ tank water	0.038	0.96944868	0.96944868	ns
	tank wall ↔ underwater rock	0.515	0.60673714	1.00000000	ns
	tank water ↔ underwater rock	0.457	0.64784982	1.00000000	ns

Table S2-2. Which frog individuals harbor differences in alpha diversity? Following significant results of Kruskal-Wallis tests (Shannon: chi-squared=29.950, df=8, $p<0.001$; Observed: chi-squared=40.841, df=8, $p<0.001$), results of *post hoc* Dunn tests for Shannon diversity and Observed richness in pairwise comparisons of frog individuals (*i.e.*, “TahoeID”) are shown; p-values were adjusted using the Benjamini-Hochberg procedure to control the false discovery rate (FDR) with multiple comparisons; for significance, “****” = $p<0.001$, “***” = $p<0.01$, “*” = $p<0.05$, “ns” = not significant.

	Comparison	Z	Unadjusted p-value	Adjusted p-value	Significance
Shannon	Tahoe178 ↔ Tahoe181	3.193	0.00141005	0.01269048	*
	Tahoe159 ↔ Tahoe181	3.270	0.00107693	0.01292313	*
	Tahoe163 ↔ Tahoe181	3.535	0.00040785	0.01468248	*
	Tahoe159 ↔ Tahoe183	3.021	0.00251608	0.01811576	*
	Tahoe163 ↔ Tahoe183	3.287	0.00101356	0.01824401	*
	Tahoe178 ↔ Tahoe183	2.944	0.00323615	0.01941689	*
	Tahoe160 ↔ Tahoe163	-2.850	0.00436894	0.02246885	*
	Tahoe123 ↔ Tahoe181	2.748	0.00600503	0.02702266	*
	Tahoe159 ↔ Tahoe160	2.585	0.00974125	0.03896500	*
	Tahoe123 ↔ Tahoe183	2.499	0.01244427	0.04072670	*
	Tahoe160 ↔ Tahoe178	-2.508	0.01214687	0.04372875	*
	Tahoe172 ↔ Tahoe181	2.157	0.03101183	0.09303549	ns
	Tahoe162 ↔ Tahoe163	-2.037	0.04164083	0.10707642	ns
	Tahoe123 ↔ Tahoe160	2.063	0.03913453	0.10837253	ns
	Tahoe172 ↔ Tahoe183	1.909	0.05630031	0.13512075	ns
	Tahoe159 ↔ Tahoe162	1.772	0.07643500	0.17197874	ns
	Tahoe162 ↔ Tahoe178	-1.695	0.09012788	0.19085903	ns
	Tahoe160 ↔ Tahoe172	-1.472	0.14097117	0.26710327	ns
	Tahoe162 ↔ Tahoe181	1.498	0.13416918	0.26833836	ns
	Tahoe163 ↔ Tahoe172	1.378	0.16819317	0.30274770	ns
	Tahoe123 ↔ Tahoe162	1.250	0.21142939	0.34597536	ns
	Tahoe162 ↔ Tahoe183	1.250	0.21142939	0.36245038	ns
	Tahoe159 ↔ Tahoe172	1.113	0.26583846	0.41609499	ns
	Tahoe172 ↔ Tahoe178	-1.036	0.30035874	0.45053811	ns

	Tahoe123 ↔ Tahoe163	-0.787	0.43102006	0.59679701	ns
	Tahoe160 ↔ Tahoe162	-0.813	0.41614643	0.59925086	ns
	Tahoe162 ↔ Tahoe172	-0.659	0.50985778	0.65553143	ns
	Tahoe160 ↔ Tahoe181	0.685	0.49351003	0.65801337	ns
	Tahoe123 ↔ Tahoe172	0.591	0.55479820	0.68871500	ns
	Tahoe123 ↔ Tahoe159	-0.522	0.60159249	0.72191099	ns
	Tahoe160 ↔ Tahoe183	0.437	0.66245971	0.74526717	ns
	Tahoe123 ↔ Tahoe178	-0.445	0.65626273	0.76211156	ns
	Tahoe163 ↔ Tahoe178	0.342	0.73207367	0.79862582	ns
	Tahoe181 ↔ Tahoe183	-0.248	0.80396645	0.82693692	ns
	Tahoe159 ↔ Tahoe163	-0.265	0.79075102	0.83726578	ns
	Tahoe159 ↔ Tahoe178	0.077	0.93859738	0.93859738	ns
Observed	Tahoe163 ↔ Tahoe183	5.032	0.00000048	0.00001745	***
	Tahoe163 ↔ Tahoe181	4.514	0.00000637	0.00011474	***
	Tahoe160 ↔ Tahoe163	-4.141	0.00003463	0.00041556	***
	Tahoe123 ↔ Tahoe163	-3.399	0.00067604	0.00608434	**
	Tahoe172 ↔ Tahoe183	3.211	0.00132497	0.00953980	**
	Tahoe162 ↔ Tahoe183	3.078	0.00208649	0.01073052	*
	Tahoe178 ↔ Tahoe183	3.116	0.00183189	0.01099134	*
	Tahoe172 ↔ Tahoe181	2.692	0.00710546	0.03197458	*
	Tahoe162 ↔ Tahoe181	2.559	0.01049791	0.03435680	*
	Tahoe159 ↔ Tahoe163	-2.568	0.01024185	0.03687067	*
	Tahoe178 ↔ Tahoe181	2.598	0.00938888	0.03755550	*
	Tahoe159 ↔ Tahoe183	2.465	0.01371351	0.04114054	*
	Tahoe160 ↔ Tahoe172	-2.319	0.02039809	0.05648702	ns
	Tahoe160 ↔ Tahoe178	-2.225	0.02610523	0.06712772	ns
	Tahoe160 ↔ Tahoe162	-2.186	0.02881084	0.06914600	ns
	Tahoe159 ↔ Tahoe181	1.946	0.05165154	0.10937972	ns
	Tahoe163 ↔ Tahoe178	1.916	0.05536231	0.11072463	ns
	Tahoe162 ↔ Tahoe163	-1.955	0.05063036	0.11391832	ns
	Tahoe163 ↔ Tahoe172	1.822	0.06849733	0.12978441	ns
	Tahoe123 ↔ Tahoe183	1.633	0.10244391	0.18439903	ns
	Tahoe159 ↔ Tahoe160	1.573	0.11569343	0.18931652	ns
	Tahoe123 ↔ Tahoe172	-1.577	0.11470442	0.19663615	ns
	Tahoe123 ↔ Tahoe178	-1.483	0.13804911	0.21607687	ns
	Tahoe123 ↔ Tahoe162	-1.445	0.14859343	0.22289015	ns

	Tahoe123 ↔ Tahoe181	1.114	0.26507992	0.38171508	ns
	Tahoe160 ↔ Tahoe183	0.892	0.37262255	0.51593891	ns
	Tahoe123 ↔ Tahoe159	-0.832	0.40565619	0.54087491	ns
	Tahoe123 ↔ Tahoe160	0.742	0.45836148	0.56900045	ns
	Tahoe159 ↔ Tahoe172	-0.746	0.45576769	0.58598703	ns
	Tahoe159 ↔ Tahoe178	-0.652	0.51470238	0.61764285	ns
	Tahoe159 ↔ Tahoe162	-0.613	0.53990574	0.62698731	ns
	Tahoe181 ↔ Tahoe183	0.519	0.60400157	0.67950176	ns
	Tahoe160 ↔ Tahoe181	0.373	0.70921021	0.77368387	ns
	Tahoe162 ↔ Tahoe172	-0.133	0.89428949	0.94689476	ns
	Tahoe172 ↔ Tahoe178	0.094	0.92487017	0.95129504	ns
	Tahoe162 ↔ Tahoe178	-0.039	0.96922714	0.96922714	ns

Table S2-3. Which frog body regions harbor differences in Shannon diversity? Following significant results of Kruskal-Wallis test for differences in Shannon diversity (Shannon diversity: chi-squared=21.543, df=9, p=0.01; Observed richness not significantly different: chi-squared=8.674, df=9, p=0.468), results of *post hoc* Dunn tests for Shannon diversity in pairwise comparisons of frog body regions (“SampleType”) are shown; p-values were adjusted using Benjamini-Hochberg procedure to control the false discovery rate (FDR) with multiple comparisons; for significance, “****” = p<0.001, “***” = p<0.01, “*” = p<0.05, “ns” = not significant.

	Comparison	Z	Unadjusted p-value	Adjusted p-value	Significance
Shannon	abdomen ↔ forefeet	-3.762	0.00016839	0.00757741	**
	forefeet ↔ hindfeet	3.320	0.00089963	0.02024164	*
	back ↔ forefeet	-2.941	0.00326904	0.04903565	*
	forefeet ↔ inner hindlimbs	2.589	0.00961514	0.10817030	ns
	abdomen ↔ snout	-2.382	0.01722542	0.12919061	ns
	abdomen ↔ vocal sack	-2.445	0.01448457	0.13036113	ns
	cloaca ↔ forefeet	-2.156	0.03105981	0.17471141	ns
	forefeet ↔ outer hindlimbs	2.174	0.02967886	0.19079265	ns
	hindfeet ↔ vocal sack	-2.003	0.04518499	0.22592495	ns
	forefeet ↔ inner forelimbs	1.877	0.06057054	0.22713953	ns
	hindfeet ↔ snout	-1.940	0.05240730	0.23583285	ns
	abdomen ↔ inner forelimbs	-1.886	0.05934353	0.24276898	ns
	back ↔ snout	-1.561	0.11856139	0.33345391	ns
	abdomen ↔ outer hindlimbs	-1.588	0.11230735	0.33692205	ns
	abdomen ↔ cloaca	-1.606	0.10828457	0.34805755	ns
	back ↔ vocal sack	-1.624	0.10437669	0.36130392	ns
	hindfeet ↔ inner forelimbs	-1.444	0.14886506	0.39405458	ns
	forefeet ↔ snout	1.380	0.16746460	0.41866149	ns
	forefeet ↔ vocal sack	1.317	0.18775782	0.44468957	ns
	inner hindlimbs ↔ vocal sack	-1.272	0.20332689	0.45748551	ns
	hindfeet ↔ outer hindlimbs	-1.146	0.25186992	0.47225610	ns
	cloaca ↔ hindfeet	1.164	0.24447925	0.47832896	ns

inner hindlimbs ↔ snout	-1.209	0.22667256	0.48572691	ns
abdomen ↔ inner hindlimbs	-1.173	0.24084156	0.49263046	ns
back ↔ inner forelimbs	-1.065	0.28704814	0.51668665	ns
back ↔ outer hindlimbs	-0.767	0.44314874	0.62317791	ns
inner forelimbs ↔ inner hindlimbs	0.713	0.47599815	0.62999755	ns
hindfeet ↔ inner hindlimbs	-0.731	0.46490246	0.63395789	ns
cloaca ↔ snout	-0.776	0.43780263	0.63551995	ns
back ↔ cloaca	-0.785	0.43249382	0.64874073	ns
abdomen ↔ back	-0.821	0.41163480	0.66155592	ns
outer hindlimbs ↔ snout	-0.794	0.42722248	0.66293143	ns
cloaca ↔ vocal sack	-0.839	0.40143309	0.66905516	ns
outer hindlimbs ↔ vocal sack	-0.857	0.39138468	0.67739656	ns
inner forelimbs ↔ vocal sack	-0.559	0.57590489	0.74044915	ns
inner forelimbs ↔ snout	-0.496	0.61973852	0.77467315	ns
inner hindlimbs ↔ outer hindlimbs	-0.415	0.67812641	0.78245354	ns
cloaca ↔ inner hindlimbs	0.433	0.66496714	0.78746108	ns
back ↔ hindfeet	0.379	0.70473808	0.79283034	ns
back ↔ inner hindlimbs	-0.352	0.72493893	0.79566468	ns
abdomen ↔ hindfeet	-0.442	0.65842570	0.80078801	ns
cloaca ↔ inner forelimbs	-0.280	0.77971675	0.81598264	ns
inner forelimbs ↔ outer hindlimbs	0.298	0.76590728	0.82061494	ns
snout ↔ vocal sack	-0.063	0.94964276	0.97122555	ns
cloaca ↔ outer hindlimbs	0.018	0.98560343	0.98560343	ns

Table S2-4. Do Beta diversity metrics differ among frogs and environment sample types?

Permutational multivariate analysis of variance (PERMANOVA) model outputs based on unweighted Unifrac distances, weighted Unifrac distances, and Bray-Curtis dissimilarities are shown; “EnvironType_FrogCategory” groupings include frog, rock perch, tank wall, tank water, and underwater rock samples.

	Factor	Df	Sum of squares	Mean squares	F Model	R²	Pr(>F)
Unweighted Unifrac	EnvironType_FrogCategory	4	8.471	2.118	11.171	0.291	0.0001
	Residuals	109	20.664	0.190		0.709	
	Total	113	29.136			1.000	
Weighted Unifrac	EnvironType_FrogCategory	4	5.564	1.391	62.000	0.695	0.0001
	Residuals	109	2.446	0.022		0.305	
	Total	113	8.009			1.000	
Bray-Curtis	EnvironType_FrogCategory	4	10.214	2.553	22.456	0.452	0.0001
	Residuals	109	12.394	0.114		0.548	
	Total	113	22.608			1.000	

Table S2-5. For which pairwise comparisons of frogs and environment sample types do Beta diversity metrics differ? Pairwise permutational multivariate analysis of variance (PERMANOVA) model outputs based on unweighted Unifrac distances, weighted Unifrac distances, and Bray-Curtis dissimilarities for frog, rock perch, tank wall, tank water, and underwater rock samples are shown; p-values were adjusted using the Benjamini-Hochberg procedure to control the false discovery rate (FDR) with multiple comparisons; for significance, “****” = $p < 0.001$, “***” = $p < 0.01$, “*” = $p < 0.05$.

	Combination	Sum of Squares	Mean Squares	F	R ²	Unadj. p-value	Adj. p-value	Signif.
Unweighted Unifrac	frog ↔ rock perch	2.101	2.101	10.850	0.103	0.0001	0.0003	***
	frog ↔ tank wall	3.470	3.470	18.187	0.158	0.0001	0.0003	***
	frog ↔ tank water	2.797	2.797	14.629	0.135	0.0001	0.0003	***
	frog ↔ underwater rock	1.191	1.191	6.149	0.063	0.0001	0.0003	***
	rock perch ↔ tank wall	0.514	0.514	2.936	0.184	0.0002	0.0004	***
	rock perch ↔ tank water	0.785	0.785	4.502	0.310	0.0025	0.0036	**
	rock perch ↔ underwater rock	0.412	0.412	2.061	0.227	0.0125	0.0125	*
	tank wall ↔ tank water	0.659	0.659	4.179	0.243	0.0003	0.0005	***
	tank wall ↔ underwater rock	0.491	0.491	2.880	0.224	0.0053	0.0066	**
	tank water ↔ underwater rock	0.507	0.507	3.032	0.302	0.0118	0.0125	*
Weighted Unifrac	frog ↔ rock perch	1.678	1.678	80.771	0.462	0.0001	0.0003	***
	frog ↔ tank wall	1.791	1.791	95.433	0.496	0.0001	0.0003	***

	frog ↔ tank water	1.757	1.757	96.948	0.508	0.0001	0.0003	***
	frog ↔ underwater rock	1.093	1.093	62.537	0.407	0.0001	0.0003	***
	rock perch ↔ tank wall	0.399	0.399	7.493	0.366	0.0002	0.0004	***
	rock perch ↔ tank water	0.346	0.346	6.019	0.376	0.0025	0.0036	**
	rock perch ↔ underwater rock	0.176	0.176	2.664	0.276	0.0352	0.0352	*
	tank wall ↔ tank water	0.411	0.411	12.082	0.482	0.0003	0.0005	***
	tank wall ↔ underwater rock	0.420	0.420	12.748	0.560	0.0053	0.0066	**
	tank water ↔ underwater rock	0.287	0.287	9.429	0.574	0.0118	0.0131	*
Bray-Curtis	frog ↔ rock perch	2.786	2.786	27.980	0.229	0.0001	0.0003	***
	frog ↔ tank wall	3.529	3.529	37.594	0.279	0.0001	0.0003	***
	frog ↔ tank water	3.123	3.123	33.940	0.265	0.0001	0.0003	***
	frog ↔ underwater rock	1.867	1.867	20.802	0.186	0.0001	0.0003	***
	rock perch ↔ tank wall	1.058	1.058	4.288	0.248	0.0002	0.0004	***
	rock perch ↔ tank water	1.132	1.132	4.116	0.292	0.0025	0.0036	**
	rock perch ↔ underwater rock	0.792	0.792	2.444	0.259	0.0125	0.0125	*
	tank wall ↔ tank water	1.087	1.087	5.661	0.303	0.0003	0.0005	***
	tank wall ↔ underwater rock	1.080	1.080	5.364	0.349	0.0053	0.0066	**
	tank water ↔ underwater rock	0.922	0.922	4.141	0.372	0.0118	0.0125	*

Table S2-6. Do frog and environment samples harbor different group dispersions in Beta diversity metrics? Permutation test for homogeneity of multivariate dispersions model output based on unweighted Unifrac, weighted Unifrac, and Bray-Curtis dissimilarities across frog, rock perch, tank wall, tank water, and underwater rock samples (*i.e.*, “EnvironType_FrogCategory” groupings) are shown; “ns” = not significant, “*” = $p < 0.05$, “**” = $p < 0.01$, “***” = $p < 0.001$.

	Factor	Df	Sum of Squares	Mean Squares	F	No. Perm	Pr(>F)
Unweighted Unifrac Dispersion	Groups: EnvironType_FrogCategory	4	0.056	0.014	1.709	9999	0.1583; ns
	Residuals	109	0.890	0.008			
Weighted Unifrac Dispersion	Groups: EnvironType_FrogCategory	4	0.136	0.034	12.755	9999	0.0001; ***
	Residuals	109	0.290	0.003			
Bray Curtis Dispersion	Groups: EnvironType_FrogCategory	4	0.638	0.159	12.443	9999	0.0001; ***
	Residuals	109	1.397	0.013			

Table S2-7. Which pairwise comparisons of frog and environment samples harbor different group dispersions in Beta diversity metrics? *Post hoc* Tukey multiple comparisons of mean dispersions for weighted Unifrac and Bray-Curtis dissimilarities across frog, rock perch, tank wall, tank water, and underwater rock samples are shown; p-values were adjusted using Benjamini-Hochberg procedure to control the false discovery rate (FDR) with multiple comparisons; for significance, “ns” = not significant, “*” = $p < 0.05$, “**” = $p < 0.01$, “***” = $p < 0.001$.

	Combination	Difference	Lower 95% CI	Upper 95% CI	Adjusted p-value	Significance
Weighted Unifrac Dispersion	rock perch ↔ frog	0.142	0.082	0.202	0.00000002	***
	tank wall ↔ frog	0.054	0.004	0.104	0.02957302	*
	tank water ↔ frog	0.044	-0.016	0.105	0.25749055	ns
	underwater rock ↔ frog	0.008	-0.076	0.091	0.99914303	ns
	tank wall ↔ rock perch	-0.089	-0.164	-0.013	0.01278466	*
	tank water ↔ rock perch	-0.098	-0.180	-0.015	0.01168291	*
	underwater rock ↔ rock perch	-0.135	-0.236	-0.033	0.00318957	**
	tank water ↔ tank wall	-0.009	-0.085	0.066	0.99697405	ns
	underwater rock ↔ tank wall	-0.046	-0.141	0.049	0.66816610	ns
	underwater rock ↔ tank water	-0.037	-0.138	0.064	0.85225254	ns
Bray Curtis Dispersion	rock perch ↔ frog	0.270	0.137	0.402	0.00000128	***
	tank wall ↔ frog	0.133	0.023	0.243	0.00949130	**
	tank water ↔ frog	0.145	0.012	0.277	0.02476239	*
	underwater rock ↔ frog	0.149	-0.035	0.333	0.17145704	ns
	tank wall ↔ rock perch	-0.137	-0.302	0.029	0.15428667	ns
	tank water ↔ rock perch	-0.125	-0.306	0.056	0.31732904	ns
	underwater rock ↔ rock perch	-0.121	-0.343	0.101	0.56071676	ns

	tank water ↔ tank wall	0.012	-0.154	0.178	0.99963007	ns
	underwater rock ↔ tank wall	0.016	-0.193	0.226	0.99949948	ns
	underwater rock ↔ tank water	0.004	-0.218	0.226	0.99999793	ns

Table S2-8. Which metadata factors explain variation in Beta diversity metrics across frog samples? Permutational multivariate analysis of variance (PERMANOVA) model outputs based on unweighted Unifrac, weighted Unifrac, and Bray-Curtis dissimilarities are shown; “SampleType” represents samples grouped by frog body region; “TahoeID” represents samples grouped by frog individual identity; for significance, “ns” = not significant, “*” = $p < 0.05$, “**” = $p < 0.01$, “***” = $p < 0.001$.

	Factor	Df	Sums Of Squares	Mean Squares	F Model	R ²	Pr(>F)	Significance
Unweighted Unifrac	SampleType	9	1.811	0.201	1.253	0.105	0.0440	*
	TahoeID	8	3.844	0.481	2.993	0.223	0.0001	***
	Residuals	72	11.561	0.161		0.672		
	Total	89	17.216			1.000		
Weighted Unifrac	SampleType	9	0.431	0.048	7.091	0.279	0.0001	***
	TahoeID	8	0.625	0.078	11.574	0.405	0.0001	***
	Residuals	72	0.486	0.007		0.315		
	Total	89	1.541			1.000		
Bray Curtis	SampleType	9	2.474	0.275	8.403	0.324	0.0001	***
	TahoeID	8	2.800	0.350	10.703	0.367	0.0001	***
	Residuals	72	2.355	0.033		0.309		
	Total	89	7.629			1.000		

Table S2-9. Which frog individuals harbor differences in Beta diversity? Pairwise permutational multivariate analysis of variance (PERMANOVA) model outputs based on unweighted Unifrac distances, weighted Unifrac distances, and Bray-Curtis dissimilarities for frog individuals (*i.e.*, “TahoeID”) are shown; p-values were adjusted using the Benjamini-Hochberg procedure to control the false discovery rate (FDR) with multiple comparisons; “Signif.” indicates significance of adjusted p-values: “****” = $p < 0.001$, “***” = $p < 0.01$, “**” = $p < 0.05$, “ns” = not significant.

	Combination	Sums of Squares	Mean Squares	F Model	R ²	Unadj. p-value	Adj. p-value	Signif.
Unweighted Unifrac	Tahoe123 ↔ Tahoe159	0.527	0.527	3.380	0.158	0.0008	0.0021	**
	Tahoe123 ↔ Tahoe160	0.725	0.725	4.750	0.209	0.0001	0.0007	***
	Tahoe123 ↔ Tahoe162	0.331	0.331	1.700	0.086	0.0694	0.0923	ns
	Tahoe123 ↔ Tahoe163	0.572	0.572	3.005	0.143	0.0002	0.0012	**
	Tahoe123 ↔ Tahoe172	0.331	0.331	1.828	0.092	0.0109	0.0194	*
	Tahoe123 ↔ Tahoe178	0.540	0.540	3.379	0.158	0.0011	0.0025	**
	Tahoe123 ↔ Tahoe181	0.440	0.440	2.890	0.138	0.0039	0.0074	**
	Tahoe123 ↔ Tahoe183	0.194	0.194	1.201	0.063	0.2489	0.2658	ns
	Tahoe159 ↔ Tahoe160	0.169	0.169	1.249	0.065	0.2319	0.2658	ns
	Tahoe159 ↔ Tahoe162	0.642	0.642	3.609	0.167	0.0005	0.0015	**
	Tahoe159 ↔ Tahoe163	0.750	0.750	4.328	0.194	0.0005	0.0015	**
	Tahoe159 ↔ Tahoe172	0.317	0.317	1.936	0.097	0.0288	0.0415	*
	Tahoe159 ↔ Tahoe178	0.118	0.118	0.826	0.044	0.6171	0.6171	ns

Tahoe159 ↔ Tahoe181	0.164	0.164	1.214	0.063	0.2510	0.2658	ns
Tahoe159 ↔ Tahoe183	0.592	0.592	4.102	0.186	0.0001	0.0007	***
Tahoe160 ↔ Tahoe162	0.870	0.870	4.987	0.217	0.0001	0.0007	***
Tahoe160 ↔ Tahoe163	1.057	1.057	6.219	0.257	0.0003	0.0013	**
Tahoe160 ↔ Tahoe172	0.524	0.524	3.260	0.153	0.0014	0.0028	**
Tahoe160 ↔ Tahoe178	0.182	0.182	1.306	0.068	0.2224	0.2658	ns
Tahoe160 ↔ Tahoe181	0.127	0.127	0.967	0.051	0.4380	0.4505	ns
Tahoe160 ↔ Tahoe183	0.664	0.664	4.706	0.207	0.0006	0.0017	**
Tahoe162 ↔ Tahoe163	0.350	0.350	1.651	0.084	0.0718	0.0923	ns
Tahoe162 ↔ Tahoe172	0.402	0.402	1.979	0.099	0.0307	0.0425	*
Tahoe162 ↔ Tahoe178	0.601	0.601	3.308	0.155	0.0010	0.0024	**
Tahoe162 ↔ Tahoe181	0.630	0.630	3.616	0.167	0.0012	0.0025	**
Tahoe162 ↔ Tahoe183	0.498	0.498	2.714	0.131	0.0113	0.0194	*
Tahoe163 ↔ Tahoe172	0.365	0.365	1.840	0.093	0.0197	0.0308	*
Tahoe163 ↔ Tahoe178	0.685	0.685	3.863	0.177	0.0004	0.0015	**
Tahoe163 ↔ Tahoe181	0.864	0.864	5.094	0.221	0.0001	0.0007	***
Tahoe163 ↔ Tahoe183	0.865	0.865	4.836	0.212	0.0001	0.0007	***
Tahoe172 ↔ Tahoe178	0.261	0.261	1.553	0.079	0.0908	0.1127	ns
Tahoe172 ↔ Tahoe181	0.350	0.350	2.186	0.108	0.0160	0.0262	*
Tahoe172 ↔ Tahoe183	0.456	0.456	2.693	0.130	0.0005	0.0015	**

	Tahoe178 ↔ Tahoe181	0.174	0.174	1.247	0.065	0.2400	0.2658	ns
	Tahoe178 ↔ Tahoe183	0.628	0.628	4.232	0.190	0.0003	0.0013	**
	Tahoe181 ↔ Tahoe183	0.333	0.333	2.368	0.116	0.0218	0.0327	*
Weighted Unifrac	Tahoe123 ↔ Tahoe159	0.016	0.016	1.573	0.080	0.2062	0.2395	ns
	Tahoe123 ↔ Tahoe160	0.045	0.045	3.685	0.170	0.0432	0.0676	ns
	Tahoe123 ↔ Tahoe162	0.090	0.090	10.802	0.375	0.0016	0.0058	**
	Tahoe123 ↔ Tahoe163	0.229	0.229	13.470	0.428	0.0001	0.0012	**
	Tahoe123 ↔ Tahoe172	0.020	0.020	2.466	0.120	0.0852	0.1136	ns
	Tahoe123 ↔ Tahoe178	0.108	0.108	9.588	0.348	0.0031	0.0093	**
	Tahoe123 ↔ Tahoe181	0.010	0.010	1.658	0.084	0.1882	0.2258	ns
	Tahoe123 ↔ Tahoe183	0.063	0.063	6.405	0.262	0.0126	0.0267	*
	Tahoe159 ↔ Tahoe160	0.019	0.019	1.396	0.072	0.2424	0.2567	ns
	Tahoe159 ↔ Tahoe162	0.052	0.052	5.464	0.233	0.0234	0.0443	*
	Tahoe159 ↔ Tahoe163	0.121	0.121	6.663	0.270	0.0019	0.0062	**
	Tahoe159 ↔ Tahoe172	0.043	0.043	4.741	0.208	0.0195	0.0390	*
	Tahoe159 ↔ Tahoe178	0.037	0.037	3.002	0.143	0.0638	0.0883	ns
	Tahoe159 ↔ Tahoe181	0.028	0.028	3.750	0.172	0.0419	0.0676	ns
	Tahoe159 ↔ Tahoe183	0.045	0.045	4.027	0.183	0.0418	0.0676	ns
	Tahoe160 ↔ Tahoe162	0.016	0.016	1.426	0.073	0.2301	0.2541	ns
	Tahoe160 ↔ Tahoe163	0.115	0.115	5.789	0.243	0.0053	0.0139	*

	Tahoe160 ↔ Tahoe172	0.086	0.086	7.859	0.304	0.0058	0.0139	*
	Tahoe160 ↔ Tahoe178	0.032	0.032	2.250	0.111	0.1250	0.1607	ns
	Tahoe160 ↔ Tahoe181	0.034	0.034	3.641	0.168	0.0546	0.0819	ns
	Tahoe160 ↔ Tahoe183	0.009	0.009	0.721	0.039	0.4172	0.4172	ns
	Tahoe162 ↔ Tahoe163	0.070	0.070	4.338	0.194	0.0056	0.0139	*
	Tahoe162 ↔ Tahoe172	0.163	0.163	23.319	0.564	0.0002	0.0018	**
	Tahoe162 ↔ Tahoe178	0.018	0.018	1.715	0.087	0.1735	0.2154	ns
	Tahoe162 ↔ Tahoe181	0.084	0.084	15.810	0.468	0.0009	0.0041	**
	Tahoe162 ↔ Tahoe183	0.008	0.008	0.844	0.045	0.3826	0.3935	ns
	Tahoe163 ↔ Tahoe172	0.303	0.303	19.364	0.518	0.0001	0.0012	**
	Tahoe163 ↔ Tahoe178	0.027	0.027	1.428	0.073	0.2329	0.2541	ns
	Tahoe163 ↔ Tahoe181	0.270	0.270	19.292	0.517	0.0001	0.0012	**
	Tahoe163 ↔ Tahoe183	0.111	0.111	6.293	0.259	0.0014	0.0056	**
	Tahoe172 ↔ Tahoe178	0.164	0.164	16.478	0.478	0.0008	0.0041	**
	Tahoe172 ↔ Tahoe181	0.021	0.021	4.314	0.193	0.0278	0.0500	ns
	Tahoe172 ↔ Tahoe183	0.129	0.129	15.101	0.456	0.0009	0.0041	**
	Tahoe178 ↔ Tahoe181	0.134	0.134	16.137	0.473	0.0004	0.0029	**
	Tahoe178 ↔ Tahoe183	0.036	0.036	3.051	0.145	0.0592	0.0852	ns
	Tahoe181 ↔ Tahoe183	0.056	0.056	8.117	0.311	0.0086	0.0194	*
Bray Curtis	Tahoe123 ↔ Tahoe159	0.092	0.092	1.478	0.076	0.2220	0.2649	ns

Tahoe123 ↔ Tahoe160	0.228	0.228	3.470	0.162	0.0570	0.0892	ns
Tahoe123 ↔ Tahoe162	0.553	0.553	13.148	0.422	0.0009	0.0046	**
Tahoe123 ↔ Tahoe163	0.808	0.808	9.708	0.350	0.0002	0.0018	**
Tahoe123 ↔ Tahoe172	0.146	0.146	3.371	0.158	0.0436	0.0713	ns
Tahoe123 ↔ Tahoe178	0.461	0.461	8.633	0.324	0.0042	0.0151	*
Tahoe123 ↔ Tahoe181	0.040	0.040	1.154	0.060	0.2993	0.3265	ns
Tahoe123 ↔ Tahoe183	0.373	0.373	7.246	0.287	0.0110	0.0283	*
Tahoe159 ↔ Tahoe160	0.116	0.116	1.459	0.075	0.2281	0.2649	ns
Tahoe159 ↔ Tahoe162	0.359	0.359	6.408	0.263	0.0147	0.0331	*
Tahoe159 ↔ Tahoe163	0.395	0.395	4.064	0.184	0.0236	0.0472	*
Tahoe159 ↔ Tahoe172	0.262	0.262	4.584	0.203	0.0269	0.0510	ns
Tahoe159 ↔ Tahoe178	0.177	0.177	2.626	0.127	0.0838	0.1257	ns
Tahoe159 ↔ Tahoe181	0.103	0.103	2.086	0.104	0.1414	0.1818	ns
Tahoe159 ↔ Tahoe183	0.276	0.276	4.208	0.189	0.0427	0.0713	ns
Tahoe160 ↔ Tahoe162	0.096	0.096	1.611	0.082	0.2048	0.2542	ns
Tahoe160 ↔ Tahoe163	0.330	0.330	3.275	0.154	0.0417	0.0713	ns
Tahoe160 ↔ Tahoe172	0.533	0.533	8.749	0.327	0.0052	0.0170	*
Tahoe160 ↔ Tahoe178	0.099	0.099	1.389	0.072	0.2510	0.2824	ns
Tahoe160 ↔ Tahoe181	0.121	0.121	2.291	0.113	0.1327	0.1769	ns
Tahoe160 ↔ Tahoe183	0.056	0.056	0.812	0.043	0.3788	0.4011	ns

Tahoe162 ↔ Tahoe163	0.284	0.284	3.681	0.170	0.0228	0.0472	*
Tahoe162 ↔ Tahoe172	1.068	1.068	28.689	0.614	0.0001	0.0018	**
Tahoe162 ↔ Tahoe178	0.107	0.107	2.256	0.111	0.0924	0.1306	ns
Tahoe162 ↔ Tahoe181	0.396	0.396	13.583	0.430	0.0019	0.0076	**
Tahoe162 ↔ Tahoe183	0.030	0.030	0.656	0.035	0.4333	0.4333	ns
Tahoe163 ↔ Tahoe172	1.181	1.181	15.060	0.456	0.0002	0.0018	**
Tahoe163 ↔ Tahoe178	0.083	0.083	0.940	0.050	0.4007	0.4121	ns
Tahoe163 ↔ Tahoe181	0.781	0.781	11.115	0.382	0.0002	0.0018	**
Tahoe163 ↔ Tahoe183	0.364	0.364	4.199	0.189	0.0109	0.0283	*
Tahoe172 ↔ Tahoe178	0.819	0.819	16.847	0.483	0.0004	0.0029	**
Tahoe172 ↔ Tahoe181	0.189	0.189	6.255	0.258	0.0107	0.0283	*
Tahoe172 ↔ Tahoe183	0.866	0.866	18.553	0.508	0.0008	0.0046	**
Tahoe178 ↔ Tahoe181	0.406	0.406	10.020	0.358	0.0011	0.0050	**
Tahoe178 ↔ Tahoe183	0.135	0.135	2.380	0.117	0.0943	0.1306	ns
Tahoe181 ↔ Tahoe183	0.266	0.266	6.885	0.277	0.0145	0.0331	*

Table S2-10. Which frog body regions harbor differences in Beta diversity? Pairwise permutational multivariate analysis of variance (PERMANOVA) model outputs based on unweighted Unifrac distances, weighted Unifrac distances, and Bray-Curtis dissimilarities for frog body regions (*i.e.*, “SampleType”) are shown; p-values were adjusted using the Benjamini-Hochberg procedure to control the false discovery rate (FDR) with multiple comparisons; “Signif.” indicates significance of adjusted p-values: “****” = $p < 0.001$, “***” = $p < 0.01$, “*” = $p < 0.05$, “ns” = not significant.

	Combination	Sums of Squares	Mean Squares	F Model	R ²	Unadj. p-value	Adj. p-value	Signif.
Unweighted Unifrac	abdomen ↔ back	0.435	0.435	2.145	0.118	0.015	0.693	ns
	abdomen ↔ cloaca	0.188	0.188	1.164	0.068	0.284	0.697	ns
	abdomen ↔ forefeet	0.285	0.285	1.727	0.097	0.062	0.697	ns
	abdomen ↔ hindfeet	0.204	0.204	1.297	0.075	0.217	0.697	ns
	abdomen ↔ inner forelimbs	0.175	0.175	1.107	0.065	0.336	0.697	ns
	abdomen ↔ inner hindlimbs	0.139	0.139	0.790	0.047	0.674	0.919	ns
	abdomen ↔ outer hindlimbs	0.260	0.260	1.365	0.079	0.180	0.697	ns
	abdomen ↔ snout	0.286	0.286	1.529	0.087	0.091	0.697	ns
	abdomen ↔ vocal sack	0.259	0.259	1.511	0.086	0.098	0.697	ns
	back ↔ cloaca	0.247	0.247	1.163	0.068	0.288	0.697	ns
	back ↔ forefeet	0.235	0.235	1.087	0.064	0.334	0.697	ns
	back ↔ hindfeet	0.231	0.231	1.108	0.065	0.317	0.697	ns

back ↔ inner forelimbs	0.279	0.279	1.334	0.077	0.185	0.697	ns
back ↔ inner hindlimbs	0.249	0.249	1.095	0.064	0.328	0.697	ns
back ↔ outer hindlimbs	0.265	0.265	1.097	0.064	0.337	0.697	ns
back ↔ snout	0.117	0.117	0.492	0.030	0.966	0.966	ns
back ↔ vocal sack	0.270	0.270	1.212	0.070	0.241	0.697	ns
cloaca ↔ forefeet	0.119	0.119	0.682	0.041	0.799	0.946	ns
cloaca ↔ hindfeet	0.113	0.113	0.678	0.041	0.772	0.946	ns
cloaca ↔ inner forelimbs	0.064	0.064	0.381	0.023	0.956	0.966	ns
cloaca ↔ inner hindlimbs	0.169	0.169	0.912	0.054	0.514	0.826	ns
cloaca ↔ outer hindlimbs	0.301	0.301	1.505	0.086	0.126	0.697	ns
cloaca ↔ snout	0.121	0.121	0.616	0.037	0.885	0.966	ns
cloaca ↔ vocal sack	0.140	0.140	0.774	0.046	0.694	0.919	ns
forefeet ↔ hindfeet	0.096	0.096	0.565	0.034	0.915	0.966	ns
forefeet ↔ inner forelimbs	0.140	0.140	0.815	0.048	0.649	0.912	ns
forefeet ↔ inner hindlimbs	0.225	0.225	1.186	0.069	0.273	0.697	ns
forefeet ↔ outer hindlimbs	0.379	0.379	1.860	0.104	0.052	0.697	ns
forefeet ↔ snout	0.110	0.110	0.551	0.033	0.942	0.966	ns
forefeet ↔ vocal sack	0.178	0.178	0.964	0.057	0.497	0.826	ns

hindfeet ↔ inner forelimbs	0.175	0.175	1.071	0.063	0.379	0.741	ns
hindfeet ↔ inner hindlimbs	0.151	0.151	0.828	0.049	0.590	0.912	ns
hindfeet ↔ outer hindlimbs	0.280	0.280	1.428	0.082	0.157	0.697	ns
hindfeet ↔ snout	0.098	0.098	0.510	0.031	0.955	0.966	ns
hindfeet ↔ vocal sack	0.146	0.146	0.829	0.049	0.635	0.912	ns
inner forelimbs ↔ inner hindlimbs	0.128	0.128	0.700	0.042	0.765	0.946	ns
inner forelimbs ↔ outer hindlimbs	0.304	0.304	1.542	0.088	0.122	0.697	ns
inner forelimbs ↔ snout	0.182	0.182	0.939	0.055	0.505	0.826	ns
inner forelimbs ↔ vocal sack	0.183	0.183	1.031	0.061	0.419	0.785	ns
inner hindlimbs ↔ outer hindlimbs	0.172	0.172	0.799	0.048	0.629	0.912	ns
inner hindlimbs ↔ snout	0.146	0.146	0.691	0.041	0.794	0.946	ns
inner hindlimbs ↔ vocal sack	0.191	0.191	0.973	0.057	0.484	0.826	ns
outer hindlimbs ↔ snout	0.268	0.268	1.186	0.069	0.265	0.697	ns
outer hindlimbs ↔ vocal sack	0.229	0.229	1.089	0.064	0.341	0.697	ns
snout ↔ vocal sack	0.123	0.123	0.596	0.036	0.934	0.966	ns

Weighted Unifrac								
	abdomen ↔ back	0.117	0.117	8.998	0.360	0.003	0.019	*
	abdomen ↔ cloaca	0.009	0.009	0.613	0.037	0.541	0.652	ns
	abdomen ↔ forefeet	0.053	0.053	3.193	0.166	0.055	0.145	ns
	abdomen ↔ hindfeet	0.008	0.008	0.595	0.036	0.462	0.630	ns
	abdomen ↔ inner forelimbs	0.010	0.010	0.613	0.037	0.499	0.641	ns
	abdomen ↔ inner hindlimbs	0.004	0.004	0.267	0.016	0.707	0.723	ns
	abdomen ↔ outer hindlimbs	0.007	0.007	0.594	0.036	0.522	0.652	ns
	abdomen ↔ snout	0.072	0.072	4.068	0.203	0.025	0.081	ns
	abdomen ↔ vocal sack	0.026	0.026	1.433	0.082	0.229	0.368	ns
	back ↔ cloaca	0.160	0.160	14.861	0.482	0.000	0.007	**
	back ↔ forefeet	0.177	0.177	13.637	0.460	0.001	0.008	**
	back ↔ hindfeet	0.184	0.184	19.896	0.554	0.000	0.007	**
	back ↔ inner forelimbs	0.103	0.103	8.172	0.338	0.002	0.015	*
	back ↔ inner hindlimbs	0.138	0.138	12.648	0.441	0.001	0.011	*
	back ↔ outer hindlimbs	0.106	0.106	12.131	0.431	0.001	0.011	*
	back ↔ snout	0.022	0.022	1.581	0.090	0.172	0.322	ns
	back ↔ vocal sack	0.064	0.064	4.407	0.216	0.012	0.055	ns
	cloaca ↔ forefeet	0.023	0.023	1.568	0.089	0.222	0.368	ns

	cloaca ↔ hindfeet	0.005	0.005	0.514	0.031	0.627	0.674	ns
	cloaca ↔ inner forelimbs	0.010	0.010	0.682	0.041	0.492	0.641	ns
	cloaca ↔ inner hindlimbs	0.002	0.002	0.197	0.012	0.882	0.882	ns
	cloaca ↔ outer hindlimbs	0.009	0.009	0.891	0.053	0.413	0.599	ns
	cloaca ↔ snout	0.085	0.085	5.558	0.258	0.008	0.040	*
	cloaca ↔ vocal sack	0.030	0.030	1.884	0.105	0.155	0.302	ns
	forefeet ↔ hindfeet	0.050	0.050	3.881	0.195	0.031	0.094	ns
	forefeet ↔ inner forelimbs	0.022	0.022	1.357	0.078	0.256	0.397	ns
	forefeet ↔ inner hindlimbs	0.032	0.032	2.229	0.122	0.117	0.264	ns
	forefeet ↔ outer hindlimbs	0.036	0.036	2.920	0.154	0.067	0.167	ns
	forefeet ↔ snout	0.081	0.081	4.615	0.224	0.022	0.075	ns
	forefeet ↔ vocal sack	0.036	0.036	1.974	0.110	0.147	0.300	ns
	hindfeet ↔ inner forelimbs	0.022	0.022	1.790	0.101	0.186	0.335	ns
	hindfeet ↔ inner hindlimbs	0.006	0.006	0.515	0.031	0.550	0.652	ns
	hindfeet ↔ outer hindlimbs	0.018	0.018	2.054	0.114	0.138	0.295	ns
	hindfeet ↔ snout	0.117	0.117	8.477	0.346	0.002	0.015	*
	hindfeet ↔ vocal sack	0.052	0.052	3.566	0.182	0.044	0.125	ns

	inner forelimbs ↔ inner hindlimbs	0.006	0.006	0.453	0.028	0.591	0.665	ns
	inner forelimbs ↔ outer hindlimbs	0.007	0.007	0.551	0.033	0.568	0.655	ns
	inner forelimbs ↔ snout	0.044	0.044	2.582	0.139	0.093	0.220	ns
	inner forelimbs ↔ vocal sack	0.007	0.007	0.394	0.024	0.666	0.697	ns
	inner hindlimbs ↔ outer hindlimbs	0.005	0.005	0.461	0.028	0.629	0.674	ns
	inner hindlimbs ↔ snout	0.077	0.077	4.980	0.237	0.016	0.065	ns
	inner hindlimbs ↔ vocal sack	0.024	0.024	1.480	0.085	0.225	0.368	ns
	outer hindlimbs ↔ snout	0.053	0.053	3.989	0.200	0.021	0.075	ns
	outer hindlimbs ↔ vocal sack	0.016	0.016	1.150	0.067	0.310	0.464	ns
	snout ↔ vocal sack	0.016	0.016	0.839	0.050	0.445	0.625	ns
Bray Curtis	abdomen ↔ back	0.682	0.682	10.461	0.395	0.004	0.017	*
	abdomen ↔ cloaca	0.036	0.036	0.471	0.029	0.639	0.670	ns
	abdomen ↔ forefeet	0.202	0.202	2.343	0.128	0.112	0.213	ns
	abdomen ↔ hindfeet	0.049	0.049	0.697	0.042	0.424	0.564	ns
	abdomen ↔ inner forelimbs	0.054	0.054	0.632	0.038	0.479	0.599	ns

abdomen ↔ inner hindlimbs	0.017	0.017	0.217	0.013	0.734	0.751	ns
abdomen ↔ outer hindlimbs	0.024	0.024	0.373	0.023	0.641	0.670	ns
abdomen ↔ snout	0.501	0.501	5.808	0.266	0.012	0.043	*
abdomen ↔ vocal sack	0.190	0.190	1.961	0.109	0.153	0.266	ns
back ↔ cloaca	0.867	0.867	19.843	0.554	0.000	0.004	**
back ↔ forefeet	0.717	0.717	13.167	0.451	0.001	0.006	**
back ↔ hindfeet	1.098	1.098	28.898	0.644	0.000	0.004	**
back ↔ inner forelimbs	0.549	0.549	10.347	0.393	0.002	0.010	**
back ↔ inner hindlimbs	0.794	0.794	16.407	0.506	0.001	0.006	**
back ↔ outer hindlimbs	0.655	0.655	19.525	0.550	0.000	0.004	**
back ↔ snout	0.075	0.075	1.383	0.080	0.244	0.379	ns
back ↔ vocal sack	0.269	0.269	4.139	0.206	0.019	0.056	ns
cloaca ↔ forefeet	0.103	0.103	1.590	0.090	0.203	0.338	ns
cloaca ↔ hindfeet	0.035	0.035	0.726	0.043	0.477	0.599	ns
cloaca ↔ inner forelimbs	0.043	0.043	0.675	0.040	0.520	0.616	ns
cloaca ↔ inner hindlimbs	0.015	0.015	0.249	0.015	0.860	0.860	ns
cloaca ↔ outer hindlimbs	0.029	0.029	0.668	0.040	0.569	0.640	ns
cloaca ↔ snout	0.584	0.584	9.010	0.360	0.000	0.004	**

	cloaca ↔ vocal sack	0.221	0.221	2.936	0.155	0.058	0.145	ns
	forefeet ↔ hindfeet	0.269	0.269	4.553	0.222	0.025	0.067	ns
	forefeet ↔ inner forelimbs	0.050	0.050	0.681	0.041	0.502	0.610	ns
	forefeet ↔ inner hindlimbs	0.157	0.157	2.267	0.124	0.114	0.213	ns
	forefeet ↔ outer hindlimbs	0.129	0.129	2.368	0.129	0.094	0.208	ns
	forefeet ↔ snout	0.362	0.362	4.786	0.230	0.012	0.043	*
	forefeet ↔ vocal sack	0.133	0.133	1.542	0.088	0.210	0.338	ns
	hindfeet ↔ inner forelimbs	0.151	0.151	2.616	0.141	0.102	0.208	ns
	hindfeet ↔ inner hindlimbs	0.042	0.042	0.784	0.047	0.400	0.563	ns
	hindfeet ↔ outer hindlimbs	0.086	0.086	2.255	0.124	0.124	0.223	ns
	hindfeet ↔ snout	0.836	0.836	14.151	0.469	0.000	0.004	**
	hindfeet ↔ vocal sack	0.389	0.389	5.597	0.259	0.016	0.051	ns
	inner forelimbs ↔ inner hindlimbs	0.053	0.053	0.779	0.046	0.426	0.564	ns
	inner forelimbs ↔ outer hindlimbs	0.031	0.031	0.575	0.035	0.555	0.640	ns
	inner forelimbs ↔ snout	0.319	0.319	4.306	0.212	0.020	0.056	ns

inner forelimbs ↔ vocal sack	0.082	0.082	0.973	0.057	0.370	0.537	ns
inner hindlimbs ↔ outer hindlimbs	0.021	0.021	0.433	0.026	0.623	0.670	ns
inner hindlimbs ↔ snout	0.558	0.558	8.029	0.334	0.004	0.017	*
inner hindlimbs ↔ vocal sack	0.197	0.197	2.458	0.133	0.099	0.208	ns
outer hindlimbs ↔ snout	0.448	0.448	8.189	0.339	0.001	0.006	**
outer hindlimbs ↔ vocal sack	0.146	0.146	2.234	0.123	0.102	0.208	ns
snout ↔ vocal sack	0.102	0.102	1.189	0.069	0.345	0.517	ns

Table S2-11. Do frog individuals harbor differences in group dispersions of Beta diversity?

Permutation test for homogeneity of multivariate dispersions model output based on unweighted Unifrac, weighted Unifrac, and Bray-Curtis dissimilarities across frog individuals (*i.e.*, “TahoeID” groupings) are shown; for significance, “ns” = not significant, “*” = $p < 0.05$, “***” = $p < 0.01$, “****” = $p < 0.001$.

	Factor	Df	Sum of Squares	Mean Squares	F	No. Permutations	Pr(>F)	Significance
Unweighted Unifrac Dispersion	Groups:							
	TahoeID	8	0.119	0.015	2.198	9999	0.038	*
	Residuals	81	0.548	0.007				
Weighted Unifrac Dispersion	Groups:							
	TahoeID	8	0.050	0.006	1.746	9999	0.096	ns
	Residuals	81	0.290	0.004				
Bray Curtis Dispersion	Groups:							
	TahoeID	8	0.246	0.031	1.572	9999	0.143	ns
	Residuals	81	1.586	0.020				

Table S2-12. Which frog individuals harbor different group dispersions in Beta diversity?

Post hoc Tukey multiple comparisons of mean dispersions for unweighted Unifrac across frog individuals (*i.e.*, “TahoeID”) are shown; p-values were adjusted using Benjamini-Hochberg procedure to control the false discovery rate (FDR) with multiple comparisons; for significance, “ns” = not significant, “*” = $p < 0.05$, “**” = $p < 0.01$, “****” = $p < 0.001$.

	Combination	Difference	Lower 95% CI	Upper 95% CI	Adjusted p- value	Significance
Unweighted Unifrac Dispersion	Tahoe159 ↔ Tahoe123	-0.050	-0.167	0.067	0.911	ns
	Tahoe160 ↔ Tahoe123	-0.057	-0.174	0.061	0.834	ns
	Tahoe162 ↔ Tahoe123	0.045	-0.072	0.163	0.946	ns
	Tahoe163 ↔ Tahoe123	0.031	-0.087	0.148	0.996	ns
	Tahoe172 ↔ Tahoe123	0.011	-0.106	0.128	1.000	ns
	Tahoe178 ↔ Tahoe123	-0.037	-0.154	0.080	0.984	ns
	Tahoe181 ↔ Tahoe123	-0.055	-0.172	0.063	0.860	ns
	Tahoe183 ↔ Tahoe123	-0.032	-0.149	0.085	0.994	ns
	Tahoe160 ↔ Tahoe159	-0.007	-0.124	0.111	1.000	ns
	Tahoe162 ↔ Tahoe159	0.095	-0.022	0.213	0.207	ns
	Tahoe163 ↔ Tahoe159	0.080	-0.037	0.198	0.425	ns
	Tahoe172 ↔ Tahoe159	0.061	-0.057	0.178	0.774	ns
	Tahoe178 ↔ Tahoe159	0.013	-0.104	0.130	1.000	ns

Tahoe181 ↔ Tahoe159	-0.005	-0.122	0.113	1.000	ns
Tahoe183 ↔ Tahoe159	0.018	-0.100	0.135	1.000	ns
Tahoe162 ↔ Tahoe160	0.102	-0.015	0.219	0.140	ns
Tahoe163 ↔ Tahoe160	0.087	-0.030	0.204	0.315	ns
Tahoe172 ↔ Tahoe160	0.067	-0.050	0.185	0.660	ns
Tahoe178 ↔ Tahoe160	0.020	-0.098	0.137	1.000	ns
Tahoe181 ↔ Tahoe160	0.002	-0.115	0.119	1.000	ns
Tahoe183 ↔ Tahoe160	0.025	-0.093	0.142	0.999	ns
Tahoe163 ↔ Tahoe162	-0.015	-0.132	0.102	1.000	ns
Tahoe172 ↔ Tahoe162	-0.035	-0.152	0.083	0.990	ns
Tahoe178 ↔ Tahoe162	-0.082	-0.200	0.035	0.390	ns
Tahoe181 ↔ Tahoe162	-0.100	-0.217	0.017	0.158	ns
Tahoe183 ↔ Tahoe162	-0.078	-0.195	0.040	0.476	ns
Tahoe172 ↔ Tahoe163	-0.020	-0.137	0.098	1.000	ns
Tahoe178 ↔ Tahoe163	-0.068	-0.185	0.050	0.658	ns
Tahoe181 ↔ Tahoe163	-0.085	-0.202	0.032	0.345	ns
Tahoe183 ↔ Tahoe163	-0.063	-0.180	0.055	0.743	ns
Tahoe178 ↔ Tahoe172	-0.048	-0.165	0.069	0.928	ns

	Tahoe181 ↔ Tahoe172	-0.065	-0.183	0.052	0.695	ns
	Tahoe183 ↔ Tahoe172	-0.043	-0.160	0.074	0.961	ns
	Tahoe181 ↔ Tahoe178	-0.018	-0.135	0.100	1.000	ns
	Tahoe183 ↔ Tahoe178	0.005	-0.112	0.122	1.000	ns
	Tahoe183 ↔ Tahoe181	0.023	-0.095	0.140	0.999	ns

Table S2-13. Do frog body regions harbor different group dispersions in Beta diversity?

Permutation test for homogeneity of multivariate dispersions model output based on unweighted Unifrac, weighted Unifrac, and Bray-Curtis dissimilarities across frog body regions (*i.e.*, “SampleType” groupings) are shown; “Signif.” indicates significance of p-values: “ns” = not significant, “*” = $p < 0.05$, “**” = $p < 0.01$, “***” = $p < 0.001$.

	Factor	Df	Sum of Squares	Mean Squares	F	No. Permutations	Pr(>F)	Signif.
Unweighted Unifrac Dispersion	Groups: SampleType	9	0.110	0.012	1.369	9999	0.211	ns
	Residuals	80	0.715	0.009				
Weighted Unifrac Dispersion	Groups: SampleType	9	0.030	0.003	0.820	9999	0.605	ns
	Residuals	80	0.321	0.004				
Bray Curtis Dispersion	Groups: SampleType	9	0.185	0.021	1.191	9999	0.311	ns
	Residuals	80	1.378	0.017				

Table S2-14. Which pairwise comparisons of frogs and environment sample types harbor different relative abundance of the family Burkholderiaceae? Following significant results of Kruskal-Wallis test (Burkholderiaceae relative abundance: chi-squared=357.31, df=4, $p<0.001$), results of *post hoc* Dunn tests for significant differences in relative abundance of the family Burkholderiaceae in pairwise comparisons of frog, rock perch, tank wall, tank water, and underwater rock samples are shown; p-values were adjusted using Benjamini-Hochberg procedure to control the false discovery rate (FDR) with multiple comparisons; for significance, “****” = $p<0.001$, “**” = $p<0.01$, “*” = $p<0.05$, “ns” = not significant.

Comparison	Z	Unadjusted p-value	Adjusted p-value	Significance
frog ↔ rock perch	-14.531	7.73E-48	7.73E-47	***
frog ↔ tank wall	-7.349	1.99E-13	6.65E-13	***
rock perch ↔ tank wall	6.750	1.48E-11	2.96E-11	***
frog ↔ tank water	-9.939	2.83E-23	1.41E-22	***
rock perch ↔ tank water	3.354	7.98E-04	1.33E-03	**
tank wall ↔ tank water	-3.076	2.10E-03	3.00E-03	**
frog ↔ underwater rock	-6.984	2.87E-12	7.18E-12	***
rock perch ↔ underwater rock	2.868	4.13E-03	5.16E-03	**
tank wall ↔ underwater rock	-2.294	2.18E-02	2.42E-02	*
tank water ↔ underwater rock	0.130	8.97E-01	8.97E-01	ns

Table S2-15. Which pairwise comparisons of frog and environment sample types harbor different relative abundances of amplicon sequence variants? Following significant Kruskal-Wallis tests (SV2 relative abundance: chi-squared=56.492, df=4, $p<0.001$; SV1 relative abundance: chi-squared=45.626, df=4, $p<0.001$), results of *post hoc* Dunn tests for significant differences in relative abundance of SV1 (family Rubritaleaceae) and SV2 (family Burkholderiaceae) in pairwise comparisons of frog, rock perch, tank wall, tank water, and underwater rock samples are shown; p-values were adjusted using Benjamini-Hochberg procedure to control the false discovery rate (FDR) with multiple comparisons; for significance, “***” = $p<0.001$, “**” = $p<0.01$, “*” = $p<0.05$, “ns” = not significant.

	Comparison	Z	Unadjusted p-value	Adjusted p-value	Significance
SV2	frog ↔ rock perch	4.335	0.000015	0.000073	***
	frog ↔ tank wall	4.736	0.000002	0.000022	***
	rock perch ↔ tank wall	-0.327	0.743755	1.000000	ns
	frog ↔ tank water	3.982	0.000068	0.000227	***
	rock perch ↔ tank water	-0.258	0.796680	1.000000	ns
	tank wall ↔ tank water	0.045	0.964388	1.000000	ns
	frog ↔ underwater rock	3.093	0.001981	0.004953	**
	rock perch ↔ underwater rock	-0.018	0.985776	0.985776	ns
	tank wall ↔ underwater rock	0.240	0.810706	1.000000	ns
	tank water ↔ underwater rock	0.193	0.847320	1.000000	ns
SV1	frog ↔ rock perch	4.233	0.000023	0.000230	***
	frog ↔ tank wall	3.459	0.000543	0.001809	**
	rock perch ↔ tank wall	-1.092	0.274712	0.457854	ns
	frog ↔ tank water	3.575	0.000350	0.001748	**
	rock perch ↔ tank water	-0.480	0.630973	0.701081	ns
	tank wall ↔ tank water	0.566	0.571346	0.714183	ns
	frog ↔ underwater rock	3.320	0.000899	0.002247	**
	rock perch ↔ underwater rock	0.232	0.816724	0.816724	ns
	tank wall ↔ underwater rock	1.109	0.267284	0.534568	ns
	tank water ↔ underwater rock	0.624	0.532647	0.760924	ns

Table S2-16. Which frog individuals harbor different relative abundances of amplicon sequence variants? Following significant results of Kruskal-Wallis test (SV2 relative abundance: chi-squared=27.863, df=8, $p<0.001$; SV1 relative abundance: chi-squared=40.828, df=8, $p<0.001$), results of *post hoc* Dunn tests for significant differences in relative abundance of SV1 (Rubritaleaceae) and SV2 (Burkholderiaceae) in pairwise comparisons of frog individuals (*i.e.*, “TahoeID”) are shown; p-values were adjusted using Benjamini-Hochberg procedure to control the false discovery rate (FDR) with multiple comparisons; for significance, “****” = $p<0.001$, “***” = $p<0.01$, “*” = $p<0.05$, “ns” = not significant.

	Comparison	Z	Unadjusted p-value	Adjusted p-value	Significance
SV2	Tahoe123 ↔ Tahoe159	-0.629	0.5292789	0.6146465	ns
	Tahoe123 ↔ Tahoe160	-1.823	0.0682842	0.1638821	ns
	Tahoe159 ↔ Tahoe160	-1.194	0.2324703	0.4184465	ns
	Tahoe123 ↔ Tahoe162	-2.979	0.0028954	0.0208469	*
	Tahoe159 ↔ Tahoe162	-2.350	0.0187975	0.0845888	ns
	Tahoe160 ↔ Tahoe162	-1.156	0.2478846	0.4249450	ns
	Tahoe123 ↔ Tahoe163	-1.682	0.0925885	0.2083241	ns
	Tahoe159 ↔ Tahoe163	-1.053	0.2924366	0.4211087	ns
	Tahoe160 ↔ Tahoe163	0.141	0.8876897	0.9399068	ns
	Tahoe162 ↔ Tahoe163	1.297	0.1947238	0.4123562	ns
	Tahoe123 ↔ Tahoe172	1.220	0.2225796	0.4451592	ns
	Tahoe159 ↔ Tahoe172	1.849	0.0644860	0.1934581	ns
	Tahoe160 ↔ Tahoe172	3.043	0.0023437	0.0210932	*
	Tahoe162 ↔ Tahoe172	4.198	0.0000269	0.0009680	****
	Tahoe163 ↔ Tahoe172	2.902	0.0037127	0.0222760	*
	Tahoe123 ↔ Tahoe178	-1.836	0.0663627	0.1706470	ns
	Tahoe159 ↔ Tahoe178	-1.207	0.2274866	0.4310273	ns
	Tahoe160 ↔ Tahoe178	-0.013	0.9897563	0.9897563	ns
	Tahoe162 ↔ Tahoe178	1.143	0.2531782	0.4142915	ns
	Tahoe163 ↔ Tahoe178	-0.154	0.8775569	0.9573348	ns
	Tahoe172 ↔ Tahoe178	-3.056	0.0022456	0.0269476	*
	Tahoe123 ↔ Tahoe181	-0.740	0.4590708	0.5508850	ns

SV1	Tahoe159 ↔ Tahoe181	-0.111	0.9114017	0.9374418	ns
	Tahoe160 ↔ Tahoe181	1.083	0.2789199	0.4183798	ns
	Tahoe162 ↔ Tahoe181	2.238	0.0252046	0.1008186	ns
	Tahoe163 ↔ Tahoe181	0.942	0.3464380	0.4796834	ns
	Tahoe172 ↔ Tahoe181	-1.960	0.0499870	0.1799532	ns
	Tahoe178 ↔ Tahoe181	1.096	0.2732592	0.4277100	ns
	Tahoe123 ↔ Tahoe183	-2.585	0.0097406	0.0500948	ns
	Tahoe159 ↔ Tahoe183	-1.956	0.0504892	0.1652375	ns
	Tahoe160 ↔ Tahoe183	-0.762	0.4461937	0.5736777	ns
	Tahoe162 ↔ Tahoe183	0.394	0.6937827	0.7805055	ns
	Tahoe163 ↔ Tahoe183	-0.903	0.3665237	0.4886983	ns
	Tahoe172 ↔ Tahoe183	-3.805	0.0001420	0.0025566	**
	Tahoe178 ↔ Tahoe183	-0.749	0.4538952	0.5634561	ns
	Tahoe181 ↔ Tahoe183	-1.845	0.0651067	0.1802954	ns
	Tahoe123 ↔ Tahoe159	0.907	0.3642548	0.4521784	ns
	Tahoe123 ↔ Tahoe160	1.451	0.1468345	0.2298278	ns
	Tahoe159 ↔ Tahoe160	0.544	0.5867742	0.6401173	ns
	Tahoe123 ↔ Tahoe162	2.491	0.0127470	0.0382410	*
	Tahoe159 ↔ Tahoe162	1.583	0.1133136	0.2039645	ns
	Tahoe160 ↔ Tahoe162	1.040	0.2983600	0.4131138	ns
	Tahoe123 ↔ Tahoe163	4.066	0.0000479	0.0005747	***
	Tahoe159 ↔ Tahoe163	3.158	0.0015864	0.0095187	**
	Tahoe160 ↔ Tahoe163	2.615	0.0089261	0.0321340	*
	Tahoe162 ↔ Tahoe163	1.575	0.1152763	0.1976165	ns
	Tahoe123 ↔ Tahoe172	-0.942	0.3464360	0.4454178	ns
	Tahoe159 ↔ Tahoe172	-1.849	0.0644849	0.1365563	ns
	Tahoe160 ↔ Tahoe172	-2.392	0.0167418	0.0463620	*
	Tahoe162 ↔ Tahoe172	-3.432	0.0005985	0.0043093	**
	Tahoe163 ↔ Tahoe172	-5.007	0.0000006	0.0000199	***
	Tahoe123 ↔ Tahoe178	2.615	0.0089261	0.0292128	*
	Tahoe159 ↔ Tahoe178	1.708	0.0877137	0.1661943	ns
	Tahoe160 ↔ Tahoe178	1.164	0.2443969	0.3519315	ns
	Tahoe162 ↔ Tahoe178	0.124	0.9012281	0.9012281	ns
	Tahoe163 ↔ Tahoe178	-1.451	0.1468345	0.2402746	ns
	Tahoe172 ↔ Tahoe178	3.556	0.0003760	0.0033838	**
	Tahoe123 ↔ Tahoe181	-0.265	0.7907485	0.8133413	ns

Tahoe159 ↔ Tahoe181	-1.173	0.2409457	0.3614186	ns
Tahoe160 ↔ Tahoe181	-1.716	0.0861359	0.1722718	ns
Tahoe162 ↔ Tahoe181	-2.756	0.0058495	0.0233981	*
Tahoe163 ↔ Tahoe181	-4.331	0.0000148	0.0002672	***
Tahoe172 ↔ Tahoe181	0.676	0.4989226	0.5793940	ns
Tahoe178 ↔ Tahoe181	-2.880	0.0039741	0.0204383	*
Tahoe123 ↔ Tahoe183	1.849	0.0644849	0.1450911	ns
Tahoe159 ↔ Tahoe183	0.942	0.3464360	0.4619147	ns
Tahoe160 ↔ Tahoe183	0.398	0.6906242	0.7312491	ns
Tahoe162 ↔ Tahoe183	-0.642	0.5209065	0.5860198	ns
Tahoe163 ↔ Tahoe183	-2.217	0.0266325	0.0684835	ns
Tahoe172 ↔ Tahoe183	2.790	0.0052653	0.0236940	*
Tahoe178 ↔ Tahoe183	-0.766	0.4436414	0.5323696	ns
Tahoe181 ↔ Tahoe183	2.114	0.0345024	0.0828057	ns

Table S2-17. Which frog body regions harbor different relative abundances of amplicon sequence variants? Following significant results of Kruskal-Wallis test (SV2 relative abundance: chi-squared=35.697, df=9, $p<0.001$; SV1 relative abundance: chi-squared=27.941, df=9, $p<0.001$), results of *post hoc* Dunn tests for significant differences in relative abundance of SV1 (family Rubritaleaceae) and SV2 (family Burkholderiaceae) in pairwise comparisons of frog body regions (*i.e.*, “SampleType”) are shown; p-values were adjusted using Benjamini-Hochberg procedure to control the false discovery rate (FDR) with multiple comparisons; for significance, “***” = $p<0.001$, “**” = $p<0.01$, “*” = $p<0.05$, “ns” = not significant.

	Comparison	Z	Unadjusted p-value	Adjusted p-value	Significance
SV2	abdomen ↔ back	3.406	0.000659	0.007419	**
	abdomen ↔ cloaca	-0.054	0.956829	0.978575	ns
	back ↔ cloaca	-3.460	0.000540	0.008101	**
	abdomen ↔ forefeet	1.416	0.156630	0.293681	ns
	back ↔ forefeet	-1.989	0.046656	0.139967	ns
	cloaca ↔ forefeet	1.471	0.141391	0.276635	ns
	abdomen ↔ hindfeet	-0.807	0.419382	0.555065	ns
	back ↔ hindfeet	-4.213	0.000025	0.001132	**
	cloaca ↔ hindfeet	-0.753	0.451234	0.580158	ns
	forefeet ↔ hindfeet	-2.224	0.026149	0.090516	ns
	abdomen ↔ inner forelimbs	0.875	0.381487	0.553771	ns
	back ↔ inner forelimbs	-2.531	0.011382	0.042682	*
	cloaca ↔ inner forelimbs	0.929	0.352737	0.529105	ns
	forefeet ↔ inner forelimbs	-0.541	0.588276	0.735345	ns
	hindfeet ↔ inner forelimbs	1.683	0.092442	0.218942	ns
	abdomen ↔ inner hindlimbs	0.036	0.971211	0.971211	ns
	back ↔ inner hindlimbs	-3.370	0.000752	0.006770	**
	cloaca ↔ inner hindlimbs	0.090	0.928110	0.971278	ns
	forefeet ↔ inner hindlimbs	-1.380	0.167461	0.301430	ns
	hindfeet ↔ inner hindlimbs	0.844	0.398903	0.560957	ns
	inner forelimbs ↔ inner hindlimbs	-0.839	0.401429	0.547403	ns
	abdomen ↔ outer hindlimbs	0.370	0.711447	0.800378	ns

	back ↔ outer hindlimbs	-3.036	0.002397	0.011987	*
	cloaca ↔ outer hindlimbs	0.424	0.671532	0.774844	ns
	forefeet ↔ outer hindlimbs	-1.047	0.295292	0.474576	ns
	hindfeet ↔ outer hindlimbs	1.177	0.239033	0.413711	ns
	inner forelimbs ↔ outer hindlimbs	-0.505	0.613385	0.746009	ns
	inner hindlimbs ↔ outer hindlimbs	0.334	0.738512	0.810562	ns
	abdomen ↔ snout	3.081	0.002062	0.013258	*
	back ↔ snout	-0.325	0.745331	0.798569	ns
	cloaca ↔ snout	3.135	0.001717	0.012879	*
	forefeet ↔ snout	1.665	0.095991	0.215979	ns
	hindfeet ↔ snout	3.889	0.000101	0.002269	**
	inner forelimbs ↔ snout	2.206	0.027388	0.088032	ns
	inner hindlimbs ↔ snout	3.045	0.002327	0.013088	*
	outer hindlimbs ↔ snout	2.711	0.006704	0.027426	*
	abdomen ↔ vocal sack	1.917	0.055208	0.146140	ns
	back ↔ vocal sack	-1.489	0.136573	0.279354	ns
	cloaca ↔ vocal sack	1.971	0.048682	0.136918	ns
	forefeet ↔ vocal sack	0.501	0.616557	0.730133	ns
	hindfeet ↔ vocal sack	2.725	0.006435	0.028960	*
	inner forelimbs ↔ vocal sack	1.042	0.297378	0.461449	ns
	inner hindlimbs ↔ vocal sack	1.881	0.059952	0.149881	ns
	outer hindlimbs ↔ vocal sack	1.547	0.121786	0.260970	ns
	snout ↔ vocal sack	-1.164	0.244475	0.407459	ns
SV1	abdomen ↔ back	-3.095	0.001970	0.017734	*
	abdomen ↔ cloaca	0.469	0.638955	0.798694	ns
	back ↔ cloaca	3.564	0.000366	0.008224	**
	abdomen ↔ forefeet	0.311	0.755596	0.829312	ns
	back ↔ forefeet	3.406	0.000659	0.009891	**
	cloaca ↔ forefeet	-0.158	0.874543	0.915220	ns
	abdomen ↔ hindfeet	0.911	0.362162	0.626819	ns
	back ↔ hindfeet	4.006	0.000062	0.002780	**
	cloaca ↔ hindfeet	0.442	0.658422	0.779710	ns
	forefeet ↔ hindfeet	0.600	0.548517	0.747978	ns
	abdomen ↔ inner forelimbs	-0.442	0.658422	0.800783	ns
	back ↔ inner forelimbs	2.653	0.007988	0.044935	*
	cloaca ↔ inner forelimbs	-0.911	0.362162	0.651891	ns

forefeet ↔ inner forelimbs	-0.753	0.451232	0.700187	ns
hindfeet ↔ inner forelimbs	-1.353	0.175945	0.416712	ns
abdomen ↔ inner hindlimbs	0.144	0.885219	0.905337	ns
back ↔ inner hindlimbs	3.239	0.001199	0.013494	*
cloaca ↔ inner hindlimbs	-0.325	0.745330	0.838496	ns
forefeet ↔ inner hindlimbs	-0.167	0.867439	0.929399	ns
hindfeet ↔ inner hindlimbs	-0.767	0.443143	0.712194	ns
inner forelimbs ↔ inner hindlimbs	0.586	0.557573	0.737964	ns
abdomen ↔ outer hindlimbs	-0.397	0.691381	0.797747	ns
back ↔ outer hindlimbs	2.698	0.006983	0.044888	*
cloaca ↔ outer hindlimbs	-0.866	0.386412	0.644021	ns
forefeet ↔ outer hindlimbs	-0.708	0.478789	0.718184	ns
hindfeet ↔ outer hindlimbs	-1.308	0.190794	0.429286	ns
inner forelimbs ↔ outer hindlimbs	0.045	0.964018	0.964018	ns
inner hindlimbs ↔ outer hindlimbs	-0.541	0.588274	0.756353	ns
abdomen ↔ snout	-2.138	0.032493	0.121850	ns
back ↔ snout	0.956	0.338888	0.635415	ns
cloaca ↔ snout	-2.607	0.009122	0.045610	*
forefeet ↔ snout	-2.450	0.014303	0.064364	ns
hindfeet ↔ snout	-3.050	0.002292	0.017189	*
inner forelimbs ↔ snout	-1.696	0.089849	0.252700	ns
inner hindlimbs ↔ snout	-2.283	0.022451	0.091846	ns
outer hindlimbs ↔ snout	-1.741	0.081630	0.244890	ns
abdomen ↔ vocal sack	-1.042	0.297376	0.581823	ns
back ↔ vocal sack	2.053	0.040114	0.138855	ns
cloaca ↔ vocal sack	-1.511	0.130728	0.346045	ns
forefeet ↔ vocal sack	-1.353	0.175945	0.439862	ns
hindfeet ↔ vocal sack	-1.953	0.050780	0.163223	ns
inner forelimbs ↔ vocal sack	-0.600	0.548517	0.771352	ns
inner hindlimbs ↔ vocal sack	-1.186	0.235451	0.504538	ns
outer hindlimbs ↔ vocal sack	-0.645	0.518866	0.753192	ns
snout ↔ vocal sack	1.096	0.272987	0.558382	ns

Table S2-18. Is SV1 relative abundance different from SV2 relative abundance within each frog individual? Results of Wilcoxon signed rank tests for significant differences between relative abundances of SV1 (family Rubritaleaceae) and SV2 (family Burkholderiaceae) by frog individual (*i.e.*, “TahoeID”) are shown; p-values were adjusted using the Benjamini-Hochberg procedure to control the false discovery rate (FDR) with multiple comparisons; for significance, “****” = $p < 0.001$, “**” = $p < 0.01$, “*” = $p < 0.05$, “ns” = not significant.

TahoeID	Vstats	Estimate	Lower 95% CI	Upper 95% CI	p-value	Adjusted p-value	Significance
Tahoe172	2	-44.609	-64.081	-20.389	0.0059	0.0410	*
Tahoe181	14	-12.486	-34.855	7.572	0.1934	0.2486	ns
Tahoe123	11	-21.250	-42.153	2.512	0.1055	0.1582	ns
Tahoe159	25	-4.254	-36.450	31.170	0.8457	0.8457	ns
Tahoe160	38	13.348	-22.241	57.774	0.3223	0.3625	ns
Tahoe183	45	45.545	-1.393	58.214	0.0840	0.1512	ns
Tahoe162	52	48.607	14.191	61.606	0.0098	0.0410	*
Tahoe178	46	32.618	-0.183	47.048	0.0645	0.1450	ns
Tahoe163	51	41.291	10.524	67.235	0.0137	0.0410	*

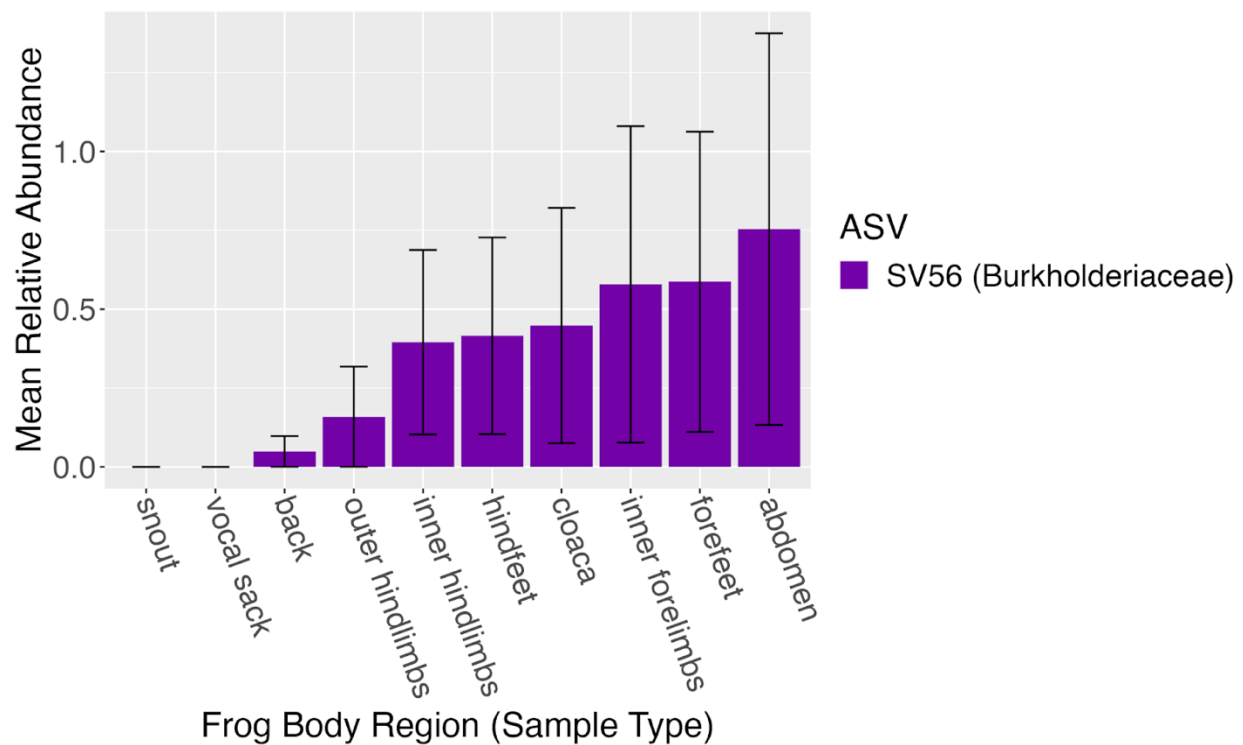
Table S2-19. Is SV1 relative abundance different from SV2 relative abundance within each frog body region? Results of Wilcoxon signed rank tests for significant differences between relative abundances of SV1 (family Rubritaleaceae) and SV2 (family Burkholderiaceae) by frog body region (*i.e.*, “SampleType”) are shown; p-values were adjusted using the Benjamini-Hochberg procedure to control the false discovery rate (FDR) with multiple comparisons; for significance, “***” = $p < 0.001$, “**” = $p < 0.01$, “*” = $p < 0.05$, “ns” = not significant.

SampleType	Vstats	Estimate	Lower 95% CI	Upper 95% CI	p-value	Adjusted p-value	Significance
back	0	-52.365	-67.638	-29.941	0.0039	0.0391	*
snout	1	-33.975	-59.223	-9.974	0.0078	0.0391	*
vocal sack	18	-5.079	-43.766	31.060	0.6523	0.6523	ns
inner forelimbs	30	9.259	-29.886	48.588	0.4258	0.4731	ns
outer hindlimbs	37	21.287	-5.464	42.263	0.0977	0.1953	ns
abdomen	33	32.050	-22.351	71.489	0.2500	0.3125	ns
inner hindlimbs	36	38.357	-4.584	56.619	0.1289	0.2148	ns
forefeet	33	15.823	-16.978	43.014	0.2500	0.3125	ns
cloaca	42	32.435	5.684	55.501	0.0195	0.0651	ns
hindfeet	39	36.652	-2.237	71.452	0.0547	0.1367	ns

Table S2-20. Which amplicon sequence variants are differentially abundant between ventral body regions and the dorsal back region? Differentially abundant amplicon sequence variants identified by DESeq2 (*i.e.*, those that had significant log₂ fold difference with p<0.05) are shown for each contrast; contrasts examined include abdomen – back, inner hindlimbs – back, forefeet – back, and hindfeet – back; “ASV” indicates the differentially abundant amplicon sequence variant and its associated taxonomy; the "baseMean" is the mean DESeq2 normalized counts for the amplicon sequence variant across all samples; the "Estimate" is the log₂ fold difference estimate for the specified contrast; the "Stat." is the Wald statistic for the specified contrast; p-values are adjusted for multiple comparisons using the Benjamini-Hochberg procedure.

Contrast	ASV	Base Mean	Estimate (log ₂ fold change)	Standard Error	Stat.	p-value	Adjusted p-value
Abdomen ↔ Back	SV56 (Family Burkholderiaceae)	10.237	24.127	6.488	3.719	0.0002	0.0498

Figure S2-1. Mean relative abundance of Burkholderiaceae taxon identified to be differentially abundant between body regions. SV56 had significant $\log_2$ fold change in DESeq2 normalized read counts between the abdomen and back (estimate of $\log_2$ fold change = 24.18, $p < 0.05$); Plot shows mean relative abundance of SV56 (family Burkholderiaceae) on frogs across all body regions (*i.e.*, “SampleType”) calculated from the rarefied dataset; Frog body regions are ordered from lowest to highest mean relative abundance of SV56; Standard errors of means are represented with error bars; Mean relative abundance of SV56 was not significantly different between body regions (Kruskal Wallis, chi-squared = 5.5597, $df = 9$, $p = 0.783$).



Chapter Three:

To rinse or not to rinse? An evaluation of rinsing frogs prior to microbiome sampling reveals that impacts are largely insignificant

Abstract

Studies of the amphibian skin microbiome have helped to illuminate the complex interactions between beneficial, commensal, and pathogenic microorganisms that contribute to host health and disease susceptibility. Rinsing amphibians with sterile water prior to collection of skin microbiome samples is a commonly applied approach, with the goal of removing microbes that are not directly associated with the host (*i.e.*, “transient” microbes). However, there has been limited verification of the necessity and validity of this pre-sampling rinse protocol. Considering that this protocol may be infeasible to conduct when collecting samples from field sites in the backcountry, we sought to evaluate whether rinsing impacts our ability to characterize the amphibian skin microbiome and differentiate it from the microbiome of the surrounding environment. We conducted 16S rRNA gene amplicon sequencing from skin swab samples collected from the same animals before and after rinsing with sterile water and from water collected from amphibian tank enclosures. While there were small differences in microbial community dissimilarity to water between pre-rinse and post-rinse frogs, we found no differences in microbial diversity or community structure between pre-rinse and post-rinse frogs, and microbiomes of both pre- and post-rinse frog samples were distinct from tank water samples. We conclude that rinsing amphibians with sterile water prior to sample collection is not required to accurately characterize the skin microbiome and distinguish it from the environment.

Introduction

The amphibian skin microbiome has become an active area of research over the past two decades, especially in relation to infection by the amphibian fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*) and the role of the microbiome in susceptibility to chytridiomycosis, the disease caused by *Bd* (Bates et al., 2022; Becker et al., 2015; Ellison et al., 2019; Harris et al., 2009, 2006; Jani et al., 2017; Woodhams et al., 2014). Rinsing amphibians with sterile water prior to microbiome sample collection has become a common practice, with the goal of removing “transient” microbes (*i.e.*, microbes associated with the environment and not directly associated with the host) (Bates et al., 2022; Culp et al., 2007; Ellison et al., 2021, 2019; Lauer et al., 2007; McKenzie et al., 2012; Walke et al., 2014). However, studies that implement this pre-sampling rinse protocol present limited evidence that it is necessary and/or effective, with most citing a single study that found differences between microbes present in frog rinse water and post-rinse amphibian skin swabs (based on denaturant gradient gel electrophoresis) (Lauer et al., 2007). Such differences could relate to variation in the ability of microbes to attach to the host rather than their degree of association to the host. Additionally, sterilization of water does not necessarily signify that DNA is absent from water; standard methods for filtering and UV- or heat-treating water reduces the amount of contaminant DNA but does not eliminate it entirely (McFeters et al., 1993; Shaw et al., 2008; Suyama and Kawaharasaki, 2013; Tamariz et al., 2006). Therefore, differences between rinse water and skin swabs may in part be the result of contaminant DNA present in sterile water. Furthermore, one study found no difference between microbial profiles of amphibian swab samples before and after rinsing with sterile water based on a molecular profiling technique (terminal restriction fragment length polymorphism) (Jani et al., 2017), which calls into question the necessity of the pre-sampling rinse protocol. In addition, we are unaware of any existing

published studies evaluating whether the pre-sampling rinse protocol significantly impacts the microbial community composition and diversity detected on the skin of amphibians when applying high-throughput sequencing approaches that are commonly utilized for microbiome characterization.

Rinsing animals with sterile water prior to sample collection is certainly more difficult and may not be feasible when sampling amphibians in the wild. For example, sterilized water would either need to be carried out to field sites in the backcountry or prepared at the field sites by filtering and boiling lake water for this purpose. Further, when attempting to sterilize water in the backcountry, it is even more likely that water will not be free of DNA without access to UV filtration systems or autoclaves, which could impact communities detected on rinsed frogs or in rinse water. Therefore, the pre-sampling rinse protocol should be evaluated to determine whether it is necessary to accurately describe the amphibian skin-associated microbiome and to distinguish this community from the microbiome of the surrounding environment using a next generation sequencing approach. We sought to test whether the pre-sampling rinse protocol alters characterization of the amphibian skin microbiome by comparing bacterial 16S rRNA gene amplicon sequencing profiles of skin swab samples collected from the same animals before and after rinsing with sterile water. We also examined whether rinsing is required to differentiate the microbiome associated with amphibians from the environmental microbiome.

Methods

Ethics Statement

Sampling of *Rana sierrae* individuals housed at the San Francisco Zoo was conducted with approval from the UC Davis IACUC (Protocol #18732) and the San Francisco Zoo Research Review Committee.

Frog Population and Handling

Frogs used in this study originated from a naturally occurring population of *Rana sierrae* in the Sierra Nevada Mountains located in the Desolation Wilderness (El Dorado County, California). Frogs were collected as egg masses by Roland Knapp and the Mountain Lakes Research Group (Sierra Nevada Aquatic Research Laboratory, Mammoth Lakes, CA) and transported to the San Francisco Zoo where they were reared to adulthood by Jessie Bushell, Director of Conservation (snout–vent length (SVL) ≥ 40 mm). Adult frogs were tagged with 8 mm unique passive integrated transponder (PIT) tags, which allow for differentiation among individuals. At the Zoo, adult frogs were housed in tanks (groups of 8-13 individuals) filled with tap water purified using biological filters and supplemented with Kent Marine R/O Right, a formulation of dissolved solids and electrolytes used to restore natural water chemistry to water that has been distilled, deionized, or purified by reverse osmosis. Partial water changes (25-50%) were conducted 2-3 times per week.

Sample Collection

We collected samples from frogs and their tank water at the San Francisco Zoo. We wore nitrile gloves during sample collection and changed gloves between handling of different individuals. Frogs were sampled using sterile synthetic fine tip dry swabs (Medical Wire &

Equipment, MW113). We collected two microbiome swab samples per individual, including one swab prior to rinsing the frog (“pre-rinse” frog samples), and another after rinsing the frog with 60 mL sterile water (prepared by running through a UV filtration system followed by autoclaving; “post-rinse” frog samples). Each microbiome swab involves 10 strokes on the back of the frog extending from the snout to vent (5 on each side), 5 strokes on the dorsal side of each hind-leg, 10 strokes of the ventral abdomen extending from the drink patch to the mouth (5 on each side), 5 strokes on each inner hind-limb, and 5 strokes of each of the hind-feet. We also recorded the sex, weight (using a Pesola spring scale), and SVL (measured using digital calipers) for each frog. We sampled tank water by passing 240 mL through a 0.22 μ m Sterivex filter (Millipore). Finally, we recorded air and water temperatures. All samples were kept on dry ice during collection and transferred to a -80 °C freezer for storage on the same day. We collected swab samples from ten frogs from the same tank (n=20 swabs) and water filter samples from five tanks housing frogs at the Zoo facility (n=10 water filters).

DNA Extraction

DNA was extracted from microbiome swabs and water filter samples using MoBio PowerSoil DNA Isolation Kits, using a modified protocol for low-biomass samples discussed with the manufacturer. This protocol has been described previously (*see Chapter Two*). Following DNA extraction, DNA concentration was quantified with a Qubit (Invitrogen, Carlsbad, CA, United States) using the dsDNA High Sensitivity Kit. DNA extracts were then stored at -80 °C.

Sequence Generation

Sequencing libraries were prepared following the protocol “16S Metagenomic Sequencing Library Preparation” (Illumina, Inc., San Diego, CA, USA) with some modifications. Briefly, we

PCR amplified the hypervariable V3-V4 region of the bacterial 16S gene using 341F and 805R primers from Klindworth et al. (2013) with overhang adaptors (forward primer with overhang = 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG; reverse primer with overhang = 5'

GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC)

from each of our samples in triplicate (“Amplicon PCR” in the Illumina protocol). We pooled replicate PCR products from each sample and purified them using magnetic beads (Axygen AxyPrep Mag PCR Clean-Up Kit) with a ratio of 0.8 beads to PCR product pool (“PCR Clean-Up” in the Illumina protocol). Next, we attached dual indices and Illumina sequencing adaptors in a second PCR step (“Index PCR” in the Illumina protocol). We then purified these PCR products using magnetic beads, this time using a ratio of 1.12 beads to PCR product (“PCR Clean-Up 2” in the Illumina protocol). Next, we quantified DNA using a Qubit (Invitrogen, Carlsbad, CA, United States) and pooled libraries in equimolar quantities. Pooled libraries were sent to the UC Davis Genome Center DNA Technology Core for sequencing on an Illumina MiSeq (Illumina, Inc., San Diego, CA, USA) with v2 chemistry and 2 x 250 bp paired end cycles.

Sequence Processing

We used a modified version of a custom script designed by G. Jospin (https://github.com/gjospin/scripts/blob/master/Demul_trim_prep.pl) to demultiplex our sequence data. Primers were removed using cutadapt v. 3.5 (Martin, 2011) with Python v. 3.9.10 (Van Rossum and Drake, 2009), setting the maximum error rate to 0.2 and discarding any reads for which primers were not present. The resulting sequences were processed using the DADA2 v.1.24.0 (Callahan et al., 2016) workflow in R v. 4.2.1 (R Core Team, 2022) with RStudio v. 2022.07.0-548 (Posit Team, 2022)). Forward and reverse reads were trimmed at 225 base pairs,

truncated at the first quality score of 2, and removed if the expected errors were greater than 4. We then merged reads and inferred amplicon sequence variants (ASVs). Next, 0.8% of merged reads were identified as chimeric and removed. Taxonomy was assigned to genus level using the Ribosomal Database Project (RDP) naïve Bayesian classifier algorithm and the SILVA high quality ribosomal RNA database v. 132 (Quast et al., 2012; Wang et al., 2007; Yilmaz et al., 2014). Based on taxonomic classification, we removed ASVs that were non-bacterial at the domain level (including Eukaryota, Archaea, and those unclassified to domain), chloroplasts, and mitochondria, which resulted in 1208 unique ASVs in our dataset. We implemented the Decontam (v. 1.16.0) prevalence method with a probability threshold of 0.5 (which identifies sequences that have a higher prevalence in negative controls than in true samples) to identify putative contaminants based on one PCR negative control, however no contaminants were detected in our samples (Davis et al., 2018).

Sequence Analysis and Visualization

We compared sample depth among the three types of samples (pre-rinse and post-rinse frog samples, and tank water samples) to ascertain if there were differences in raw read counts after filtering steps described above. While sample depths for both types of frog samples were normally distributed, library sizes for water samples were not (determined using Shapiro-Wilk tests of normality, using the `shapiro.test` function in base R v.4.2.1 (R Core Team, 2022)). We therefore used nonparametric Kruskal-Wallis rank sum tests, implementing the `kruskal.test` function in base R, followed by post-hoc Dunn tests using the `dunnTest` function in FSA v. 0.9.3 (Ogle et al., 2022) and correcting resultant p-values using the Benjamini-Hochberg procedure to control the false discovery rate (FDR) with multiple comparisons. Next, we rarefied samples at an

even sampling depth of 1586, which was the minimum depth of a true sample. Our resulting dataset included 30 true samples and 743 unique ASVs.

To assess within-sample diversity, we used the `estimate_richness` function in `phyloseq` v. 1.40.0 (McMurdie and Holmes, 2013) to calculate alpha diversity metrics including observed richness and Shannon diversity for the rarefied dataset. We first split the data by sample types so that we could compare between frogs before rinsing with sterile water (“pre-rinse”), frogs after rinsing (“post-rinse”), and tank water samples, and then we estimated the alpha diversity values for each of these groups. Alpha diversity estimates were not normally distributed (based on Shapiro-Wilk tests of normality). Therefore, we again used Kruskal-Wallis rank sum tests, here to test for significant differences in alpha diversity values between sample types, followed by post-hoc Dunn tests with Benjamini-Hochberg corrected p-values.

To assess community structure we compared between-sample diversity (*i.e.*, beta diversity). We used the `ordinate` function in `phyloseq` to calculate Bray-Curtis dissimilarities from the rarefied dataset and visualized using non-metric multidimensional scaling (NMDS). Bray-Curtis dissimilarities range from zero (*i.e.*, no difference) to one (*i.e.*, entirely dissimilar). To test for significant differences in community structure between sample types we ran a permutational multivariate analysis of variance (PERMANOVA; (Anderson, 2001)) using the `adonis` function in `vegan` v. 2.6.2 with 9999 permutations (Oksanen et al., 2022). Next we performed post-hoc pairwise PERMANOVA tests using the `adonis.pair` function in `EcolUtils` v. 0.1 (Salazar, 2022) with 9999 permutations, and correcting p-values for multiple comparisons using the Benjamini-Hochberg procedure. We calculated mean dispersion by sample type and tested for significant differences using the `betadisper` and `permutest.betadisper` functions in `vegan`, followed by a post-

hoc Tukey Honest Significant Differences test (using the TukeyHSD function in base R, which corrects for multiple comparisons using the family-wise error rate).

We tested whether average Bray-Curtis dissimilarity to tank water samples was significantly different between pre-rinse and post-rinse frog sample groups using a two-tailed paired t-test (t.test function in base R). We applied a second t-test to assess whether average Bray-Curtis dissimilarity between pre-rinse frog samples and water samples was significantly different from dissimilarity between pre- and post-rinse frog samples.

Results

Overall, we found few differences between pre- and post-rinse frog samples, while both consistently differed from tank water samples.

Prior to rarefaction, the mean sample depth of water samples was 20,971.2 reads (± 7336.5 standard error; $n=10$), the mean sample depth of pre-rinse frog samples was 5248.2 reads (± 1160.1 standard error; $n=10$), and the mean sample depth of post-rinse frog samples was 4894.1 (± 902.1 standard error; $n=10$). There were significant differences in the sample depth among these sample types (Kruskal-Wallis, chi-squared = 14, $p < 0.001$), however these differences were between water samples and each of the two frog sample types (Dunn, $p < 0.01$, $p < 0.01$; Table S3-1). There was no significant difference between sample depths of pre-rinse and post-rinse frogs (Dunn, $p = 0.90$; Table S3-1). Because of the uneven sample depth between water and frog samples, all subsequent analyses were performed on rarefied data.

While we found significant differences among sample types for observed richness (Kruskal-Wallis, chi-squared = 19.63, $p < 0.001$) and Shannon diversity (Kruskal-Wallis, chi-squared = 18.90, $p < 0.001$), neither the observed richness nor the Shannon diversity differed

between pre-rinse and post-rinse frogs (Dunn, $p=0.63$ and $p=0.58$, respectively; Figure 3-1; Table S3-2; Table S3-3). Differences detected in observed richness and Shannon diversity were due to significant differences between tank water and both pre- and post- rinse frogs (Dunn, $p<0.001$, $p<0.001$, $p<0.001$; Figure 3-1; Table S3-2; Table S3-3).

Community structure (measured in terms of Bray-Curtis dissimilarities) was not significantly different between pre-rinse and post-rinse frogs (PERMANOVA, $p=0.19$; Table S3-5), but was significantly different between tank water and both pre- and post- rinse frogs (PERMANOVA, $p<0.001$, $p<0.001$; Figure 3-2; Table S3-4; Table S3-5). The mean dispersion (*i.e.*, within group variance) did not differ between pre-rinse and post-rinse frogs (TukeyHSD, $p=0.44$; Table S3-7), but dispersion was significantly different between tank water samples and both pre- and post-rinse frog sample groups (TukeyHSD, $p<0.001$, $p<0.001$; Figure 3-2; Table S3-6; Table S3-7). This indicates that the differences between tank water and frog community structure may have been due to differences in dispersion. However, given that the observed clustering in the NMDS visualization showed a clear separation between frog samples and tank water samples (Figure 3-2), mean centroids also appeared to differ between tank water and frog sample communities. Therefore, it is likely that microbial community structure differed between tank water and frog samples in terms of both dispersion and mean centroid.

The average Bray-Curtis dissimilarity between post-rinse frog microbiome samples and water microbiome samples (0.994) was significantly greater than the average dissimilarity between pre-rinse frog microbiome samples and water samples (0.987), though this difference was very small (T-test, $p<0.001$, mean difference = 0.0065, 0.0049–0.0081 95% CI). The average Bray-Curtis dissimilarity between pre-rinse frog microbiomes and tank water microbiomes (0.987) was

over 5.5 times greater than the average dissimilarity between pre-rinse frog microbiomes and post-rinse frog microbiomes (0.179; T-test, $p < 0.001$, mean difference = 0.809, 0.787–0.830 95% CI).

Discussion

We conclude that rinsing amphibians with sterile water prior to swab sampling the microbiome is not required for accurate characterization of the skin microbiome. We found no differences in raw sample depths, microbial diversity, or community structure between pre-rinse and post-rinse frogs, indicating that the process of rinsing frogs does not significantly impact characterization of the microbiome. In addition, we found that microbiomes of both pre- and post-rinse frog samples were distinct from tank water samples in terms of raw sample depths, diversity, and community structure. Therefore, rinsing is not needed in order to distinguish the amphibian associated community from the local environmental microbial community. These results are consistent with a previous study that compared microbial profiles of pre- and post-rinse amphibian skin swabs and pond water (Jani et al., 2017).

We did find that pre-rinse frog microbiomes were more similar on average to tank water microbiomes than were post-rinse frog microbiomes, which supports the idea that there were transient environmental microbes on the skin prior to rinsing that were removed during rinsing. This finding is consistent with previous evidence that microbes in rinse water were distinct from skin microbiome swabs (Lauer et al., 2007). However, the average difference in dissimilarity to tank water microbiomes between pre- and post-rinse frog microbiomes was very small, especially considering that average dissimilarities to tank water microbiomes were close to one for both frog sample groups. Furthermore, the average dissimilarity between pre-rinse frog microbiomes and tank water microbiomes was over five times greater than average dissimilarity between pre- and

post-rinse frog microbiomes. These findings suggest that while microbes shared with the environment are removed by rinsing, those microbes make up only a very small portion of the amphibian skin community. This is supported by the fact that the slight difference in average dissimilarity to tank water observed between pre-rinse and post-rinse frog groups did not result in differences in overall community structure or diversity between pre-rinse and post-rinse frogs. Therefore, if the main interest of a study is to describe the general pattern of microbial community structure, then rinsing has little benefit. However, if the interest is in removing the small percentage of microbes that appear to be loosely associated, then rinsing might help.

Forgoing the pre-sampling rinse protocol would make sampling the microbiome more straightforward, especially facilitating ease of sample collection in the backcountry where the rinse protocol is less feasible. To collect frog skin samples using this protocol at remote field sites, sterile water would need to be carried out or lake water would need to be sterilized at field sites, and sterilized water may still contain contaminant DNA molecules. Considering that frog skin swab samples are very low biomass, adding additional sources of potential contamination may compromise the integrity of sample characterization. In our study, when we did not rinse away microbes from the skin, the microbiome remained distinct from the surrounding environment and differed only slightly from rinsed frogs. Thus, we conclude that the benefit of removing some transient microbes prior to sampling may not outweigh the cost of procuring sterile (or ideally, DNA free) water and conducting the rinsing protocol in the field. Further, if the decision is made to omit the pre-sampling rinse protocol for animals collected in the backcountry, then the rinse protocol should also be omitted in captivity so that sampling protocols remain consistent for comparison between captivity and the wild.

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Figures

Figure 3-1. Within-sample diversity is not affected by rinsing frogs with sterile water and varies between frogs and tank water. Here we depict (A) Observed richness (number of ASVs) and (B) Shannon diversity for samples of each sample type (frog pre-rinse, frog post-rinse, and tank water) using boxplots. *Post hoc* Dunn test results for pairwise comparisons of sample types are indicated (“ns” = not significant; “***” = $p < 0.001$).

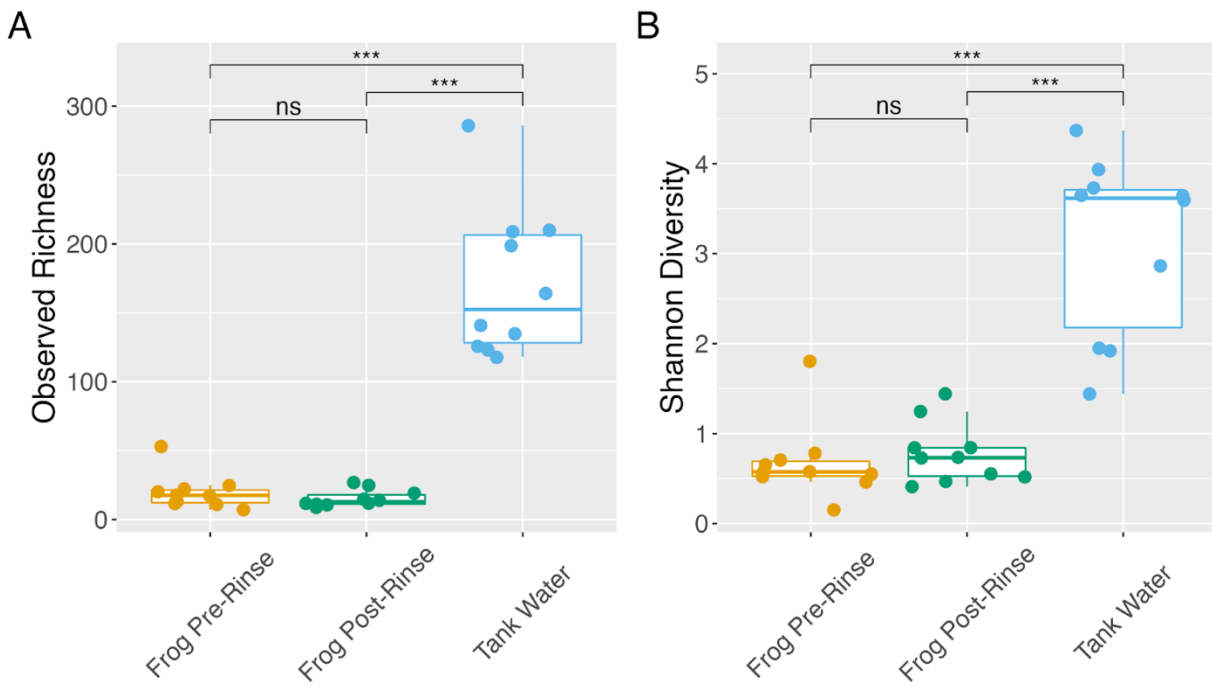
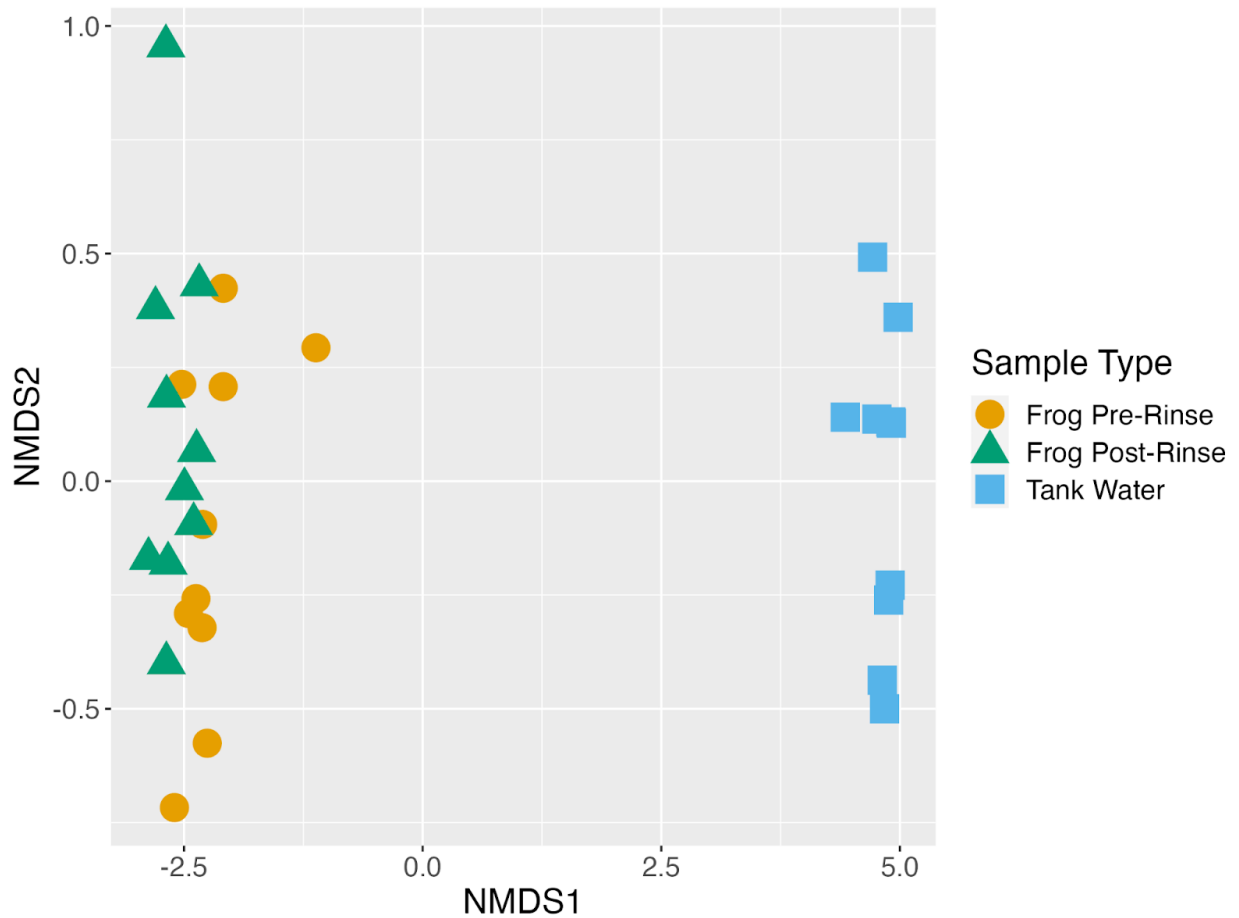


Figure 3-2. Community structure does not differ between unrinsed and rinsed frog samples but is distinct between tank water and frog samples. PERMANOVA, $p=0.19$, $p<0.001$, $p<0.001$, respectively; Non-metric multidimensional scaling (NMDS) visualization of Bray-Curtis dissimilarities of bacterial communities associated with pre-rinse frogs (orange circles), post-rinse frogs (green triangles), and tank water (blue squares).



Supplementary Material

Table S3-1. Does mean unrarefied sample depth differ by sample type? Following significant results of Kruskal-Wallis test (chi-squared=14, df=2, p=0.0009), *post hoc* Dunn test results are shown; P-values are adjusted using Benjamini-Hochberg procedure to control the false discovery rate (FDR) with multiple comparisons.

Dunn Test Comparison	Z	p-value (unadjusted)	p-value (adjusted)
Frog Post-Rinse ↔ Frog Pre-Rinse	-0.13	0.89894	0.89894
Frog Post-Rinse ↔ Tank Water	-3.30	0.00096	0.00288
Frog Pre-Rinse ↔ Tank Water	-3.18	0.00150	0.00225

Table S3-2. Does observed richness differ by sample type? Following significant results of Kruskal-Wallis test ($\chi^2=19.627$, $df=2$, $p=5.471e-05$), *post hoc* Dunn test results are shown; p-values are adjusted using Benjamini-Hochberg procedure to control the false discovery rate (FDR) with multiple comparisons.

Dunn Test Comparison	Z	p-value (unadjusted)	p-value (adjusted)
Frog Post-Rinse ↔ Frog Pre-Rinse	-0.48	.62904	0.62904
Frog Post-Rinse ↔ Tank Water	-4.06	.00005	0.00015
Frog Pre-Rinse ↔ Tank Water	-3.57	.00035	0.00053

TableS3-3. Does Shannon diversity differ by sample type? Following significant results of Kruskal-Wallis test (chi-squared=18.901, df=2, p=7.866e-05), *post hoc* Dunn test results are shown; p-values are adjusted using Benjamini-Hochberg procedure to control the false discovery rate (FDR) with multiple comparisons.

Dunn Test Comparison	Z	p-value (unadjusted)	p-value (adjusted)
Frog Post-Rinse ↔ Frog Pre-Rinse	0.56	0.57630	0.57630
Frog Post-Rinse ↔ Tank Water	-3.45	0.00055	0.00083
Frog Pre-Rinse ↔ Tank Water	-4.01	0.00006	0.00018

Table S3-4. Does community structure differ by sample type? Permutational multivariate analysis of variance (PERMANOVA) model output based on Bray-Curtis dissimilarities; Sample types include pre-rinse frog samples, post-rinse frog samples, and tank water samples.

Factor	df	Sum of squares	Mean squares	F Model	R ²	Pr(>F)
Sample Type	2	5.36	2.68	36.23	0.729	0.0001***
Residuals	27	2.00	0.074		0.271	
Total	29	7.35	7.35		1	

Table S3-5. Which sample types harbor distinct community structure? *Post hoc* pairwise

PERMANOVA model output based on Bray-Curtis dissimilarities.

Combination	Sums of squares	Mean squares	F Model	R ²	p-value	p-value (adjusted)
Frog Post-Rinse ↔ Frog Pre-Rinse	0.032	0.032	1.574	0.080	0.1894	0.18940
Frog Post-Rinse ↔ Tank Water	4.006	4.006	38.622	0.682	0.0001	0.00015
Frog Pre-Rinse ↔ Tank Water	3.995	3.995	40.860	0.694	0.0001	0.00015

Table S3-6. Does community dispersion differ by sample type? Permutation test for homogeneity of multivariate dispersions; model output based on Bray-Curtis dissimilarities.

Factor	df	Sum of squares	Mean squares	F Model	No. Permutations	Pr(>F)
Sample Type	2	0.55870	0.279	41.94	9999	0.0001***
Residuals	27	0.17984	0.007			

Table S3-7. Which sample types differ in community dispersion? *Post hoc* Tukey multiple comparisons of mean dispersions in Bray-Curtis dissimilarities; p-values are adjusted using Benjamini-Hochberg procedure to control the false discovery rate (FDR) with multiple comparisons.

Combination	Difference	Lower 95% CI	Upper 95% CI	p-value (adjusted)
Frog Pre-Rinse ↔ Frog Post-Rinse	-0.045	-0.136	0.045	0.4440599
Tank Water ↔ Frog Post-Rinse	0.264	0.174	0.355	0.0000003
Tank Water ↔ Frog Pre-Rinse	0.309	0.219	0.400	0.0000000

Chapter Four:
Effects of experimental *Batrachochytrium dendrobatidis* inoculation
and itraconazole treatment on the skin microbiome of
Sierra Nevada yellow-legged frogs (*Rana sierrae*)

Abstract

Hundreds of amphibian species are currently threatened by the emerging fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*). Amphibian skin microbiomes have been shown to alter disease dynamics, in some cases promoting host survival. Treatments applied to the skin including *Bd* inoculation and application of antifungal agents have been used in an attempt to mitigate the impacts of *Bd* during reintroduction efforts and in natural populations. However, the effects of these combined management strategies on the entire skin microbiome are largely unknown. Here, we characterized the skin microbiome of captive-reared *Rana sierrae* throughout experimental *Bd* inoculation and subsequent antifungal treatment with itraconazole to assess changes driven by each intervention. We found that impacts of *Bd* and itraconazole to the skin microbiome were greater in magnitude than natural temporal variation alone. Further, we identified changes in microbial diversity, community structure, and normalized relative abundance of several taxa associated with each intervention. We also identified differences among frog microbiomes after itraconazole treatment depending upon whether frogs were exposed to *Bd*, suggesting that legacy effects of *Bd* exposure may persist after antifungal treatment. Considering the importance of the skin microbiome to disease dynamics, effects on the microbiome should be taken into account when planning and executing management strategies. Our results indicate that currently applied

mitigation strategies impact skin microbiomes, which may have implications for long term success of current management practices.

Introduction

Amphibians currently face worldwide declines unprecedented among vertebrates, with 41% of amphibians considered globally threatened (Blaustein and Wake, 1990; Stuart et al., 2004; “The IUCN Red List of Threatened Species,” 2021). The emerging infectious disease chytridiomycosis, caused by the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*), has been implicated as the cause of declines and extinctions for hundreds of species worldwide (Fisher and Garner, 2020; Scheele et al., 2019; Skerratt et al., 2007). Research has focused on possible conservation approaches for susceptible amphibians to mitigate the profoundly negative effects that *Bd* has had on populations across the globe. Several mitigation strategies have focused on treatments applied to the skin of amphibians, since the *Bd* pathogen infects and proliferates in host epidermal cells (Berger et al., 1999; Longcore et al., 1999; van Rooij et al., 2012).

One strategy has been to investigate and manipulate skin-associated microbiota that may interact with *Bd*. Previous work has shown that disease outcome is associated with the composition of the host skin microbiome (Becker et al., 2015; Walke and Belden, 2016). Particular skin microbes have been found to inhibit *Bd* growth (Harris et al., 2009a; Woodhams et al., 2015, 2007), and several studies have utilized this information to test probiotics for frogs with varying success (Becker et al., 2021, 2015, 2011, 2009; Harris et al., 2009a, 2009b; Knapp et al., 2022; Kueneman et al., 2016; Woodhams et al., 2020, 2012). A better understanding of microbial community dynamics on the skin is needed before effective and reliable probiotics can be developed, but it is

clear that the microbiome plays an important role in disease dynamics (Garner et al., 2016; Walke and Belden, 2016).

Other mitigation strategies to manipulate amphibian skin and reduce infections have included (1) inoculation of hosts with *Bd* in captivity in order to prime the host immune system, based on the finding that previous exposure can reduce infection load of subsequent exposures (McMahon et al., 2014), and (2) use of the antifungal drug itraconazole to clear existing *Bd* infections in captivity and in natural populations with the goal of reducing *Bd* prevalence and infection loads (Bosch et al., 2015; Hardy et al., 2015; Knapp et al., 2022). Generally, when these protocols have been implemented, effects on the entire microbiome have not been examined. Considering the importance of the microbiome to surviving disease, an investigation of the impacts of these skin treatments to the amphibian skin microbiome is justified. Whether the microbiome is resistant to disturbance associated with these protocols, and if not, whether there is recovery of the microbiome after such treatments (*i.e.*, microbiome resilience), could impact host survival (Shade et al., 2012). Further, if microbiomes do not recover from disturbance of the pathogen and/or the fungicide, work on probiotic treatments to reverse these effects may be warranted. For example, in mice treated with antibiotics, administration of probiotic bacterial isolates resulted in increased recovery to pre-treatment microbial composition compared to antibiotic-treated mice with no intervention (Shi et al., 2018).

Previous work has shown that the amphibian skin microbiome is not resistant to disturbance by the *Bd* pathogen, evidenced by significant impacts to the microbiome following *Bd* exposure (Jani and Briggs, 2014; Walke et al., 2015). In contrast, the potential impacts of the antifungal drug itraconazole to the skin microbiome have not been well examined. Itraconazole is an azole-derivative compound with a broad spectrum of activity against fungi (Odds, 1993). While it

is applied to amphibians for the purpose of clearing *Bd* infection, it is likely to have nontarget effects on the fungal component of the microbiome. Although most work on amphibian microbiomes have focused on bacteria, there are fungi present within these communities (Alexiev et al., 2023; Bates et al., 2022). It is unclear whether itraconazole will directly impact the bacterial component of the microbiome, however it is likely to have indirect effects, considering that fungi can play a role in regulation of bacteria within microbial communities and vice versa (MacAlpine et al., 2023; Qiu et al., 2015; Sam et al., 2017).

Here, we focus on the impacts of *Bd* inoculation and itraconazole treatment to the bacterial microbiome of Sierra Nevada yellow-legged frogs (*Rana sierrae*) using 16S rRNA gene amplicon sequence data. *R. sierrae* and its sister species, *Rana muscosa*, have both been highly threatened due to invasive fish and *Bd* epizootics (*i.e.*, epidemics in wildlife). These species have gone extinct in >93% of their historic range in the Sierra Nevada mountains, and remaining populations have persisted with the pathogen in an enzootic state (Vredenburg et al., 2010, 2007). Efforts to repopulate extirpated sites have included rearing of frogs in captivity, inoculating with *Bd*, and clearing infections using itraconazole prior to reintroduction (Mountain Yellow-legged Frog Interagency Technical Team, 2018).

We sought to investigate the impacts of *Bd* infection, itraconazole treatment, and the combination of both treatments to the skin microbiome of captive-reared *R. sierrae*. One previous study examined the microbiome throughout an equivalent inoculation regime for *R. muscosa* (Jani et al., 2021), which provides a relevant point of comparison to consider our results in the sister species. While that study focused on microbial response to and recovery from *Bd* infection, we were also interested in describing putative impacts of itraconazole. Our goals were to (1) test whether shifts in the microbiome due to infection and anti-fungal treatment were greater than shifts

due to natural temporal variation, (2) investigate changes to the microbiome during *Bd* infection relative to the pre-treatment state, (3) investigate changes to the microbiome after itraconazole treatment relative to the pre-treatment state, and (4) identify whether the microbiome experiences recovery or legacy effects of *Bd* after itraconazole treatment relative to impacts of itraconazole alone.

Methods

Ethics Statement

Non-invasive sampling of *Rana sierrae* individuals housed at the San Francisco Zoo was conducted with approval from the UC Davis IACUC (Protocol #18732) and the San Francisco Zoo Research Review Committee.

Frog Population and Handling

Frogs used in this study came from a source population of *R. sierrae* in the Sierra Nevada Mountains. More specifically, this source site is located in the Desolation Wilderness (El Dorado County, California), in the Lake Tahoe basin, at about 2500 m elevation. Frogs were collected as egg masses in the summer of 2014 by Roland Knapp and the Mountain Lakes Research Group (Sierra Nevada Aquatic Research Laboratory, Mammoth Lakes, CA) and transported to the San Francisco Zoo, where they hatched approximately one week later and were reared to adulthood over the following year by Jessie Bushell, Director of Conservation at the SF Zoo. Once frogs reached adulthood (snout–vent length (SVL) ≥ 40 mm) individuals were tagged with 8 mm unique passive integrated transponder (PIT) tags, which allow for differentiation among individuals. At the Zoo, adult frogs were housed in tanks (in groups of 8-13 individuals per tank) filled with tap

water purified using biological filters (*i.e.*, filters containing bacteria that break down nitrogenous compounds) and supplemented with Kent Marine R/O Right, a formulation of dissolved solids and electrolytes used to restore natural water chemistry to purified water. Partial water changes (25-50%) were conducted 2-3 times per week, unless a spike in ammonia levels was detected during biweekly tests, warranting changing a higher percent of the water (75%) and/or more frequent water changes. One day before commencing experimental inoculations, frogs were transferred into smaller tank enclosures containing water treated as described above, each housing 5-6 individuals. After initial inoculation, frogs remained in the same water until day 3 when the water was changed. The water was changed again 2 days later (day 5 of infection trial) and then every day after that through the end of the experiment.

Experimental Design

We sampled the microbiome during three phases of the inoculation protocol (*i.e.*, experimental *Bd* infection and anti-fungal treatment) conducted at the San Francisco Zoo by J. Bushell (Figure 4-1): during the pre-treatment phase (prior to any experimental manipulation), the post-infection phase (after *Bd* exposure and prior to anti-fungal treatment), and the post-treatment phase (after anti-fungal treatment). Frogs were randomly assigned to one of two treatment categories: the *Bd*⁺ group (*i.e.*, those exposed to *Bd*) and the *Bd*⁻ group (*i.e.*, those not exposed to *Bd*).

As these frogs are highly endangered, we were limited in terms of how often we could sample and how many animals we could sample. We collected frog samples during the pre-treatment phase from 34 individuals on 2/8/2016 and 4/26/2016 (T1 and T2), which included 13 *Bd*⁻ group frogs and 21 *Bd*⁺ group frogs. Frogs were cohoused across their original tanks during

the pre-treatment phase. On 5/3/2016, all animals were transferred from large tank aquariums to smaller tank enclosures. *Bd*- group frogs were housed in a separate room from *Bd*+ group frogs in order to prevent cross-contamination. On 5/4/2016 (day 1 of infection) *Bd*+ group frogs were exposed to one million zoospore counts (eight days after T2 of the pre-treatment phase). *Bd*+ group animals were exposed to the same zoospore dosage on days 2 and 3 of infection. On day 3, water was changed prior to inoculating with *Bd* zoospores. Water was changed again on day 5, and then every day after until the end of the experiment. The *Bd*- group were exposed to a sham preparation containing no zoospores on each day that the *Bd*+ group were exposed to *Bd* zoospores, and their water was changed on the same schedule as the *Bd*+ group. J. Bushell collected post-infection phase frog samples for us from 20 *Bd*+ group frogs on 5/24/2016 (T3; day 21 of infection). We were unable to obtain samples from the *Bd*- group during the post-infection phase. On 5/28/2016, all frogs (*Bd*+ and *Bd*- groups) were exposed to 0.0025% itraconazole (Sporonox), and this treatment was repeated every 24 hours for 14 consecutive days. The water was changed and tub enclosures were cleaned with a 10% bleach solution prior to applying itraconazole each day. We collected post-treatment phase frog samples from 13 *Bd*- group frogs and 17 *Bd*+ group frogs on 6/20/2016, which was 10 days after the final itraconazole application. There were four fewer *Bd*+ group frogs sampled from the post-treatment phase than we had sampled during the pre-treatment phase because those animals experienced mortality prior to sample collection post-treatment.

Sample Collection

We collected samples for this study at the San Francisco Zoo. We wore nitrile gloves while sampling frogs using sterile synthetic fine tip dry swabs (Medical Wire & Equipment, MW113). From each frog, we collected one swab for microbiome analysis (“microbiome swabs”) and one

swab for assessing *Bd* presence and infection intensity (“*Bd* swabs”). *Bd* swabs involve 30 strokes of ventral surfaces (10 strokes of ventral abdomen, 10 strokes of inner hindlimbs, and 5 strokes on each hindfoot) (“Chytrid Swabbing Protocol,” 2009; Hyatt et al., 2007). Each microbiome swab involves 10 strokes on the back of the frog extending from the snout to vent (5 on each side), 5 strokes on the dorsal side of each hind-leg, 10 strokes of the ventral abdomen extending from the drink patch to the mouth (5 on each side), 5 strokes on each inner hind-limb, and 5 strokes of each of the hind-feet. We chose not to rinse frogs with sterile water prior to collecting microbiome samples based on our previous finding that this practice does not significantly alter the community detected (*see Chapter Three*). We recorded the sex, weight (using a Pesola spring scale), SVL (measured using digital calipers), tank identity, and the individual identity (recording both the PIT tag number and the unique “Tahoe ID” assigned to each frog by the Zoo, *e.g.*, individual Tahoe124 with PIT# 900067000060949) for each frog sampled. We also took note of any abnormalities in the physical appearance of animals during pre-treatment phase time points and at the post-treatment time point. We recorded air and water temperatures. We kept samples on dry ice during collection and transferred them to a -80 °C freezer for storage on the same day.

Molecular Methods

***Bd* samples**

We extracted DNA from *Bd* swab samples using the PrepMan Ultra Sample Preparation Reagent following the manufacturer’s protocol (40 µL used per sample). We then stored DNA extracts at -20 °C or -80 °C. We used a standard quantitative PCR (qPCR) assay with *Bd*-specific primers for ITS1 (Boyle et al., 2004; Hyatt et al., 2007). qPCR assays of DNA extracts were completed in Roland Knapp’s laboratory at the Sierra Nevada Aquatic Research Laboratory

(Mammoth Lakes, California, USA). Plasmid *Bd* standards were used in assays, which are based on single ITS1 PCR amplicons (Joseph and Knapp, 2018; Longo et al., 2013). 5 µL of each 40 µL DNA extract ($\frac{1}{8}$ of the volume) was diluted 1:10 before use in qPCR assays, resulting in a dilution factor of 1/80. We therefore multiplied quantities detected by 80 to determine the number of ITS1 copies in the original DNA extract (Briggs et al., 2010; Vredenburg et al., 2010). It was previously determined from populations of *R. sierrae* in eastern Yosemite National Park (California, USA) that *Bd* in the region has an average of 60 ITS1 copies per zoospore (Joseph and Knapp, 2018). We therefore divided the quantity of ITS1 copies by 60 to convert to zoospore equivalents (ZE). We then log transformed ZE quantities by adding one to each quantity and taking the logarithm to produce units of logZE. Any sample for which we detected logZE greater than zero was considered *Bd*-positive (*i.e.*, *Bd*-infected).

Microbiome samples

We extracted DNA from microbiome swabs using MoBio PowerSoil DNA Isolation Kits or Qiagen DNeasy PowerSoil Kits, using a modified protocol for low-biomass samples discussed with the manufacturer. We described the methods for this protocol previously (*see Chapter Two*). Following DNA extraction, we quantified DNA concentration using a Qubit (Invitrogen, Carlsbad, CA, United States) and the dsDNA High Sensitivity Kit, and stored DNA extracts at -80 °C.

We randomized DNA extracts from microbiome swab samples across 96-well plates and shipped them to the Integrated Microbiome Resource (IMR) at Dalhousie University (Halifax, Nova Scotia, Canada) for 16S rRNA gene library preparation and sequencing. Due to the low-biomass nature of our skin swab samples, IMR performed an additional PCR step prior to their standard library preparation for 16S rRNA gene amplicons. Samples were first amplified using

bacteria-specific primers targeting the hypervariable V3-V4 region of the 16S rRNA gene (341F: CCTACGGGNGGCWGCAG; 805R: GACTACHVGGGTATCTAATCC) (Klindworth et al., 2013). Next, libraries were prepared and sequenced according to the IMR SOP for amplicon sequencing (Comeau et al., 2017; see published wet-lab protocols at <https://www.protocols.io/workspaces/integrated-microbiome-resource-imr/publications>). Briefly, a dual indexing PCR step was performed for each sample in duplicate using “fusion primers” that include Illumina Nextera adaptors, indices, and the V3-V4 primers (341F and 805R) (Klindworth et al., 2013). Duplicate PCRs were combined and verified using a high-throughput 96-well E-gel (Invitrogen). PCR products were cleaned and normalized using the high-throughput SequalPrep 96-well plate kit (Invitrogen). Purified, normalized, and indexed PCR products were pooled into a single library, which was quantified using the Invitrogen Qubit double-stranded DNA high-sensitivity (dsDNA HS) fluorescence-based method. This library contained 118 true samples and 17 controls for the present study. Control samples included three dry swab controls (processed in the same way as true samples), six blank DNA extraction kit controls (for which no sample was added to the PowerBead tube, but otherwise processed in the same way as true samples), and eight PCR negative controls (for which no sample DNA was added to the second PCR step, and subsequent processing was the same as for true samples). The pooled library was sequenced across two runs of an Illumina Miseq (Illumina, Inc., San Diego, CA, USA) using 2 x 300 bp paired-end v3 chemistry. The resulting sequence data was demultiplexed by IMR. We downloaded the sequence read files for downstream analysis.

Microbiome Sequence Processing

We used Cutadapt v. 3.5 (Martin, 2011) with Python v. 3.9.10 (Van Rossum and Drake, 2009) to remove primer sequences from forward and reverse reads separately (specifying $e=0.2$ for forward reads and $e=0.1$ for reverse reads), discarding reads for which primers were not present. The resulting sequences were processed using the DADA2 v.1.22.0 (Callahan et al., 2016) workflow in R v. 4.2.1 with RStudio v. 1.1.419 (Posit Team, 2022; R Core Team, 2022). Our preliminary assessment revealed the low quality of reverse reads, so we proceeded to analyze only the forward reads. Forward reads were trimmed at 250 base pairs, truncated at the first quality score of 2, and removed if the expected errors were greater than 4. After inferring amplicon sequence variants (ASVs) and constructing the sequence tables for each Miseq run separately, we merged sequences from the two runs using the mergeSequenceTables function. We then identified and removed chimeric sequences. We assigned taxonomy to genus level using the Ribosomal Database Project (RDP) naive Bayesian classifier algorithm and the SILVA high quality ribosomal RNA database v. 132, and then made species level assignments based on exact matching of ASVs to reference strains in the SILVA database (Quast et al., 2012; Wang et al., 2007; Yilmaz et al., 2014). We then assigned ASVs unique names beginning with “SV” (sequence variant) followed by a number (*e.g.*, SV1, SV2, etc.).

Next, we used phyloseq v. 1.40.0 (McMurdie and Holmes, 2013) to merge the sequence table, taxa table, and metadata into a phyloseq object for further processing. We filtered out ASVs that had a total read count summed across samples of less than 0.1% of the mean sample read depth (based on the IMR Amplicon SOP: [https://github.com/LangilleLab/microbiome_helper/wiki/Amplicon-SOP-v2-\(qiime2-2020.8\)#41-filter-out-rare-asvs](https://github.com/LangilleLab/microbiome_helper/wiki/Amplicon-SOP-v2-(qiime2-2020.8)#41-filter-out-rare-asvs)). The mean sample depth from the run with higher total depth was

16,828 reads, and we therefore removed ASVs with a sum across samples of less than 17. We then removed ASVs based on taxonomic classification that were (1) non-bacterial at the domain level (including Eukaryota, Archaea, and those unclassified to domain), (2) chloroplasts, and (3) mitochondria. We then removed samples with zero reads remaining after filtering (this removed four of the PCR negative controls). We used Decontam v. 1.14.0 to identify putative contaminants, implementing the prevalence method with a probability threshold of 0.1 and setting the batch argument so that contaminants were identified independently within groups of samples associated with specific negative controls (Davis et al., 2018). We identified contaminants separately for each of the following three control sample groups, using batch as indicated: (1) dry swabs (n=3) using batch by the sample material so as to identify contaminants in swab samples, (2) extraction kit blanks (n=6) using batch by the PowerSoil kit used so as to identify contaminants from each kit separately, and (3) PCR negative controls (n=4 after previous filtering steps) using batch by the sequencing run so as to identify contaminants associated with each run separately. We compiled a list of putative contaminants identified using each control group (166 unique ASVs) and removed them, leaving 13,608 unique ASVs across the 118 true samples and 13 remaining control samples in the dataset.

The sample depth after removing putative contaminants ranged from 1,219 to 42,829 reads for true samples. In order to normalize sample depths across samples, we chose to rarefy the data because rarefaction was previously shown to be more effective than other normalization methods at controlling effects of sample library size when sample depths are very uneven (Schloss, 2024; Weiss et al., 2017), and because we were interested in community level comparisons that can become distorted using other normalization methods (McKnight et al., 2019). We rarefied to the minimum sampling depth for a true sample, which was 1,219, in order to preserve the most

samples. This removed six control samples from the dataset, leaving 125 samples and 1,889 unique ASVs.

We aligned ASVs using DECIPHER v. 2.22.0 (Wright, 2016) and built a maximum likelihood tree with a GTR + $\Gamma(4)$ + I model using phangorn v. 2.8.1 (Schliep et al., 2017; Schliep, 2011) on the UC Davis Bioinformatics Core High Performance Computing Cluster in R v. 4.1.0 (R Core Team, 2022). We midpoint-rooted the tree using phangorn v. 2.9.0. We then merged the rooted tree with the phyloseq object containing the sequence table, taxatable, and metadata. Finally, we removed the remaining seven control samples from the dataset, leaving the 118 true samples with 1,889 unique ASVs.

Data Analysis and Visualization

Analysis of changes in host body mass

To examine changes in frog body mass throughout the experiment, we calculated the proportional weight change for each animal between timepoints using the equation (mass final – mass initial)/(mass initial) (Jani et al., 2021). After confirming that proportional weight changes within groups of individuals were normally distributed (using Shapiro-Wilk tests), we used Welch’s two sample t-tests to test for significant differences in proportional weight change between *Bd*⁺ and *Bd*⁻ group frogs within the pre-treatment phase, and between the pre-treatment and post-treatment phases.

Microbial alpha diversity analysis

To assess within-sample microbial community diversity (*i.e.* alpha diversity), we used the estimate_richness function in phyloseq v. 1.40.0 (McMurdie and Holmes, 2013) to calculate alpha

diversity metrics including observed richness (*i.e.*, the number of ASVs present from the rarefied dataset) and Shannon diversity, which accounts for both the number of ASVs and their frequencies (*i.e.*, evenness). Prior to statistical evaluation of whether metadata groupings of samples harbored significant differences in observed richness and Shannon diversity, we conducted Shapiro-Wilk normality tests for all groups of alpha diversity estimates that we sought to compare. These tests showed that alpha diversity estimates were not normally distributed for at least one sample grouping per comparison of interest (with only one exception where a parametric test could have been applied). We therefore used nonparametric Kruskal-Wallis rank sum tests for all comparisons of diversity estimates among sample groupings, using the `kruskal.test` function in base R v. 4.2.1 (R Core Team, 2022). For significant Kruskal-Wallis results of metadata factors with more than two levels, we implemented *post hoc* Dunn tests using the `dunnTest` function in FSA v. 0.9.3 (Ogle et al., 2022) in order to identify which groups were significantly different, correcting resultant p-values using the Benjamini-Hochberg procedure to control the false discovery rate (FDR) with multiple comparisons.

We first estimated these community diversity metrics for samples within the pre-treatment phase of the experiment to assess whether we could combine datasets for samples across time points (T1 and T2) and across treatment categories (*Bd*⁺ and *Bd*⁻ groups) if there were no differences between these subsets of the data. Second, we estimated the diversity metrics for samples from the post-infection phase (those collected from *Bd*⁺ group frogs on day 21 of *Bd* infection) to assess whether there were differences based on survival, since four *Bd*⁺ group frogs died before the post-treatment phase of the experiment. Third, we estimated community diversity metrics for samples from the post-treatment phase to assess whether treatment category groups were distinct or could be combined. Next, we combined datasets for subsets of samples within

each experimental phase for which distinct groupings were not supported. We refer to the final groups as “inoculation status groups.” Finally, we compared metrics for the inoculation status groups to determine if there were differences in within-sample diversity.

Microbial Beta diversity analysis

To assess between-sample differences in community structure, we used Unweighted Unifrac, a phylogenetically informed distance metric that can be used to evaluate differences in community membership between sample communities (Lozupone and Knight, 2005). We used the ordinate function in phyloseq to calculate these distances for different subsets of the data, and visualized ordinations using principal coordinate analysis (PCoA). To test for significant differences in community structure between metadata groupings of the samples, we first calculated Unifrac distances using the distance function in phyloseq, and then ran permutational multivariate analysis of variance (PERMANOVA) tests using the `adonis2` function in `vegan` v. 2.6.4 with 9999 permutations, specifying the grouping metadata factor and specifying `by = “terms”` to implement a sequential model (Anderson, 2001; Oksanen et al., 2022). For factors that rejected the null hypothesis in these PERMANOVA tests ($p < 0.05$), we performed *post hoc* pairwise PERMANOVA tests to identify which levels within factors differed significantly, using the `adonis.pair` function in `EcolUtils` v. 0.1 with 9999 permutations (Salazar, 2022). We corrected p-values for multiple comparisons using the Benjamini-Hochberg procedure. We also calculated mean dispersion for factors included in PERMANOVA tests and tested for significant differences in dispersion using the `betadisper` and `permutest.betadisper` functions in `vegan`. For significant comparisons ($p < 0.05$), we then implemented *post hoc* Tukey Honest Significant Differences tests to identify which levels within factors differed significantly in their group dispersion using the

TukeyHSD function in base R, which adjusts p-values to control for the family-wise error rate (FWER).

As with alpha diversity, we began by examining whether metadata sample groupings within each experimental phase could be combined, here based on community structure (*i.e.*, Unifrac dissimilarities). If the groupings were not significantly different in terms of community structure, then we combined them in subsequent comparisons. We first examined community structure within the pre-treatment phase to assess whether we could combine datasets for samples across time points (T1 and T2) and/or across treatment categories (*Bd*⁺ group and *Bd*⁻ group). Next, we evaluated community structure within the post-infection phase to assess whether we could combine datasets for samples across survival outcomes (*i.e.*, those that survived to the end of the experiment and those that experienced mortality prior to the post-treatment phase). Finally, we looked at community structure within the post-treatment phase to assess whether we could combine datasets for samples across treatment category groups and *Bd* status groups (*i.e.*, those testing positive or negative for *Bd* within treated frogs). Based on these assessments, (1) we combined datasets for samples across treatment categories from the pre-treatment phase (excluding pre-treatment samples from T1, as they differed in community structure from T2), (2) we combined survival groups from the post-infection phase, and (3) maintained separate groups for treatment categories from the post-treatment phase. These final groupings were our inoculation status groups for community structure. Finally, we compared community structure among inoculation status groups.

We next sought to examine the relative importance of each treatment (*i.e.*, *Bd* inoculation and itraconazole treatment) to explaining variation in community structure across inoculation status groups. We encoded the variable “*Bd*_treatment” with two levels: “no *Bd*,” for all pre-

treatment phase samples and for *Bd*- group samples collected post-treatment, and “*Bd*,” for all *Bd*+ group samples collected during post-infection and post-treatment phases. We also defined the variable “Itraconazole_treatment” with two levels: “no itraconazole” for all pre-treatment and post-infection phase samples (*i.e.*, prior to itraconazole treatment), and “itraconazole” for all post-treatment phase samples. We then compared sequential PERMANOVA models including these treatment factors as well as factors for frog individual identity and sex. If factors were not significant, they were dropped from the model.

Owing to the fact that we obtained microbiome data for the same frog individuals across time points, we quantified the displacement in microbial community structure experienced by individuals throughout the experiment. We utilized Unifrac distances between samples from the same individuals between time points to compare the magnitude of per-individual shifts in community structure. We confirmed that all groups of Unifrac distances that we sought to compare were normally distributed using Shapiro-Wilk normality tests to ensure that parametric statistical tests were appropriate. We then tested for significant differences in the per-individual Unifrac distances between treatment categories and experimental phases by implementing two-tailed t-tests (using the `t.test` function in base R). In cases where we compared displacement in microbial community structure for the same individuals across different time points, we used paired t-tests. In cases where the individuals included in comparison groups were distinct (*e.g.*, when comparing microbial community displacement between *Bd*+ group individuals and *Bd*- group individuals), we used Welch two sample t-tests (*i.e.*, unpaired). In cases where the same subset of the data was compared in multiple t-tests, we corrected resultant p-values using the Benjamini-Hochberg procedure.

Clustering of differentially abundant microbial taxa

Next, we sought to identify groups of ASVs that shared similar patterns of change in normalized relative abundance between the inoculation status groups determined for community structure (these included pre-treatment T2, post-infection, post-treatment *Bd*+, and post-treatment *Bd*- groups). We used DESeq2 v. 1.36.0 (Love et al., 2014) on filtered but un-rarified read counts, implementing the likelihood ratio hypothesis test (LRT) to evaluate changes in abundance across the four sample groups. The likelihood ratio test compares the fit of a model for normalized ASV counts that includes the main effect grouping term (here the inoculation status groups) to the fit of a reduced model without the main effect term for each ASV in the dataset in order to test the null hypothesis that there is no difference in model fit. If the null hypothesis is rejected for an ASV ($p < 0.05$), this indicates that variation in abundance of that ASV is significantly explained by the inoculation status groups.

First, we subset the data to only include frog samples from time points T2-T4, excluding samples from healthy T1 so that differences detected would correspond to treatment groups rather than shifts in natural variation occurring between healthy T1 and T2. We used the `phyloseq_to_deseq2` function to format the raw read count data for DESeq, specifying the inoculation status grouping factor in the design. DESeq normalizes read counts for sample depth using the median of ratios method: sample specific size factors used to normalize raw counts are the median of ratios of per-sample ASV counts to the geometric mean of ASV counts across samples. Our ASV counts table was sparse with only one ASV present across all samples, therefore we calculated geometric means across samples for each ASV by ignoring samples with zero counts so that geometric means and resulting estimated size factors would not be based on only one ASV. Then, we estimated size factors for each sample based on these geometric means (applying the

estimateSizeFactors function). We filtered out low abundance ASVs with 10 or fewer reads (this filtered out 12,475 low abundance ASVs, leaving 1,133 unique ASVs across samples). We then ran the DESeq function specifying both the LRT test and the reduced model without any terms. ASVs with adjusted p-values less than 0.05 were considered to be differentially abundant across the sample groupings.

Next, to identify clusters of significantly differentially abundant ASVs that exhibited shared patterns of change in abundance across inoculation status groups through time, we utilized degPatterns from the DEGreport R package on LRT-identified significant ASVs (Pantano, 2022). We applied a regularized log (rlog) transformation to DESeq normalized counts for those ASVs. Then we implemented the degPatterns function, specifying the inoculation status grouping factor for “time” and the treatment category (*i.e.*, *Bd*⁺ group or *Bd*⁻ group) for “col.” This function first collapsed together the rlog transformed normalized read counts within each time point and treatment group to obtain a mean count. Then the function calculated pairwise correlations of counts to create a distance matrix, which it used to cluster ASVs based on the divisive coefficient of the clustering. This produced clusters of ASVs sharing patterns of change in normalized relative abundance across the time series for *Bd*⁺ group frogs (pre-treatment *Bd*⁺ → post-infection *Bd*⁺ → post-treatment *Bd*⁺) and across the time series for *Bd*⁻ group frogs (pre-treatment *Bd*⁻ → post-treatment *Bd*⁻).

Results

***Bd* Assay**

All animals tested negative for *Bd* during the pre-treatment phase (n = 68). Animals that were experimentally infected with *Bd* tested positive for *Bd* during the post-infection phase time

point ($n = 20$), with infection intensities ranging from 2.98 – 4.21 logZE (mean infection intensity = 3.62 logZE; standard deviation = 0.34 logZE). During the post-treatment phase, all *Bd*- group frogs tested negative for *Bd* ($n = 13$), however 5 out of the 17 surviving *Bd*+ group frogs tested positive for *Bd*. Infection intensities for those 5 animals were very low, ranging from 0.51 – 0.85 logZE (mean infection intensity = 0.69 logZE; standard deviation = 0.15 logZE). The remaining 12 post-treatment *Bd*+ group frogs tested negative for *Bd*.

Observations of Skin Appearance Before and After Treatment

We recorded observations of any abnormalities in the physical appearance of the skin of frogs during the pre-treatment phase (T1 and T2) and during the post-treatment phase (T4) for *Bd*- and *Bd*+ frog groups. We recorded only one observation of a skin abnormality across healthy pre-treatment frogs, which was for one individual sampled at T1 experiencing skin sloughing at the time of sample collection. We observed no skin abnormalities at T2. In contrast, we recorded observations of skin abnormalities including discoloration in most frogs post-treatment, for both *Bd*+ and *Bd*- treatment categories. All frogs appeared paler in their coloration after treatment, with several frogs having red coloration of various parts of the body including the ridges on the dorsal surface, the inner hindlimbs, the forefeet and/or the hindfeet. Images of frogs in Figure 4-2 exemplify the difference in skin coloration between pre-treatment frogs (Figure 4-2, A–C) and itraconazole treated frogs (Figure 4-2, D–H). Both post-treatment *Bd*- frogs (Figure 4-2D, E) and post-treatment *Bd*+ frogs (Figure 4-2, F–H) had less vibrant coloring of ventral surfaces, and reddened skin of the hindlimbs and hindfeet. We also noted the presence of sloughed skin in several of the *Bd*+ frog tanks post-treatment and in one of the four tanks containing *Bd*- frogs post-

treatment. The marked increase in abnormalities across both treatment categories post-treatment suggests that itraconazole application was associated with such abnormalities.

Changes in Host Body Mass

We found that the mean proportional weight change was positive (*i.e.*, on average, mass increased) between pre-treatment phase time points (T1–T2; 0.044 ± 0.017 SE; $n = 34$), but was negative (*i.e.*, on average, mass decreased) between pre-treatment and post-treatment phases (T2–T4; -0.056 ± 0.015 SE; $n = 30$). The proportional weight change of individuals did not differ between *Bd*⁺ and *Bd*⁻ treatment categories across pre-treatment time points (Welch's T-test, $p=0.97$; $n_{Bd^+} = 21$; $n_{Bd^-} = 13$), nor did it differ between treatment categories across pre- and post-treatment phases (Welch's T-test, $p=0.22$; $n_{Bd^+} = 17$; $n_{Bd^-} = 13$). This suggests that negative proportional weight change observed between pre- and post-treatment phases may have been related to itraconazole application.

Alpha Diversity

Within our pre-treatment phase samples there were no differences in either within-sample diversity metric (observed richness and Shannon diversity) between treatment categories (*Bd*⁺ group and *Bd*⁻ group; Figure S4-1C, D; Observed: Kruskal-Wallis, chi-squared = 1.33, $df = 1$, $p=0.25$; Shannon: Kruskal-Wallis, chi-squared = 0.97, $df = 1$, $p = 0.32$) nor between time points (T1 and T2; Figure S4-1A, B; Observed: Kruskal-Wallis, chi-squared = 1.64, $df = 1$, $p = 0.20$; Shannon: Kruskal-Wallis, chi-squared = 1.25, $df = 1$, $p = 0.26$).

Four of the *Bd*⁺ frogs experienced mortality during itraconazole treatment, so we looked within our post-infection phase samples to test whether there were differences in within-sample

diversity between infected frogs that survived and those that died. We found no difference in either diversity metric between these groups (Figure S4-2; Observed: Kruskal-Wallis, chi-squared = 0.44, $df = 1$, $p=0.51$; Shannon: Kruskal-Wallis, chi-squared = 2.89; $df = 1$, $p = 0.09$).

Within post-treatment phase samples, we compared treatment categories, and found no difference in either diversity metric between *Bd*⁺ and *Bd*⁻ groups (Figure S4-3; Observed: Kruskal-Wallis, chi-squared = 1.97, $df = 1$, $p=0.16$; Shannon: Kruskal-Wallis, chi-squared = 0.05, $df = 1$, $p = 0.82$). We also found that within *Bd*⁺ group frogs, there was no difference in either diversity metric based on *Bd* status (*i.e.*, the five samples with low *Bd* loads after treatment did not differ from those whose infections were cleared completely by itraconazole; Observed: Kruskal-Wallis, chi-squared = 1.88, $df = 1$, $p = 0.17$; Shannon: Kruskal-Wallis, chi-squared = 2.18, $df = 1$, $p = 0.14$).

We combined datasets for samples within each experimental phase that did not differ by alpha diversity to compare diversity among “inoculation status groups.” These included pre-treatment, post-infection, and post-treatment groups. We found significant differences among these groups for observed richness (Kruskal-Wallis, chi-squared = 9.63, $df = 2$, $p < 0.01$) and Shannon diversity (Kruskal-Wallis, chi-squared = 20.19, $p < 0.001$). Pre-treatment frogs had significantly higher richness than post-treatment frogs (Dunn, $p < 0.01$; Figure 4-3A; Table S4-1) but significantly lower Shannon diversity than both post-infection and post-treatment frogs (Dunn, $p < 0.001$ and $p < 0.01$, respectively; Figure 4-3B; Table S4-1). This suggests that both infection and itraconazole treatment were associated with reduced Shannon, but only itraconazole treatment was associated with reduced richness.

Beta Diversity

Within pre-treatment phase samples, we found no significant difference in microbial community structure between treatment categories (*i.e.*, between *Bd*⁺ and *Bd*⁻ groups), allowing us to combine datasets for these groups in subsequent comparisons (PERMANOVA, $p=0.64$; Table S4-2; Figure 4-4A). There was a significant difference, however, between pre-treatment time points (PERMANOVA, $p<0.01$; Table S4-2; Figure 4-4B). We also found that community dispersion (*i.e.*, the amount of variation in microbial community structure) was significantly different between pre-treatment time points, indicating that the difference could be due to community dispersion alone or in combination with differences in group centroids of community structure (betadispersion permutest, $p<0.05$; Table S4-3). Based on the difference detected, we chose to exclude T1 samples from downstream analyses comparing community structure among experimental phases in order to reduce sample variation due to factors other than the applied treatments.

Within post-infection phase samples (*i.e.*, samples from *Bd* infected *Bd*⁺ group frogs), we found that community structure was not different between the 16 frogs that survived through itraconazole treatment and the 4 frogs that died prior to post-treatment sample collection (PERMANOVA, $p=0.18$; Table S4-4; Figure 4-4C). We therefore combined datasets for these survival groups of post-infection phase samples in subsequent comparisons.

Within post-treatment phase samples (*i.e.*, samples from frogs after itraconazole treatment), we found significant differences between treatment categories (*Bd*⁺ and *Bd*⁻ groups; PERMANOVA, $p < 0.001$; Table S4-5; Figure 4-4D). We also found that the post-treatment *Bd*⁺ group frogs had significantly greater community dispersion (*i.e.*, variation in microbial community structure) than *Bd*⁻ group frogs (betadispersion permutest, $p<0.01$; Table S4-6). Given that these

groups appeared to cluster separately in ordination (Figure 4-4D), it is likely that both dispersion and centroids of community structure were distinct between groups. As post-treatment *Bd*⁺ and *Bd*⁻ group samples were significantly different, we defined them as separate inoculation status groups in subsequent analyses.

We also examined whether differences in *Bd* status (*i.e.*, testing positive or negative for *Bd*) among post-treatment phase samples corresponded to significant differences in microbial community structure. We compared the 5 *Bd*-positive post-treatment *Bd*⁺ group frogs to the 12 *Bd*-negative post-treatment *Bd*⁺ group frogs and the 13 post-treatment *Bd*⁻ group frogs. We found that there were significant differences in community structure (PERMANOVA, $p < 0.01$; Table S4-5), but not between *Bd*-positive and *Bd*-negative *Bd*⁺ group frogs in pairwise comparisons (PERMANOVA, $p = 0.40$; Table S4-7; Figure 4-4D). Both *Bd* status groups of post-treatment *Bd*⁺ frogs were still significantly different from post-treatment *Bd*⁻ frogs in pairwise comparisons (PERMANOVA, $p < 0.01$; Table S4-7; Figure 4-4D), and there were no differences in group dispersions (betadispersion permutest, $p = 0.08$; Table S4-6). Because community structure did not differ according to *Bd* status within post-treatment *Bd*⁺ group frogs, we combined datasets for these *Bd* status groups in subsequent comparisons.

Based on the results described above, we formed our final inoculation status groups for comparison (*i.e.*, pre-treatment T2, post-infection, post-treatment *Bd*⁺, and post-treatment *Bd*⁻ groups). We found that these inoculation status groupings explained 29.2% of variation in Unifrac distances among samples (*i.e.*, variation in community structure; PERMANOVA, $p < 0.001$; Figure 4-4E; Table S4-8). All inoculation status groups were significantly different in terms of community structure in pairwise comparisons (PERMANOVA, $p < 0.001$; Table S4-9).

We confirmed that centroid locations of community structure differed significantly between post-infection frog samples and both post-treatment frog sample groups based on finding no difference in community dispersion between post-infection and *Bd*⁺ or *Bd*⁻ post-treatment groups (TukeyHSD, $p=0.18$ and $p=0.09$, respectively; Table S4-11). There were also cases, however, where we did detect differences in community dispersion among inoculation status groups (betadispersion permutest, $p<0.001$; Table S4-10). We found that pre-treatment frog samples had higher dispersion than post-infection, post-treatment *Bd*⁻, and post-treatment *Bd*⁺ groups (TukeyHSD, $p<0.001$, $p<0.001$, $p<0.05$, respectively; Table S4-11). The clear separation of clusters in ordination suggests that not only dispersion differed between these groups, but also group centroids (Figure 4-4E). Considering that the number of pre-treatment samples was equal to the combined number of post-treatment *Bd*⁺ and *Bd*⁻ samples, we also checked whether dispersion was greater for pre-treatment samples when post-treatment samples were considered together. We found that pre-treatment frogs still had higher community dispersion than treated frog samples combined (TukeyHSD, $p<0.001$). Finally, as described above, group dispersion was greater for post-treatment *Bd*⁺ frogs than for post-treatment *Bd*⁻ frogs, however the centroids of these groups also appeared to differ in ordination space (Figure 4-4D, E). Together, the combination of statistical results and interpretations of ordination visualizations described above suggest that all inoculation status groups differed in centroid locations of community structure and that some also harbored differences in community dispersion.

Sequential PERMANOVA models revealed the amount of variation explained by treatment factors (*Bd* treatment and itraconazole treatment) across samples from inoculation status groups determined by community structure. Both individual identity and sex were dropped from the model because they did not explain a significant amount of variation even when included as the first terms

in the model. *Bd* treatment, itraconazole treatment, and the interaction of the two were all significant regardless of the order of terms. The combined variation explained by these factors and their interaction term was 29.2%, which is equal to the variation explained by the inoculation status groups examined above. When *Bd* treatment was included as the first term, it explained 12.7% of variation in community structure, while itraconazole explained 8.2%. When itraconazole treatment was included as the first term, the percent variation explained by this factor was higher at 9.3% while *Bd* treatment explained 11.5%. The interaction of *Bd* treatment and itraconazole treatment explained 8.3% of variation in community structure in both models (Table S4-12).

Because we found that community structure of pre-treatment frogs changed over time (T1–T2, 78 days), we utilized per-individual dissimilarities between pre-treatment time points to assess whether the amount of community displacement experienced by individuals due to natural temporal variation was smaller in magnitude than displacement due to treatments for the same individuals. For *Bd*- group frogs (n=13), we found that the per-individual distances between pre- and post-treatment phases (T2–T4, 55 days) were significantly greater than the distances between pre-treatment timepoints (paired T-test, $p < 0.01$, mean difference = 0.078, 0.035–0.12 95% CI). For *Bd*+ group frogs, we examined those individuals for which we had post-infection time point data (n=20 *Bd*+ group frogs), and found that the per-individual distances between pre-treatment and post-infection time points (T2–T3, 28 days) were significantly greater than the distances between pre-treatment time points (paired T-test, $p < 0.001$, mean difference = 0.14, 0.10–0.17 95% CI). We also compared the per-individual community displacement for *Bd*+ group frogs between pre- and post-treatment phases (T2–T4, 55 days; n=17) to the displacement experienced by those individuals between pre-treatment phase time points, and found that the former was significantly greater (paired T-test, $p < 0.001$, mean difference = 0.10, 0.07–0.14 95% CI). In summary, these

tests show that the magnitude of community displacement experienced by frog individuals between pre-treatment phase time points was always smaller than the community displacement experienced by those individuals between pre-treatment and post-infection or post-treatment time points, for both *Bd*⁺ and *Bd*⁻ groups. This is true despite the fact that the number of days between pre-treatment time points (78 days) was greater than between pre-treatment and post-infection (28 days), and between pre-treatment and post-treatment (55 days).

To assess whether itraconazole treatment alone caused a different impact than *Bd* infection plus treatment, we compared the magnitude of microbial community displacement for *Bd*⁺ and *Bd*⁻ groups post-treatment, utilizing per-individual dissimilarities between time points. We compared per-individual dissimilarities for *Bd*⁻ group animals (n=13) and for *Bd*⁺ group animals (n=17) between pre-treatment T2 and post-treatment T4, and found no difference in the magnitude of community displacement between these treatment categories (Welch's T-test, p=0.66). While the magnitude of community displacement of these groups relative to the pre-treatment state is the same, the community structure of these groups differs (*i.e.*, the actual shifts in the community differ between post-infection *Bd*⁻ and *Bd*⁺ groups; Figure 4-4D).

Next, we compared the magnitude of microbial community displacement between pre-treatment, post-infection, and post-treatment phases for *Bd*⁺ group frogs. We subset the data to only include dissimilarities for individuals sampled at both post-infection and post-treatment time points (n=16) to conduct paired tests for the same individuals across experimental phases. We first compared per-individual dissimilarities to pre-treatment T2 between (1) post-infection T3 samples and (2) post-treatment T4 samples. We found that the magnitude of per-individual displacement did not differ (paired T-test, p=0.18). While the degree that communities were displaced from the pre-treatment state was equal for post-infection and post-treatment *Bd*⁺ frogs, as stated above, the

actual changes in community structure differed between these groups (Figure 4-4E; Table S4-9). Second, we compared per-individual dissimilarities of *Bd*⁺ group frogs between (1) pre-treatment T2 to post-infection T3, and (2) post-infection T3 to post-treatment T4. We found that the community displacement was significantly greater between pre-treatment and post-infection than between post-infection and post-treatment (paired T-test, $p < 0.001$, mean difference = 0.11, 0.07–0.16 95% CI).

Clustering of Differentially Abundant Taxa

Likelihood ratio tests of DESeq2 normalized read counts across 1,889 ASVs identified 40 unique ASVs that differed in relative abundance among inoculation status groups determined based on community structure (*i.e.*, pre-treatment T2, post-infection, post-treatment *Bd*⁺, and post-treatment *Bd*⁻ groups; Table S4-13). These ASVs formed six groups that primarily exhibited shared patterns in abundance across *Bd*⁺ and *Bd*⁻ time series (Figure 4-5; Table 4-1). Because the method used to generate these groups involved hierarchical clustering based on a distance matrix of pairwise correlations of abundances, the cut-offs used can sometimes form groups for which all ASVs do not exhibit the exact same behavior. For example, ASVs could fall within a cluster based on shared behavior between certain time points but not others. We note, therefore, that seven of the ASVs within groups 3 and one ASV within group 5 did not follow the group's pattern for *Bd*⁺ frogs post-infection (these are indicated with an asterisk in Table 4-1). While most ASVs in these groups either declined or did not change between pre-treatment and post-infection phases for the *Bd*⁺ group, these eight ASVs in groups 3 and 5 increased during infection (Figure 4-5; Table 4-1), which we describe further below.

We identified 22 ASVs that were negatively associated with both post-infection and post-treatment phases. These ASVs decreased from pre-treatment relative abundance at post-infection and post-treatment phases across both treatment categories, and included both ASVs in group 1 and 20 ASVs from group 3 (Table 4-1). We identified ten ASVs that were positively associated with *Bd* infection, increasing only in post-infection *Bd*⁺ frogs before decreasing after treatment. These included both ASVs in group 4, seven ASVs from group 3, and one ASV from group 5 (Table 4-1). Five of these ASVs from group 3 maintained low relative abundance across all frogs pre-treatment, did not change in abundance for *Bd*⁻ frogs after treatment, and decreased to pre-treatment levels in *Bd*⁺ frogs after itraconazole treatment, indicating that they were impacted by *Bd* and recovered after *Bd* was cleared (these are indicated with a dagger symbol in Table 4-1). We also identified seven ASVs that displayed different behavior between treatment categories post-treatment: two ASVs increased in the post-treatment phase for *Bd*⁺ frogs only (group 2), while six ASVs increased in the post-treatment phase for *Bd*⁻ frogs only (group 5; one of which, in the genus *Curvibacter*, was also increased during infection but decreased after treatment for *Bd*⁺ frogs; Table 4-1). Finally, we identified one ASV that was positively associated with the post-treatment phase. This ASV increased in normalized relative abundance after itraconazole treatment for both *Bd*⁺ and *Bd*⁻ frogs (group 6; Table 4-1).

These differentially abundant bacterial ASVs spanned 8 phyla and 25 families (Table 4-1). While most bacterial families and genera only appeared in one of the six abundance pattern groups, members of the Pseudomonadaceae (genus *Pseudomonas*) were present in two groups (groups 1 and 2), and members of the Burkholderiaceae were present in four groups (groups 3, 4, 5, and 6) (Figure 4-5; Table 4-1). Within Burkholderiaceae, unclassified members were found in two groups (groups 3 and 4), members of the Comamonas were found in two groups (groups 3 and 5), and the

remaining 5 genera represented were each only found in one group (Hydrogenophaga and Rivibacter in group 3, Acidovorax in group 4, and Curvibacter and Paucibacter in group 5). Patterns of abundance were not necessarily shared among members of the same family, or even among members of the same genus. For example, a member of the genus Comamonas (Family Burkholderiaceae) from group 3 followed a different pattern of abundance across the inoculation status groups compared to a distinct member of the Comamonas in group 5. Similarly, the Pseudomonas bacterium in group 1 followed a different pattern of abundance than the distinct Pseudomonas bacterium in group 2.

Discussion

Our results suggest that impacts of *Bd* and itraconazole to the *Rana sierrae* skin microbiome were greater in magnitude than natural temporal variation. We identified shifts in microbial diversity, community structure, and normalized relative abundance of several microbes associated with *Bd* infection and itraconazole treatment. Further, we found changes in the microbiome unique to frogs that experienced both *Bd* infection and itraconazole treatment, suggesting legacy effects of *Bd* exposure post-treatment.

Limitations of the study

We identified three main limitations to our study that are important to consider when interpreting our results. First, we were limited in the quantity and frequency of microbiome sampling of *R. sierrae*. The original goal of the *Bd* inoculation protocol was to prepare the captive *R. sierrae* population for reintroduction to the wild, and because these frogs are highly endangered, we were not permitted to sample microbiomes from as many frogs as often as would have been

ideal. For example, our study would have benefitted from having samples from the *Bd*- group post-infection to quantify effects of *Bd* in the *Bd*+ group. Instead, we compared microbiomes of *Bd*+ frogs post-infection to the pre-treatment phase to quantify effects of *Bd*. Similarly, in order to quantify impacts of itraconazole, our study would have benefitted from including an additional treatment category that was not exposed to itraconazole, however this was not possible because treatment of all individuals with itraconazole before release into the wild was the standard protocol.

Second, animal enclosure is known to impact the microbiome (Hildebrand et al., 2013), however, controlling for effects of enclosure was not possible in our study. Frogs were cohoused based on their inoculation status group in the post-infection and post-treatment phases, which was necessary to prevent cross-contamination between *Bd*+ and *Bd*- treatment categories. Further, very few sampled frogs were housed in each enclosure, preventing us from controlling for enclosure effects within inoculation status groups.

Finally, the type of tank used to house frogs was different in the pre-treatment phase than in the post-infection and post-treatment phases. While frogs were housed in large tanks containing submerged rocks and perches above water level during the pre-treatment phase, all post-infection and post-treatment frogs were housed in smaller tanks containing only a low level of water. This could have impacted microbial communities, considering that amphibians select for skin microbes from their environment, and changes in housing have been shown to alter the microbiome in other systems (Breen et al., 2019; Walke et al., 2014). Therefore, some of our findings were confounded with the change in tank type. There were also cases, however, for which evidence supported the role of treatment effects.

Putative impacts of applied treatments were greater than natural temporal variation in the skin microbiome

If the microbiome experiences natural variation in community structure through time, then in order to determine whether disturbances have significant effects and to quantify microbial community response, a comparison to the expected range of natural community turnover within the microbiome is needed (Shade et al., 2012). We detected variation in community structure of the *R. sierrae* microbiome over time during our pre-treatment phase, which is consistent with previous findings that amphibian skin microbiomes do experience natural community turnover (Bletz et al., 2017; Ellison et al., 2021).

Next, we sought to assess the impact of *Bd* and itraconazole induced disturbances to microbiomes. As we had neither samples from *Bd*- frogs during the post-infection phase nor post-treatment samples from frogs that did not experience itraconazole treatment, we were unable to compare within post-infection and post-treatment phases to address associated impacts. We therefore compared the magnitude of natural microbiome displacement through time to displacement after treatments to assess the impact of these disturbances. We found that the magnitude of community displacement was always greater between pre-treatment and post-infection or post-treatment phases than was displacement within the pre-treatment phase, even though more time passed within the pre-treatment phase. These results indicate that a significant amount of change to the microbiome was incurred due to *Bd* and itraconazole treatments. However, we note that some portion of this change may have been driven by the change in tank type experienced by frogs between pre-treatment and post-infection phases.

Community structure of the *R. sierrae* skin microbiome differed significantly between the pre-treatment phase, post-infection phase, and post-treatment phase, with significant differences

between *Bd*⁺ and *Bd*⁻ treatment categories within the post-treatment phase. This provides evidence that change in tank type between pre-treatment and post-infection phases cannot account for all of the observed treatment effects, as there were differences among groups of frogs housed in smaller tanks according to experimental phase and treatment category.

We also found that pre-treatment frog microbiomes had significantly higher dispersion than post-infection or post-treatment frog microbiomes, indicating that infection and anti-fungal treatment both appear to reduce variability in microbiome structure. While this reduction in variability may have been driven in part by the change in tank type after the pre-treatment phase, it is also possible that these impacts were related to treatment-induced disturbance. Reduced dispersion in response to disturbance has been hypothesized to be evidence that the microbiome has shifted to an adaptive state since it indicates that the response was directional rather than stochastic (Woodhams et al., 2023). A comparable *Bd* inoculation experiment with *Rana mucosa*, the sister species to *R. sierrae*, also found that pre-treatment microbiome dispersion was significantly greater than for *Bd*⁺ frogs after itraconazole treatment (Jani et al., 2021). However, they did not find that pre-treatment dispersion differed from any other post-infection or post-treatment groups (Jani et al., 2021). This incongruity could indicate that post-infection and *Bd*⁻ post-treatment groups experienced reduced dispersion here due to tank type, however it may indicate that even closely related amphibian species respond differently to infection and treatment disturbances. Further, it could indicate differences in the adaptive ability of the microbiomes of closely related hosts if reduced dispersion in *R. sierrae* microbiomes post-infection were driven by selective shifts in community structure (Woodhams et al., 2023).

We identified several taxa that decreased in normalized relative abundance in all post-infection and post-treatment groups relative to the pre-treatment microbiome, which may relate to

the observed reduction in community dispersion. These included 22 diverse taxa across several bacterial phyla including the Acidobacteria, Actinobacteria, Bacteroidetes, Cyanobacteria, Nitrospirae, Planctomycetes, Proteobacteria, and Verrucomicrobia. One taxon was an undescribed member of the family Rubritaleaceae (Phylum Verrucomicrobia) that we previously found to be a dominant member of the *Rana sierrae* skin microbiome (see Chapter Two). An undescribed Rubritaleaceae was also found to decrease upon *Bd* infection of *R. muscosa*, though in that study it maintained a higher abundance in *Bd*-naïve frogs after itraconazole treatment (Jani et al., 2021). Here, the Rubritaleaceae bacterium also decreased in animals that experienced itraconazole treatment alone. Previous study of *R. sierrae* found some of the same taxa decreased during *Bd* infection as observed here, including a *Pseudomonas* (Phylum Proteobacteria, Family Pseudomonadaceae) and a *Rhodococcus* (Phylum Actinobacteria, Family Norcardiaceae) (Jani and Briggs, 2014), though in our study, these were also negatively impacted in frogs that experienced itraconazole alone.

As all 22 taxa described above were heavily and similarly impacted after *Bd* infection and itraconazole treatment, treatment effects were confounded with tank type effects. It is possible that these taxa were sensitive to any form of disturbance to the skin environment in *R. sierrae*, including change in tank type. It is also possible that they were equally impacted due to some similarity in selective pressure caused by *Bd* and itraconazole induced disturbances to the skin of *R. sierrae*. Considering that itraconazole is a broad-spectrum antifungal drug and recent evidence has shown that *Bd* infection is associated with altered community structure and reduced diversity of amphibian skin-associated fungi (Sun et al., 2023), this similarity could relate to shared impacts to the fungal portion of the microbiome that may indirectly impact associated bacteria. Indeed, several studies have shown that fungi and bacteria interact within host-associated microbiomes to

regulate growth across Kingdoms, and that maintenance of fungal community members may be important to microbial homeostasis and preventing dysbiosis (Kapitan et al., 2019; MacAlpine et al., 2023; Santus et al., 2021). Future work characterizing *R. sierrae* associated fungi, their interactions with associated bacteria, and impacts of infection and anti-fungal treatment across Kingdoms would help to elucidate our understanding of these complex dynamics.

***Bd* infection altered microbiomes relative to the pre-treatment state**

Our results were consistent with previous findings that the amphibian skin microbiome is not resistant to *Bd*-induced disturbance (Jani et al., 2021; Jani and Briggs, 2014; Walke et al., 2015). We found significant impacts to microbiome structure, reduction in community dispersion, and an increase in Shannon diversity in post-infection microbiomes relative to the pre-treatment community. We note, however, that changes coincide with the change in tank type between these experimental phases. However, despite having access to fewer environmental substrates to act as sources of skin microbes, we did not detect a change in observed richness between pre-treatment and post-infection phases, which provides some evidence that tank type may not have had major impacts. Further, community structure differed between post-infection phase frogs and post-treatment frogs also held in small tanks, suggesting that changes in community structure post-infection were driven in part by infection.

Previous studies also found that *Bd* infection did not affect microbiome richness (Jani et al., 2021; Walke et al., 2015), however others found reduced richness in infected amphibians, including in *R. sierrae* (Jani and Briggs, 2014; Muletz-Wolz et al., 2019). The previous study of *R. sierrae* found that Shannon diversity did not differ between infected and uninfected individuals (Jani and Briggs, 2014). We were unable to find reported instances in the literature of increased

Shannon diversity coinciding with *Bd* infection, making our result unique. Further, previous study of wild populations of *R. sierrae* found that infected animals with *Bd* loads greater than 3.0 logZE had significantly lower richness and evenness than uninfected animals and those with lower infection loads (Ellison et al., 2019). We found that itraconazole treated frogs maintaining very low infection intensities did not differ in microbiome structure or diversity compared to uninfected frogs whose infections were cleared, which mirrors these findings from wild *R. sierrae* (Ellison et al., 2019). However, all but one of the frogs at our post-infection timepoint were highly infected, and the frog with lower infection intensity was close to the threshold at 2.98 logZE, yet our results show that these animals harbored communities with higher abundance-weighted diversity than pre-treatment uninfected frogs. It is difficult to explain conceptually why frogs would have higher Shannon diversity upon infection with *Bd*. We do not believe that increased Shannon diversity was related to loss of rare taxa since observed richness did not change between these experimental phases. However, in our study we found that many taxa showed either reduced or increased normalized relative abundance upon *Bd* infection, which may have contributed to increasing community evenness that would boost values of Shannon diversity. Future research comparing diversity in an unchanging environment is needed to confirm our results.

All taxa that decreased in normalized relative abundance during *Bd* infection were equally reduced in itraconazole treated frogs whether they had been infected with *Bd* or not (discussed above). The ten taxa that increased in normalized relative abundance during *Bd* infection were in the phyla Bacteroidetes and Proteobacteria. For the most part, these taxa were reduced after itraconazole treatment for *Bd*⁺ frogs, and either decreased or remained at low abundance in *Bd*⁻ frogs after treatment. These differences observed according to treatment category and experimental phase provide evidence that changes in these taxa were driven by infection and not

by the change in tank type. Within the phylum Bacteroidetes, taxa that increased in normalized relative abundance during *Bd* infection included a bacterium in the genus *Pedobacter* (Family Sphingobacteriaceae) and one in the genus *Chryseobacterium* (Family Weeksellaceae). An unclassified Weeksellaceae previously showed an increased relative abundance with *Bd* infection in *R. muscosa*, though in that case the abundance remained higher after itraconazole treatment for *Bd*⁺ animals than for *Bd*⁻ animals (Jani et al., 2021). Within the Proteobacteria, several taxa that increased with infection were in the family Burkholderiaceae, including members of the genera *Acidovorax*, *Comamonas*, *Curvibacter*, and *Hydrogenophaga*. Both *Acidovorax* and *Curvibacter* were also found to increase with *Bd* infection in a previous study of *R. sierrae* (Jani and Briggs, 2014). The inoculation study for *R. muscosa*, however, found *Acidovorax* to be lower in abundance during infection (Jani et al., 2021), providing further evidence for host species specific differences in the response of associated microbiota.

The fact that bacterial taxa that increased during infection in our study showed lower normalized relative abundance in all other inoculation status groups might indicate that their growth was promoted by *Bd* or that *Bd*-induced depletion of other taxa allowed for their increased normalized relative abundance. We believe the former is more likely, considering that the taxa that decreased during infection were also reduced after treatment, while the taxa that increased during infection did not maintain higher levels after treatment. It is unclear, however, whether growth of these microbes would have been facilitated due to positive interactions with the pathogen or promoted via selection for *Bd*-inhibitory function. For example, many of the taxa that increased with *Bd* were in the family Burkholderiaceae, a group for which some members have been reported to have close beneficial associations with a broad range of fungi (Stopnisek et al., 2016), while other members have been found to inhibit fungal growth, including inhibition of *Bd* growth

(Carrión et al., 2018; Harris et al., 2009a, 2009b; Muletz Wolz et al., 2012). Functional analysis of the specific microbes that increased after *Bd* exposure in our study will be needed to elucidate their interactions with the pathogen.

Itraconazole had shared putative impacts to frog microbiomes across treatment categories

The magnitude of community displacement relative to the pre-treatment phase was the same for both *Bd*⁺ and *Bd*⁻ frogs post-treatment as it was for *Bd*⁺ frogs post-infection. This suggests that microbiomes did not experience recovery after treatment; if there had been some level of recovery, we would have expected to see less community displacement of post-treatment groups relative to the pre-treatment phase compared to displacement of post-infection microbiomes. Jani et al (2021) also found that microbiomes of *R. muscosa* did not recover to the pre-treatment community structure after itraconazole clearance of *Bd* infection. That study did not examine effects of itraconazole treatment alone relative to the pre-treatment state, but our finding that *Bd*-naïve frogs treated with itraconazole experienced the same degree of microbiome displacement as did *Bd*⁺ frogs during infection and after itraconazole treatment supports the idea that itraconazole itself disturbs the community. We note the caveat, however, that these changes were confounded with change in tank type in our study.

We found that skin microbiomes of both *Bd*⁺ and *Bd*⁻ frogs post-treatment harbored significantly lower observed richness and significantly higher Shannon diversity than did pre-treatment frogs, and that they did not differ in either metric compared to post-infection frogs. While it is possible that these changes were driven by change in tank type, considering that post-infection frog microbiomes did not differ in observed richness relative to the pre-treatment phase, it is also possible that itraconazole drove changes in richness observed in post-treatment frogs relative to

pre-treatment. Further, previous work found that fungicidal treatment with another azole derivative, fluconazole, also resulted in increased bacterial Shannon diversity and alterations to bacterial composition that were associated with worsening symptoms in mice with inflammatory bowel disease (Qiu et al., 2015). The authors proposed that reduction of the fungal community allowed proliferation of bacteria whose colonization would usually be limited by fungi (Qiu et al., 2015). Our results support the idea that there are also interactions and dependencies between bacteria and fungi within the amphibian microbiome that were altered by anti-fungal treatment.

We also observed qualitative changes in host condition post-treatment that indicate negative impacts of itraconazole. After itraconazole treatment, frogs appeared paler in color whether they had been exposed to *Bd* or not. Further, we observed sloughed skin in tanks post-treatment. While this may be expected for *Bd*⁺ frogs since skin sloughing is a common response to *Bd* exposure (Berger et al., 1999), we also noted sloughed skin in a tank housing *Bd*⁻ frogs post-treatment, which suggests that the stress of itraconazole treatment may have triggered a sloughing response. In addition, while changes in frog body weight were positive on average during the pre-treatment phase, they were negative on average between pre- and post-treatment phases. Further, the amount of weight loss did not differ between *Bd*⁺ and *Bd*⁻ treatment categories. While it is possible that changing tank type caused the shift from weight gain to weight loss, frogs were fed the same amount in both types of tanks. This suggests that itraconazole may have driven reductions in host weight. These observations are consistent with previous findings that itraconazole can have negative impacts to amphibian hosts, including mortality, skin discoloration/depigmentation, reduced growth, lethargy, and other morbidities (Brannelly et al., 2012; Garner et al., 2009; Loyau et al., 2016; Woodhams et al., 2012). These manifestations of stress to amphibian hosts could affect their immune system and alter the production of skin secretions including anti-microbial peptides

or antibodies, which in turn could affect growth of bacteria on the skin (Woodhams et al., 2023). Therefore, changes to the skin microbiome associated with itraconazole treatment could be related to its negative impacts on the host condition.

We identified a single taxon that increased in normalized relative abundance across both *Bd*⁺ and *Bd*⁻ treatment categories in the post-treatment phase. This was a member of the genus *Aquabacterium* (Phylum Proteobacteria, Family Burkholderiaceae). Considering that abundance of this bacterium was not different between pre-treatment and post-infection phases, the increase observed post-treatment was most likely driven by itraconazole and not tank type. This *Aquabacterium* could have increased post-treatment because of itraconazole-induced impacts to the fungi present that may have previously regulated its growth, impacts to the hosts' ability to regulate skin microbiota, or direct impacts to *Aquabacterium* growth. Further work is needed to determine which mechanism is taking place and would require examining changes in host-produced metabolites and host-associated fungi in addition to the bacteria.

Evidence for legacy effects of *Bd* exposure after itraconazole treatment

We found that a significant amount of variation in microbiome structure across inoculation status groups was explained by an interaction of our categorical variables for *Bd* treatment and itraconazole treatment; after itraconazole treatment, microbiomes were different depending on whether or not frogs had been exposed to *Bd*. This result was not confounded by tank type since all post-treatment frogs were housed in small tanks. Additionally, community structure of *Bd*⁻ and *Bd*⁺ treatment categories differed significantly post-treatment even though microbiome structure of these treatment groups were not different in the pre-treatment phase. These results suggest that there are detectable legacy effects of *Bd* exposure post-treatment. Legacy effects are defined as

indirect effects on current ecological processes that persist after the causal species or causal activity is no longer present (Correa-Garcia et al., 2023; Cuddington, 2011). Such effects of past events have been detected within microbiomes across many systems (Correa-Garcia et al., 2023). With legacy effects, the causal agent altered the community in such a way that it responds differently to future conditions (Cuddington, 2011).

In our study, we found several taxa that appeared to have altered dynamics within the microbiome post-treatment depending on whether they had previously been exposed to *Bd*, further supporting the idea of *Bd*-driven legacy effects. We identified two taxa in the phylum Proteobacteria that increased only in *Bd*⁺ frogs after itraconazole treatment. One was a member of the genus *Crenobacter* (Family Chromobacteriaceae) and the other a member of the genus *Pseudomonas* (Family Pseudomonadaceae). Normalized relative abundance of these taxa were low and did not change between pre-treatment and post-infection phases, and also remained low post-treatment for *Bd*⁻ frogs. They therefore only increased after itraconazole treatment for frogs that had been exposed to *Bd*, indicating that previous *Bd* infection may have predisposed the community to promote higher normalized relative abundance of these taxa after clearing infections with itraconazole. We also identified six taxa in the phylum Proteobacteria that increased only in *Bd*⁻ frogs post-treatment. These included four taxa within the family Burkholderiaceae (a *Curvibacter*, a *Comamonas*, and two *Paucibacter*) and two taxa within the family Caulobacteraceae (a *Brevundimonas* and a *Caulobacter*). While the *Curvibacter* was also increased during infection in *Bd*⁺ frogs, its normalized relative abundance returned to pre-treatment levels for these frogs after itraconazole treatment. The other five taxa maintained low normalized relative abundances during pre-treatment and post-infection phases, and post-infection for *Bd*⁺ frogs, only showing increased normalized relative abundance in *Bd*⁻ frogs post-treatment. Persisting impacts of *Bd*

exposure on communities post-treatment may also explain why these taxa did not increase after treatment in *Bd*⁺ frogs. Further work is needed to elucidate how *Bd* exposure altered the trajectory of microbiomes post-treatment. In particular, it is difficult to explain why *Curvibacter* would have higher normalized relative abundance in both *Bd*⁺ frogs during infection and *Bd*⁻ frogs after itraconazole treatment, but not in *Bd*⁺ frogs after treatment.

Conclusions

We conclude that both *Bd* infection and itraconazole treatment contribute to changes in the *R. sierrae* skin microbiome. Further, we find evidence for legacy effects of *Bd* that persist after itraconazole treatment. Current management practices to protect and restore *R. sierrae* populations implement experimental *Bd* inoculation and itraconazole treatment of captive frogs prior to reintroduction to the wild (Mountain Yellow-legged Frog Interagency Technical Team, 2018). However, despite its limitations, ours is the first study to examine impacts of both infection and itraconazole to the *R. sierrae* skin microbiome. Further, the long-term impacts of this inoculation strategy to the skin microbiome have not been studied. It is possible that such restoration efforts could be improved by incorporating knowledge of the microbiome, considering the importance of the skin microbiome to disease dynamics (Redford et al., 2012; Trevelline et al., 2019; Walke and Belden, 2016; Woodhams et al., 2023). However, research on the functional implications of impacts to the microbiome is needed to determine whether this would enhance host success. Future studies of the impacts of *Bd* and itraconazole to the skin microbiome should investigate the fungal community in addition to the bacteria in order to elucidate the complex dynamics that likely exist across Kingdoms (Kapitan et al., 2019; MacAlpine et al., 2023; Santus et al., 2021). In addition, considering both the negative effects of itraconazole to hosts observed here and elsewhere

(Brannelly et al., 2012; Garner et al., 2009; Loyau et al., 2016; Woodhams et al., 2012) and its putative impacts to skin microbiomes described in this study, more consideration is needed to determine the optimal strategy to clear *Bd* infections.

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Tables and Figures

Table 4-1. Taxa identified to differ in normalized relative abundance across inoculation status groups. Amplicon sequence variants (ASVs) included here were those with significant shifts in DESeq2 normalized read counts across inoculation status groups identified by likelihood ratio tests; Groups were generated with degPatterns and represent clusters of ASVs that exhibit shared behavior (*i.e.*, shifts in rlog transformed DESeq normalized counts) across *Bd*- and *Bd*+ time series; asterisk symbol (*) denotes an ASV that increased in abundance during *Bd* infection (*Bd*+ group) when the majority of ASVs in its respective cluster group decreased (group 3) or did not change (group 5) in abundance during infection; dagger symbol (†) denotes group 3 ASVs that did not change in abundance over the *Bd*- time series while the majority of ASVs in its cluster group decreased in abundance for *Bd*- frogs from pre- to post- treatment.

Group	Phylum	Family	Genus (ASVs)
1	Proteobacteria	Pseudomonadaceae	Pseudomonas (SV145)
	Verrucomicrobia	Rubritaleaceae	Unclassified (SV2)
2	Proteobacteria	Chromobacteriaceae	Crenobacter (SV58)
		Pseudomonadaceae	Pseudomonas (SV206)
3	Acidobacteria	Blastocatellaceae	Unclassified (SV478)
	Actinobacteria	Ilumatobacteraceae	Unclassified (SV1512)
		Nocardiaceae	Rhodococcus (SV340)
		Sporichthyaceae	Candidatus_Planktophila (SV326)
		Chitinophagaceae	Sediminibacterium (SV1320)
	Bacteroidetes	Microscillaceae	Unclassified (SV1244)
		Sphingobacteriaceae	Pedobacter (SV216) *†
		Weeksellaceae	Chryseobacterium (SV142) *†
	Cyanobacteria	Leptolyngbyaceae	Chamaesiphon_PCC-7430 (SV241)
	Nitrospirae	Nitrospiraceae	Nitrospira (SV439)
	Planctomycetes	Unclassified OM190	Unclassified (SV93; SV482)
	Proteobacteria	A0839	Unclassified (SV482)
		Burkholderiaceae	Comamonas (SV975) *†
			Hydrogenophaga (SV467) *
			Rivibacter (SV200)

		Hyphomonadaceae	Unclassified (SV730)
		Methylophilaceae	SWB02 (SV920)
		Moraxellaceae	Methylophilus (SV1069) *†
			Acinetobacter (SV174) *
			Perlucidibaca (SV208)
		Reyranellaceae	Unclassified (SV428)
		Rhizobiales_Incertae_Sedis	Unclassified (SV818)
		Rhodobacteraceae	Pseudorhodobacter (SV276) *†
			Rhodobacter (SV459)
		Xanthobacteraceae	Pseudolabrys (SV609)
		Xanthomonadaceae	Arenimonas (SV228)
4	Proteobacteria	Burkholderiaceae	Acidovorax (SV119)
			Unclassified (SV168)
5	Proteobacteria	Burkholderiaceae	Comamonas (SV377)
			Curvibacter (SV917) *
			Paucibacter (SV152; SV812)
		Caulobacteraceae	Brevundimonas (SV129)
			Caulobacter (SV209)
6	Proteobacteria	Burkholderiaceae	Aquabacterium (SV334)

Figure 4-1. Experimental design and microbiome sample collections. The diagram depicts the experimental design for *Bd* inoculation and itraconazole treatment conducted at the San Francisco Zoo, and stars indicate when microbiome samples were collected; The microbiome was sampled during three phases of the experiment: the pre-treatment phase (blue star; prior to any treatments), the post-infection phase (pink star; after inoculation with either *Bd* or sham inoculum), and the post-treatment phase (orange star; after treatment with itraconazole); Frogs were divided into two treatment categories: the *Bd*⁺ group (individuals exposed to *Bd* after the pre-treatment phase) and the *Bd*⁻ group (individuals exposed to sham inoculum after the pre-treatment phase); During the pre-treatment phase, samples from 34 individuals were collected at two time-points (T1 on 2/8/2016 and T2 on 4/26/2016), and these included 21 *Bd*⁺ and 13 *Bd*⁻ individuals; During the post-infection phase, samples from 20 *Bd*⁺ group individuals were collected on 5/24/2016 (T3; day 21 of *Bd* infection; one of the 21 *Bd*⁺ group frogs from which we collected pre-treatment healthy samples was not sampled during post-infection); we did not collect samples from *Bd*⁻ group animals during the post-infection phase; During the post-treatment phase, samples from 30 individuals were collected on 6/20/2016 (T4), and these included samples from 13 *Bd*⁻ group animals and 17 *Bd*⁺ group animals (four of the 21 *Bd*⁺ group frogs from which we collected pre-treatment samples experienced mortality during itraconazole treatment and were therefore not sampled post-treatment).

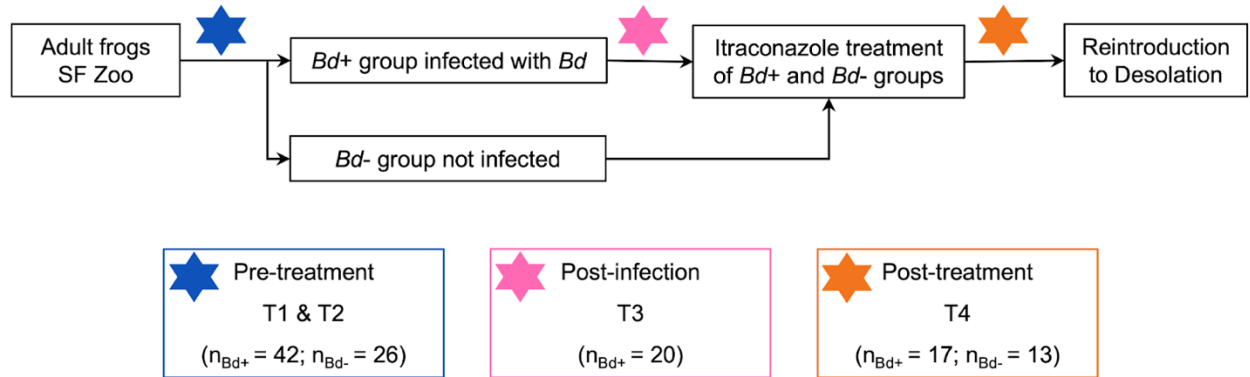


Figure 4-2. Images of *Rana sierrae* at the San Francisco Zoo before and after treatments highlight impacts to skin coloration. (A-C) representative images of pre-treatment frogs taken on 7/28/2015 (photos in B and C taken of the same individual; photo in A taken of a distinct individual); (D-E) representative images of post-treatment *Bd*- group animals taken on 6/20/2016 (2 distinct individuals are shown); (F-H) representative images of post-treatment *Bd*+ group animals taken on 6/20/2016 (3 distinct individuals are shown).

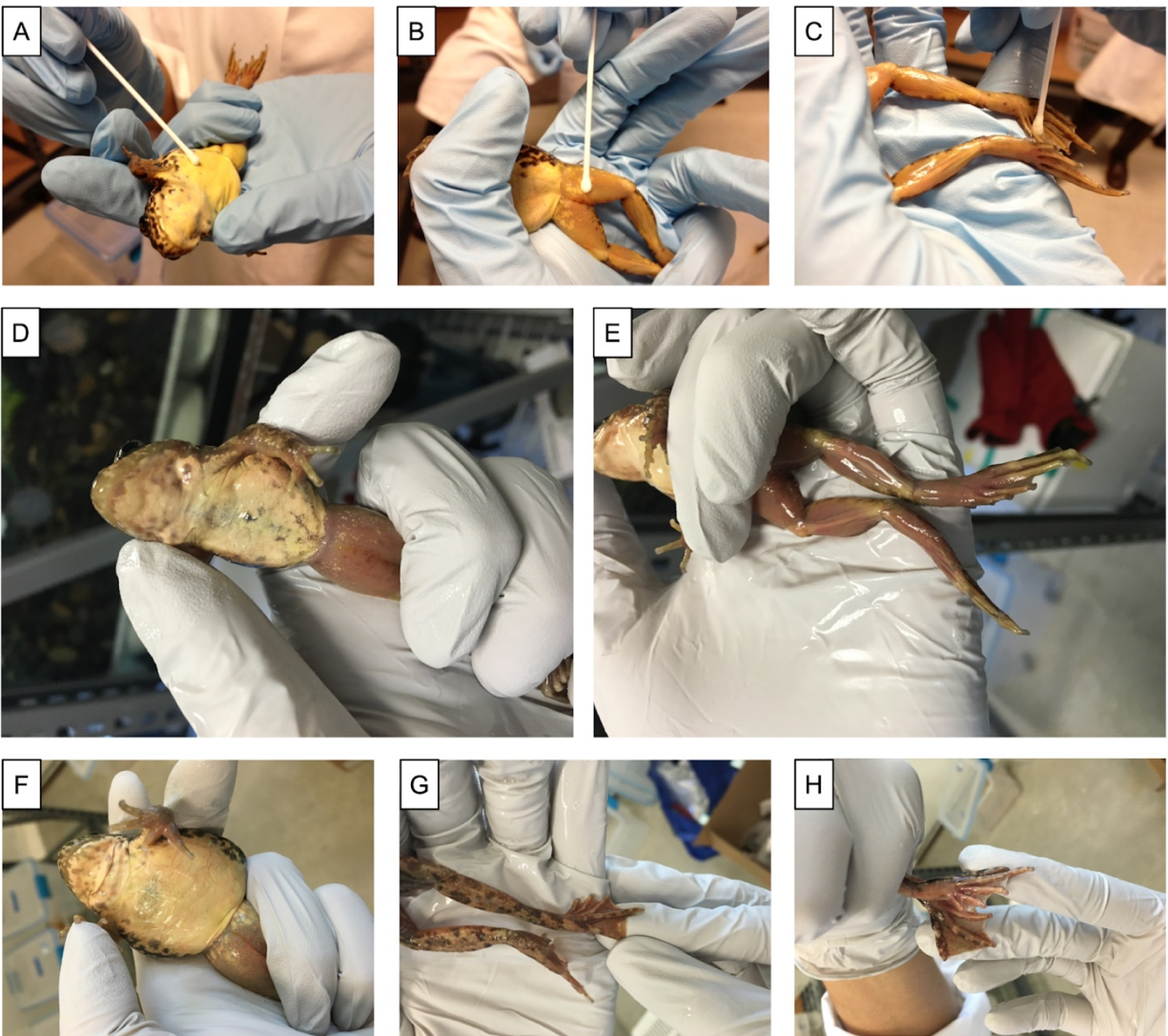


Figure 4-3. Within-sample diversity differs among inoculation status groups. We found significant differences among inoculation status groups in terms of **(A)** Observed richness (Kruskal-Wallis, chi-squared = 9.63, df = 2, $p < 0.01$) and **(B)** Shannon diversity (Kruskal-Wallis, chi-squared = 20.19, $p < 0.001$); **(A-B)** Boxplots and points are grouped and colored by inoculation status group and are shaped by treatment category; *Post hoc* Dunn test results for pairwise comparisons of inoculation status groups are indicated (“ns” = not significant; “***” = $p < 0.01$; “****” = $p < 0.001$).

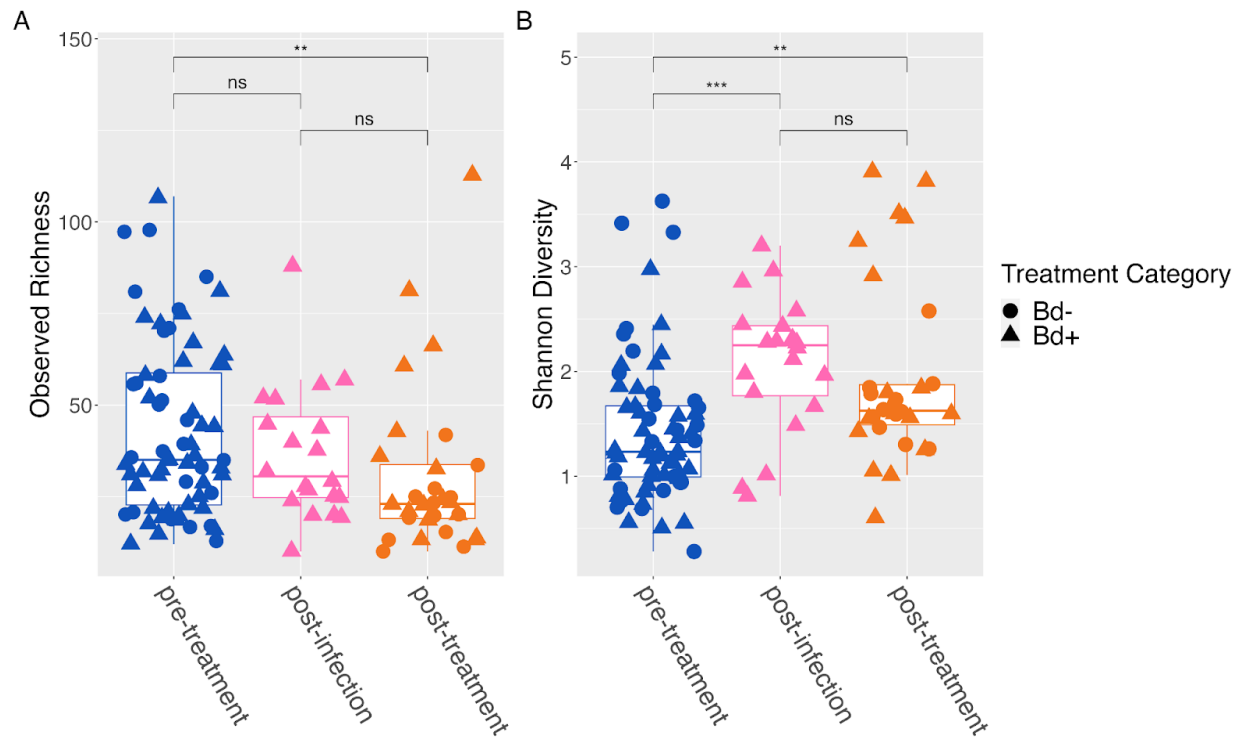


Figure 4-4. Microbial community structure within and among experimental phases. PCoAs of unweighted Unifrac distances are shown; samples collected during the pre-treatment phase are shown in shades of blue, samples collected at the post-infection time point are shown in shades of pink, and samples collected at the post-treatment time point are shown in shades of orange; ellipses represent 95% confidence intervals; **(A-B)** community structure of pre-treatment phase frog samples differed by **(A)** time point (T1 and T2; PERMANOVA, $p < 0.01$, Table S4-2; betadispersion permutest, $p < 0.05$, Table S4-3), but did not differ by **(B)** treatment category (*Bd*⁺ and *Bd*⁻ groups; PERMANOVA, $p = 0.64$; Table S4-2); **(C)** community structure of post-infection phase samples did not differ by survival outcome (PERMANOVA, $p = 0.18$, Table S4-4); **(D)** community structure of post-treatment samples differed by treatment category (*Bd*⁺ and *Bd*⁻ groups; PERMANOVA, $p < 0.001$, Table S4-5; betadispersion permutest, $p < 0.01$, Table S4-6); when post-treatment samples were grouped by treatment category and *Bd* status, pairwise comparisons revealed no significant difference between *Bd*-positive and *Bd*-negative post-treatment *Bd*⁺ samples (PERMANOVA, $p = 0.40$; Table S4-7), and both groups differed from post-treatment *Bd*⁻ samples (PERMANOVA, $p < 0.01$, $p < 0.01$; Table S4-7); **(E)** community structure ordination for inoculation status groups across experimental phases; all inoculation status groups (*i.e.*, pre-treatment T2, post-infection, post-treatment *Bd*⁻ and post-treatment *Bd*⁺ groups) harbored distinct community structure (PERMANOVA, $p < 0.001$; Table S4-9); pre-treatment frog samples had higher dispersion than post-infection, post-treatment *Bd*⁻, and post-treatment *Bd*⁺ samples (TukeyHSD, $p < 0.001$, $p < 0.001$, $p < 0.05$; Table S4-11) and post-treatment *Bd*⁺ samples had higher dispersion than post-treatment *Bd*⁻ samples (TukeyHSD, $p < 0.001$; Table S4-11); even when post-treatment *Bd*⁺ and *Bd*⁻ treatment samples were combined together, treated frogs still maintained lower community dispersion than pre-treatment samples (TukeyHSD, $p < 0.001$).

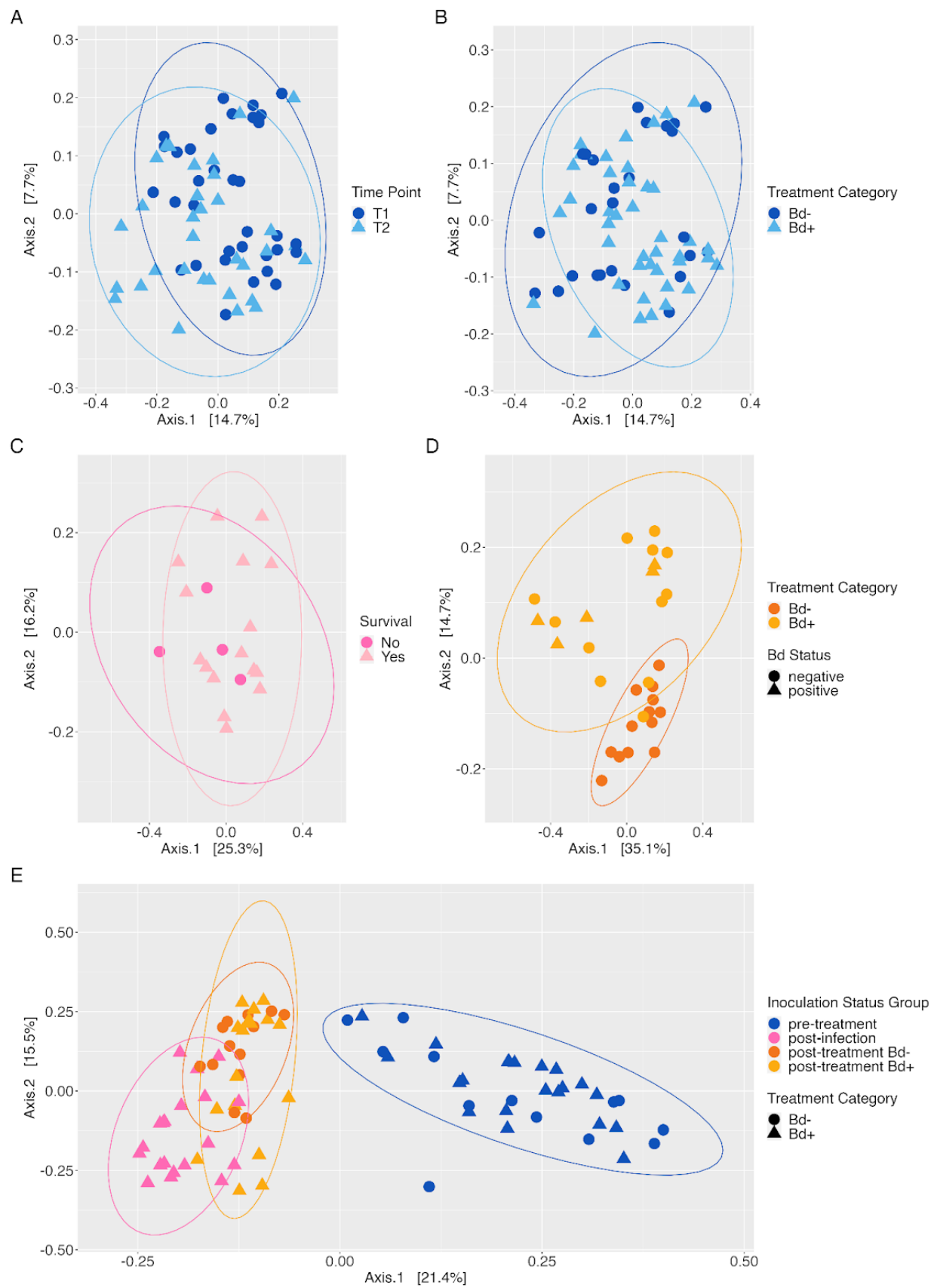
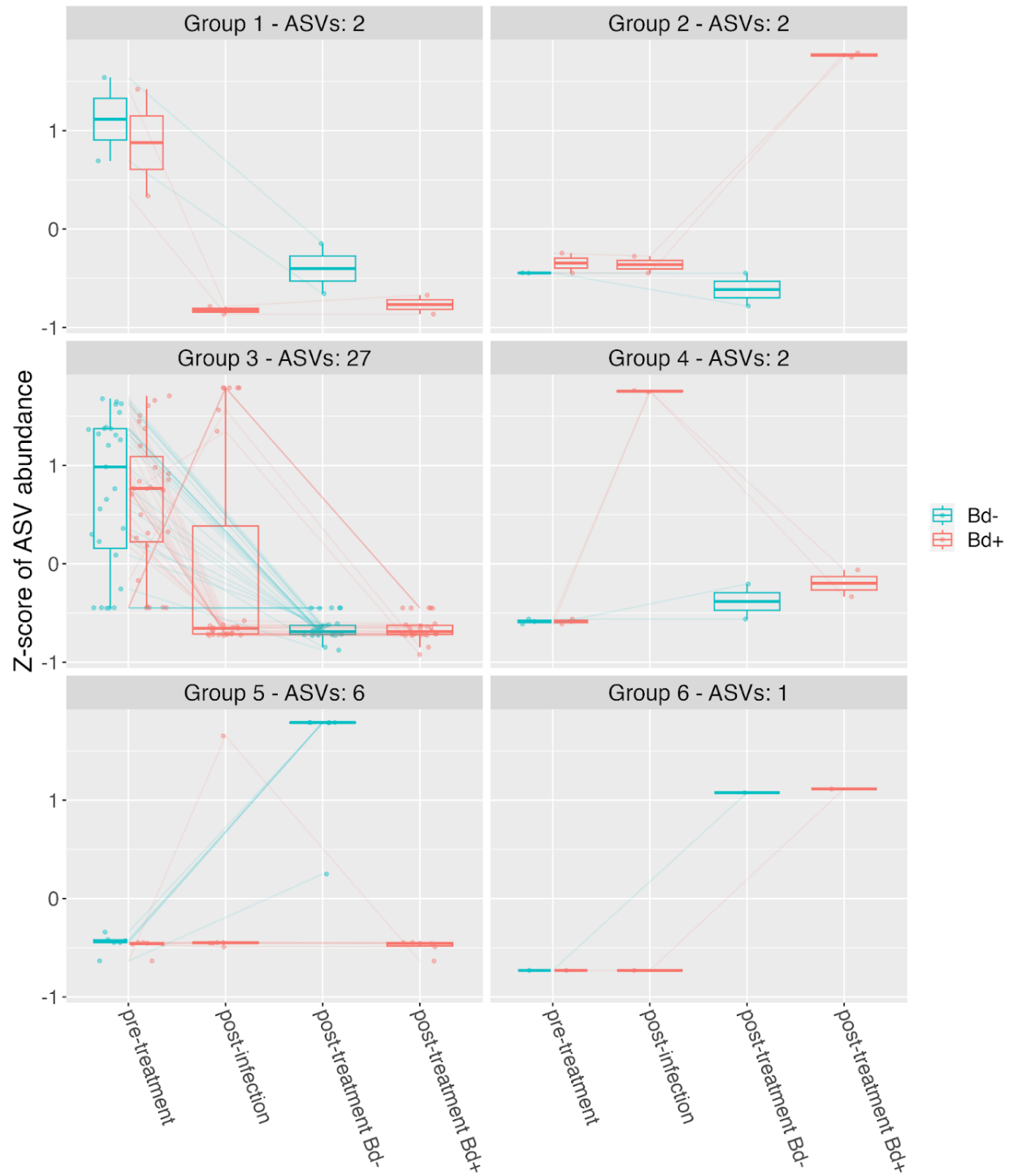


Figure 4-5. Patterns of differential relative abundance for amplicon sequence variants across inoculation status groups. ASVs included here were those with significant shifts in DESeq normalized read counts across inoculation status groups identified by likelihood ratio tests; Facets represent unique clusters of ASVs identified by degPatterns to exhibit shared patterns of rlog transformed DESeq normalized counts across *Bd*- and *Bd*+ time series; points, boxplots, and lines are colored by treatment category (*Bd*- vs. *Bd*+); lines connect each ASV across *Bd*- and *Bd*+ inoculation time series separately.



Supplementary Material

Table S4-1. For which pairwise comparisons of inoculation status groups does alpha diversity differ? Following significant results of Kruskal-Wallis tests (Observed: chi-squared=9.629, df=2, $p<0.001$; Shannon: chi-squared=20.189, df=2, $p<0.01$), results of *post hoc* Dunn tests for significant differences in Observed richness and Shannon diversity in pairwise comparisons of all pre-treatment, post-infection, and post-treatment samples are shown; p-values are adjusted using Benjamini-Hochberg procedure to control the false discovery rate (FDR) with multiple comparisons; for significance, “****” = $p<0.001$, “***” = $p<0.01$, “**” = $p<0.05$, “ns” = not significant.

Metric	Comparison	Z	Unadjusted p-value	Adjusted p-value	Signif.
Observed	pre-treatment ↔ post-infection	0.72	0.46867	0.46867	ns
	pre-treatment ↔ post-treatment	3.10	0.00192	0.00577	**
Shannon	post-infection ↔ post-treatment	1.72	0.08610	0.12915	ns
	pre-treatment ↔ post-infection	-3.93	0.00009	0.00026	****
	pre-treatment ↔ post-treatment	-3.14	0.00169	0.00253	**
	post-infection ↔ post-treatment	1.08	0.28157	0.28157	ns

Table S4-2. Does community structure differ among subgroups of pre-treatment frog samples? Permutational multivariate analysis of variance (PERMANOVA) model outputs based on unweighted Unifrac distances across pre-treatment frog samples are shown; “Treatment Category” groupings include *Bd+* and *Bd-* groups; “Time Point” groupings include T1 and T2; for significance, “***” = $p < 0.001$, “**” = $p < 0.01$, “*” = $p < 0.05$, “ns” = not significant.

Metric	Model No.	Terms	Df	Sum of squares	R²	F model	Pr(>F)	Signif.
Unweighted Unifrac	1	Treatment Category	1	0.144	0.013	0.881	0.635	ns
		Residuals	66	10.816	0.987			
		Total	67	10.960	1			
	2	Time Point	1	0.364	0.033	2.266	0.002	**
		Residuals	66	10.596	0.967			
		Total	67	10.960	1			

Table S4-3. Does community dispersion differ between pre-treatment time points?

Permutation test for homogeneity of multivariate dispersions model output based on Unweighted Unifrac distances across pre-treatment frog samples at T1 and T2 (*i.e.*, time points) is shown; for significance, “****” = $p < 0.001$, “***” = $p < 0.01$, “*” = $p < 0.05$, “ns” = not significant.

Metric	Factor	Df	Sum of squares	Mean squares	F model	No. Perm	Pr(>F)	Significance
Unweighted Unifrac	Time Point	1	0.014997	0.015	7.013	9999	0.0102	*
	Residuals	66	0.141138	0.00214				

Table S4-4. Does community structure differ between survival groups of post-infection frog samples? Permutational multivariate analysis of variance (PERMANOVA) model outputs based on unweighted Unifrac distances across infected frog samples are shown; “Survival” groupings include frogs that survived to the end of the experiment and frogs that experienced mortality during itraconazole treatment; for significance, “****” = $p < 0.001$, “***” = $p < 0.01$, “*” = $p < 0.05$, “ns” = not significant.

Metric	Terms	Df	Sum of squares	R ²	F model	Pr(>F)	Significance
Unweighted Unifrac	Survival	1	0.12948	0.06887	1.3313	0.1762	ns
	Residuals	18	1.75071	0.93113			
	Total	19	1.88019	1			

Table S4-5. Does community structure differ among subgroups of post-treatment frog samples? Permutation multivariate analysis of variance (PERMANOVA) model outputs based on unweighted Unifrac distances across treated frog samples are shown; “Treatment Category” groupings include treated *Bd*+ and *Bd*- groups; “Treatment Category & *Bd* status” groupings include *Bd*-positive post-treatment *Bd*+, *Bd*-negative post-treatment *Bd*+, and post-treatment *Bd*- (all were *Bd*-negative) groups; For significance, “***” = $p < 0.001$, “**” = $p < 0.01$, “*” = $p < 0.05$, “ns” = not significant.

Metric	Model No.	Terms	Df	Sum of squares	R ²	F model	Pr(>F)	Signif.
Unweighted Unifrac	1	Treatment						
		Category	1	0.4679	0.1328	4.2876	0.0008	***
		Residuals	28	3.0555	0.8672			
	2	Total	29	3.5234	1			
		Treatment						
		Category & <i>Bd</i> status	2	0.6001	0.1703	2.7714	0.0030	**
		Residuals	27	2.9232	0.8297			
		Total	29	3.5234	1			

Table S4-6. Does community dispersion differ among subgroups of post-treatment frog samples? Permutation tests for homogeneity of multivariate dispersions are shown; model outputs based on Unweighted Unifrac distances across treated frog samples; “Treatment Category & *Bd* status” groupings include *Bd*-positive post-treatment *Bd*+, *Bd*-negative post-treatment *Bd*+, and post-treatment *Bd*- (all were *Bd*-negative) groups; for significance, “***” = $p < 0.001$, “**” = $p < 0.01$, “*” = $p < 0.05$, “ns” = not significant.

Metric	Model No.	Factor	Df	Sum of Sq.s	Mean Sq.s	F	No. Perm	Pr(>F)	Signif.
Unweighted Unifrac	1	Treatment Category	1	0.0735	0.0735	8.585	9999	0.0077	**
		Residuals	28	0.2396	0.0086				
	2	Treatment Category & <i>Bd</i> status	2	0.0552	0.0276	2.732	9999	0.0799	ns
		Residuals	27	0.2729	0.0101				

Table S4-7. For which pairwise comparisons of post-treatment subgroups does community structure differ? Pairwise permutational multivariate analysis of variance (PERMANOVA) model outputs based on unweighted Unifrac distances across treated frog samples are shown; Model number indicates the corresponding PERMANOVA model from Table S4-5 (comparing “Treatment Category & *Bd* status” subgroups: *Bd*-positive post-treatment *Bd*+, *Bd*-negative post-treatment *Bd*+, and post-treatment *Bd*- frogs); p-values are adjusted using the Benjamini-Hochberg procedure to control the false discovery rate (FDR) with multiple comparisons; for significance, “***” = $p < 0.001$, “**” = $p < 0.01$, “*” = $p < 0.05$, “ns” = not significant.

Metric	Model No.	Combination	Sums of Sqs	Mean Sqs	F	R ²	Unadj. p-value	Adj. p-value	Sig.
Unweighted Unifrac	2	<i>Bd</i> -negative treated <i>Bd</i> + ↔ <i>Bd</i> -positive treated <i>Bd</i> +	0.13	0.13	0.95	0.06	0.397	0.397	ns
		<i>Bd</i> -negative treated <i>Bd</i> + ↔ treated <i>Bd</i> -	0.35	0.35	3.55	0.13	0.001	0.002	**
		<i>Bd</i> -positive treated <i>Bd</i> + ↔ treated <i>Bd</i> -	0.38	0.38	4.18	0.21	0.001	0.002	**

Table S4-8. Does community structure differ among inoculation status groups? Permutational multivariate analysis of variance (PERMANOVA) model outputs based on unweighted Unifrac distances across inoculation status groups including pre-treatment T2, post-infection, post-treatment *Bd*⁺, and post-treatment *Bd*⁻ groups are shown; For significance, “****” = $p < 0.001$, “***” = $p < 0.01$, “*” = $p < 0.05$, “ns” = not significant.

Metric	Terms	Df	Sum of squares	R ²	F model	Pr(>F)	Significance
Unweighted Unifrac	Inoculation status group	3	4.3907	0.292	10.999	0.0001	****
	Residuals	80	10.6445	0.708			
	Total	83	15.0352	1			

Table S4-9. For which pairwise comparisons of inoculation status groups does community structure differ? Pairwise permutational multivariate analysis of variance (PERMANOVA) model outputs based on unweighted Unifrac distances across inoculation status groups including pre-treatment T2, post-infection, post-treatment *Bd*⁺, and post-treatment *Bd*⁻ groups are shown; p-values are adjusted using the Benjamini-Hochberg procedure to control the false discovery rate (FDR) with multiple comparisons; for significance, “***” = p<0.001, “**” = p<0.01, “*” = p<0.05, “ns” = not significant.

Metric	Combination	Sum of Sq.s	Mean Sq.s	F	R ²	p-value	Adjusted p-value	Signif.
Unweighted Unifrac	pre-treatment ↔ post-infection	2.52	2.52	17.25	0.25	0.0001	0.0001	***
	pre-treatment ↔ post-treatment <i>Bd</i> ⁻	1.50	1.5	10.3	0.19	0.0001	0.0001	***
	pre-treatment ↔ post-treatment <i>Bd</i> ⁺	1.59	1.59	9.84	0.17	0.0001	0.0001	***
	post-infection ↔ post-treatment <i>Bd</i> ⁻	0.95	0.95	10.76	0.26	0.0001	0.0001	***
	post-infection ↔ post-treatment <i>Bd</i> ⁺	0.98	0.98	8.34	0.19	0.0001	0.0001	***
	post-treatment <i>Bd</i> ⁻ ↔ post-treatment <i>Bd</i> ⁺	0.47	0.47	4.29	0.13	0.0008	0.0008	***

Table S4-10. Does community dispersion differ among inoculation status groups?

Permutation tests for homogeneity of multivariate dispersions are shown; model outputs based on Unweighted Unifrac distances across inoculation status groups including pre-treatment T2, post-infection, post-treatment *Bd*⁺, and post-treatment *Bd*⁻ groups; for significance, “****” = $p < 0.001$, “***” = $p < 0.01$, “*” = $p < 0.05$, “ns” = not significant.

Metric	Factor	Df	Sum of squares	Mean squares	F model	No. Perm	Pr(>F)	Signif.
Unweighted Unifrac	Inoculation status group	3	0.28096	0.09365	21.81	9999	0.0001	***
	Residuals	80	0.34356	0.00429				

Table S4-11. Which inoculation status groups harbor differences in community dispersion?

Post hoc Tukey multiple comparisons of mean dispersions based on Unweighted Unifrac distances across inoculation status groups including pre-treatment T2, post-infection, post-treatment *Bd+*, and post-treatment *Bd-* groups are shown; p-values are adjusted using Benjamini-Hochberg procedure to control the false discovery rate (FDR) with multiple comparisons; For significance, “***” = $p < 0.001$, “**” = $p < 0.01$, “*” = $p < 0.05$, “ns” = not significant.

Metric	Comparison	Difference	Lower 95% CI	Upper 95% CI	Adjusted P-value	Significance
Unweighted Unifrac	post-infection ↔ pre-treatment	-0.102	-0.150	-0.053	2.44E-06	***
	post-treatment <i>Bd-</i> ↔ pre-treatment	-0.157	-0.214	-0.101	8.24E-10	***
	post-treatment <i>Bd+</i> ↔ pre-treatment	-0.057	-0.108	-0.006	0.021454	*
	post-treatment <i>Bd-</i> ↔ post-infection	-0.056	-0.117	0.006	0.089545	ns
	post-treatment <i>Bd+</i> ↔ post-infection	0.045	-0.012	0.101	0.175413	ns
	post-treatment <i>Bd+</i> ↔ post-treatment <i>Bd-</i>	0.100	0.037	0.163	0.000481	***

Table S4-12. What is the relative importance of treatment factors to variation in community structure? Sequential permutational multivariate analysis of variance (PERMANOVA) model outputs based on Unweighted Unifrac distances across pre-treatment T2, post-infection, and post-treatment samples are shown; “*Bd*_treatment” has levels of “no *Bd*” (for all pre-treatment samples and for *Bd*- samples post-treatment), and “*Bd*” (for *Bd*+ samples post-infection and post-treatment); “Itraconazole_treatment” has levels of “no itraconazole” (for all pre-treatment and post-infection phase samples), and “itraconazole” (for all post-treatment phase samples); for significance, “ns” = not significant, “*” = $p < 0.05$, “**” = $p < 0.01$, “***” = $p < 0.001$.

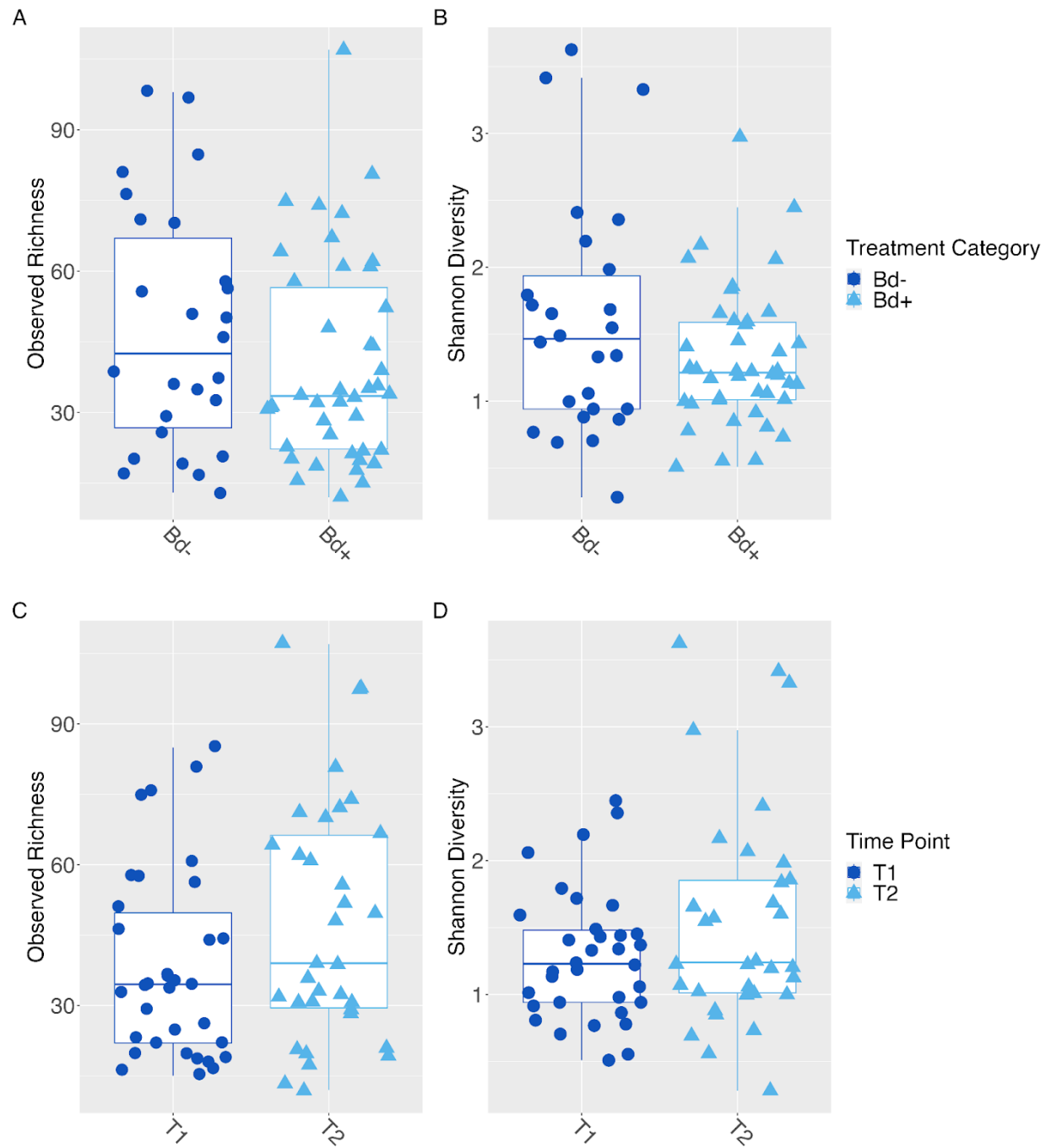
Metric	Model No.	Terms (in order)	Df	Sum of Sq.s	R ²	F	Pr(>F)	Sig.
Unweighted Unifrac	1	<i>Bd</i> _treatment	1	1.92	0.127	14.4	0.0001	***
		Itraconazole_treatment	1	1.23	0.082	9.21	0.0001	***
		<i>Bd</i> _treatment:Itraconazole_treatment	1	1.25	0.083	9.39	0.0001	***
		Residual	80	10.64	0.708			
		Total	83	15.04	1			
	2	Itraconazole_treatment	1	1.41	0.093	10.56	0.0001	***
		<i>Bd</i> _treatment	1	1.74	0.115	13.05	0.0001	***
		<i>Bd</i> _treatment:Itraconazole_treatment	1	1.25	0.083	9.39	0.0001	***
		Residual	80	10.64	0.708			
		Total	83	15.04	1			

Table S4-13. Differentially abundant amplicon sequence variants across inoculation status groups. The 40 ASVs included here were those with significant shifts in DESeq normalized read counts (adjusted p-value < 0.05) across inoculation status groups (*i.e.*, pre-treatment T2, post-infection, post-treatment *Bd*⁺, and post-treatment *Bd*⁻ groups) identified by the likelihood ratio test (LRT) function in DESeq2; DESeq2 adjusts p-values using the Benjamini-Hochberg procedure to control the false discovery rate (FDR) with multiple comparisons.

ASV	base Mean	log2Fold Change	lfcSE	stat	p-value	adjusted p-value
SV2	5692	-4.1968	0.848	94.59	2.26E-20	3.06E-18
SV58	86.702	3.02905	1.384	12.24	0.006593	0.025432
SV93	48.849	-9.9957	1.849	34.22	1.78E-07	2.67E-06
SV119	60.007	22.1679	1.454	33.04	3.16E-07	4.26E-06
SV129	14.705	-1.3551	2.188	18.77	0.000305	0.002572
SV142	71.644	-19.879	2.696	16.38	0.000948	0.006739
SV145	27.688	-24.852	2.097	14.8	0.001997	0.010637
SV152	44.406	-5.9153	2.606	15.81	0.00124	0.008371
SV168	36.902	22.6784	2.239	16.41	0.000933	0.006739
SV174	64.385	-25.883	1.946	13.16	0.004298	0.018963
SV200	43.334	-9.8235	1.479	53.44	1.48E-11	4.98E-10
SV206	16.625	8.31677	2.362	21.7	7.54E-05	0.000783
SV208	57.978	-26.341	2.94	11.64	0.008725	0.031834
SV209	7.3104	-1.1242	2.449	19.1	0.000261	0.002516
SV216	46.048	-1.8184	1.492	64.77	5.63E-14	2.53E-12
SV228	38.338	-9.6466	1.656	41.21	5.91E-09	1.60E-07
SV241	32.884	-9.4254	1.648	40.49	8.38E-09	1.89E-07
SV252	21.544	-26.176	2.558	12.61	0.005573	0.02213
SV276	33.928	-1.1242	1.494	67.04	1.84E-14	1.24E-12
SV326	5.7172	-6.8901	2.223	13.57	0.003558	0.017153
SV334	9.1864	6.6948	2.057	18.9	0.000286	0.002572
SV340	24.021	-25.639	2.57	12.8	0.005093	0.020836
SV377	11.642	-1.1242	3.328	11.36	0.009929	0.035272
SV428	13.352	-8.1254	1.586	36.64	5.48E-08	1.06E-06
SV439	9.4497	-7.6265	2.331	15.47	0.001454	0.008534
SV459	16.607	-8.4401	2.037	22.97	4.09E-05	0.000461
SV467	15.179	-7.0121	2.297	10.91	0.012246	0.042392
SV478	17.986	-8.555	1.686	34.4	1.63E-07	2.67E-06

SV482	7.6831	-7.3282	2.319	14.91	0.001896	0.010637
SV609	7.5851	-7.3089	2.087	18.37	0.000369	0.002928
SV730	6.8563	-7.1641	2.586	11.65	0.008677	0.031834
SV812	9.1223	-1.5514	2.892	13.13	0.004355	0.018963
SV818	7.4298	-7.2795	2.322	14.74	0.002049	0.010637
SV917	9.9203	-1.1242	2.519	13.45	0.003765	0.017525
SV920	5.7197	-6.9035	2.184	15.63	0.001347	0.008415
SV975	6.4989	-1.1242	2.756	12.91	0.004829	0.020371
SV1069	6.875	-1.1242	2.006	25.17	1.42E-05	0.000175
SV1244	5.5643	-6.8631	2.179	15.6	0.001371	0.008415
SV1320	5.4858	-6.8424	2.299	13.95	0.002975	0.014876
SV1512	2.4457	-5.6782	2.357	10.5	0.014758	0.049808

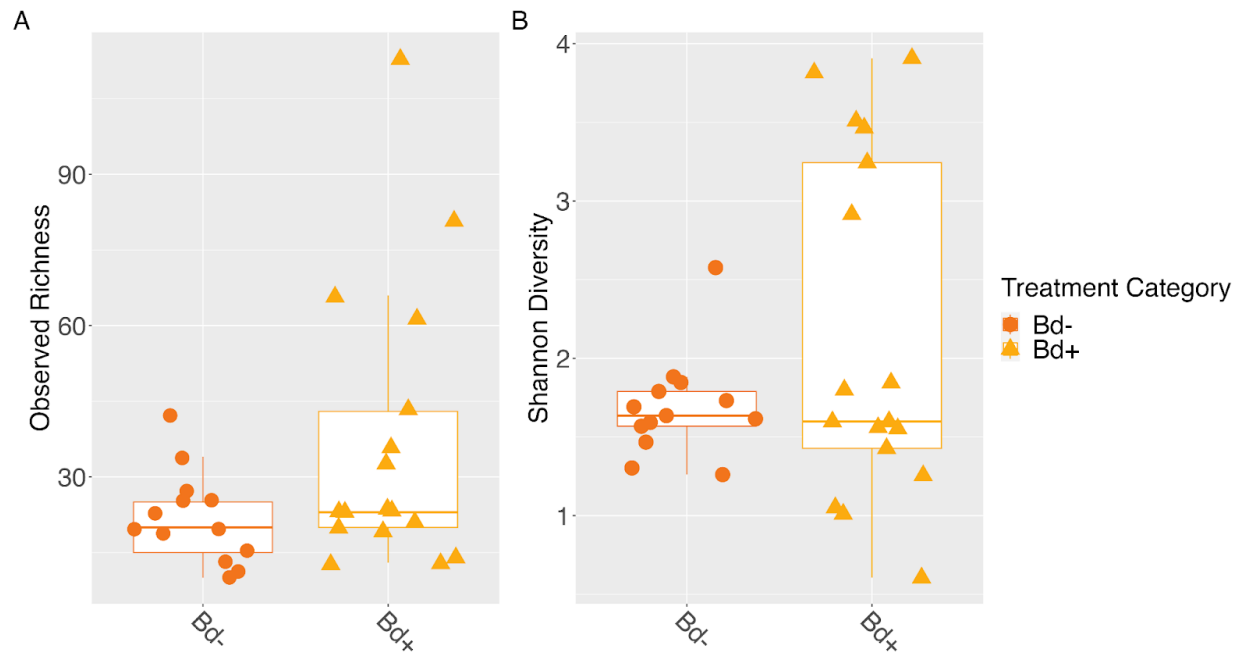
Figure S4-1. Alpha diversity of pre-treatment frog samples does not differ due to treatment category or time point. We found that pre-treatment frog samples did not differ significantly between treatment categories in terms of **(A)** Observed richness (Kruskal-Wallis, chi-squared = 1.33, df = 1, p=0.25) or **(B)** Shannon diversity (Kruskal-Wallis, chi-squared = 0.97, df = 1, p = 0.32); pre-treatment frog samples also did not differ significantly between time points in terms of **(C)** Observed richness (Kruskal-Wallis, chi-squared = 1.64, df = 1, p = 0.20) or **(D)** Shannon diversity (Kruskal-Wallis, chi-squared = 1.25, df = 1, p = 0.26); **(A-B)** Boxplots and points are grouped and colored by the treatment category (*Bd*⁺ vs. *Bd*⁻); **(C-D)** Box plots and points are grouped and colored by the time point (T1 vs. T2).



We found that infected frog samples did not differ significantly based on whether frogs survived or experienced mortality during treatment in terms of **(A)** Observed richness (Kruskal-Wallis, chi-squared = 0.44, df = 1, p=0.51) or **(B)** Shannon diversity (Kruskal-Wallis, chi-squared = 2.89; df = 1, p = 0.09). **(A-B)** Boxplots and points are grouped and colored by survival of frogs through the end of treatment.



Figure S4-3. Within-sample diversity of post-treatment frogs does not differ between treatment categories. We found that treated frog samples did not differ significantly between treatment categories in terms of **(A)** Observed richness (Kruskal-Wallis, chi-squared = 1.97, df = 1, $p=0.16$) or **(B)** Shannon diversity (Kruskal-Wallis, chi-squared = 0.05, df = 1, $p = 0.82$). **(A-B)** Boxplots and points are grouped and colored by the treatment category (*Bd*+ and *Bd*- groups).



Chapter Five:

Characterizing the impacts of restoration efforts on skin microbiomes of

Sierra Nevada yellow-legged frogs (*Rana sierrae*)

across restoration histories and sites

Abstract

In the wake of disease-driven extirpation of amphibian populations around the world, restoration efforts have become vital. Such efforts are particularly important for *Rana sierrae*, a species that is highly susceptible to the emerging fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*). *Bd* has contributed to the extinction of the majority of historic *R. sierrae* populations and remaining populations have managed to persist with *Bd* at high prevalence by enigmatically maintaining sublethal infection intensities. These populations have been used as sources to repopulate sites in the species historic range via direct translocation or via reintroduction after rearing and *Bd* inoculation in captivity. Host-associated microbiomes may play an important role in frog health and *Bd* resistance, and we therefore evaluated possible drivers of differences in microbiomes among captive, natural, and restored site types and sites. We identified some aspects of frog microbiomes that appeared to be primarily associated with factors also shaping environmental microbiomes, and others that were associated with host-related factors. For instance, similarity between frog microbiomes at two restored sites sourced from the same natural population was consistent with a possible role of host genetics. Further, putatively host-driven microbiome features often differed between natural and restored populations. *Bd* loads were generally higher at restored sites than at natural sites, and this was associated with higher

microbiome diversity and lower putative *Bd*-inhibitory microbiome function. We did not, however, find differences between translocated and reintroduced frogs, their infections, or their skin microbiomes for any metric tested. Likewise, *Bd* inoculation treatment did not result in long-term differences in frog infections or microbiomes after reintroduction. Nevertheless, we found that reintroduced frog microbiomes experienced changes greater in magnitude than natural variation years after their release at restored sites. Together, our evidence suggests that management efforts may have contributed to differences in host-pathogen-microbiome relationships observed between natural and restored populations. However, the specific restoration strategies employed did not result in long-term differences within restored populations, suggesting that current environment and host genetics may play a greater role in determining these relationships than the restoration history.

Introduction

The devastating impacts of the emerging fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*) on hundreds of amphibian species globally have been unprecedented by any other known pathogen (Scheele et al., 2019; Skerratt et al., 2007). For some susceptible amphibian species, however, population-level disease outcomes are variable. While certain populations experience *Bd* epizootics (*i.e.*, epidemics in wildlife) that induce population collapse, others enigmatically survive *Bd* invasion, maintaining sublethal infection intensities (*i.e.*, enzootic dynamics) (Brannelly et al., 2021; Briggs et al., 2010; Retallick et al., 2004). Restoration protocols for the highly endangered and federally listed mountain yellow-legged frog species complex (*Rana sierrae*, the Sierra Nevada yellow-legged frog, and its sister species, *Rana muscosa*), have thus been able to use surviving populations as sources to repopulate historically occupied sites (Briggs et al., 2010;

Joseph and Knapp, 2018; Mountain Yellow-legged Frog Interagency Technical Team, 2018; Vredenburg et al., 2007). However, while host genetics, immune response, behavior, population density, pathogen virulence, and microclimate all likely contribute to why some populations persist and others collapse, this phenomenon is still not well understood, which could impact the success of restoration efforts (Berger et al., 2016; A. Ellison et al., 2014; A. R. Ellison et al., 2014; James et al., 2015; Rowley and Alford, 2013; Savage and Zamudio, 2011).

Previous work has revealed that groups of amphibian individuals that survive *Bd* infection harbor microbiomes with distinct community structure compared to conspecific amphibians that experience mortality (Becker et al., 2015a). Further, amphibian-associated microbiome structure has been found to differ between populations experiencing epizootic and enzootic dynamics (Bates et al., 2018; Jani et al., 2017). Therefore, microbiomes may contribute to observed population-level differences in disease dynamics/outcomes. Consequently, differences among microbiomes associated with naturally occurring source populations and restored populations may be relevant to the long-term success of restored frogs. This is especially true considering that *Bd* remains in habitats long after susceptible amphibians have disappeared, and thus that frogs relocated to sites of previous *Bd*-driven extinctions will likely gain new infections (Brannelly et al., 2018; Johnson and Speare, 2003; McMahon et al., 2013).

Two distinct restoration protocols have been employed to repopulate sites with *R. sierrae* using naturally persisting populations as a frog source: (1) reintroductions, for which frogs are reared to adulthood in captivity and inoculated with *Bd* to bolster their immune system in preparation for reinfection upon release (*see Chapter Four*; McMahon et al., 2014; Mountain Yellow-legged Frog Interagency Technical Team, 2018), and (2) translocations, for which adult frogs are directly transferred from source sites to restoration sites (Mountain Yellow-legged Frog

Interagency Technical Team, 2018). We sought to assess the impacts that these restoration efforts may have on host microbiomes and *Bd* infections. In this study, we addressed five main questions, which are summarized below.

Our first question was whether features of frog microbiomes were primarily associated with host-associated factors or with local environmental factors. Frog microbiomes are distinct from local environmental microbiomes; however, their assembly is influenced by environmental conditions and their local aquatic environments in addition to host-related factors like disease dynamics (Estrada et al., 2019; Jani et al., 2017; Jani and Briggs, 2014; Kueneman et al., 2014; Walke et al., 2014). Additionally, we know that lake water microbiomes are highly associated with local environmental conditions (Sperlea et al., 2021). We therefore used a statistical examination of relationships in measures of frog and water microbiomes to try to tease apart possible models for frog microbiome dynamics.

Second, we examined whether *Bd* infections and host factors (including skin microbiomes) were distinct between natural and restored population types. In particular, we assessed frog microbiome features identified to be putatively host driven in analysis relating to question one. If *Bd* infections, host measures, and features of host microbiomes were associated with management efforts, we would expect them to differ between natural and restored frog populations. We hypothesized further that hosts and their microbiomes may exhibit distinct relationships with *Bd* between natural and restored population types.

Third, we investigated whether specific restoration protocols were associated with frog skin microbiomes, *Bd* infections, and other host factors. Recent work showed that amphibian skin microbiomes resembled microbiomes of local wild counterparts within weeks to months after release from captivity or after translocation. (Bletz et al., 2016; Estrada et al., 2022; Korpita et al.,

2023; Kueneman et al., 2022). We therefore hypothesized that relocated populations would differ from source and captive populations. We are unaware of any previous studies that have compared reintroduced and translocated frog microbiomes, however we hypothesized that we would be able to detect differences between these groups as their histories were quite disparate.

Fourth, we addressed whether history of *Bd* inoculation treatment was associated with long-term effects on reintroduced frog microbiomes and *Bd* infections. We previously found that after *Bd* inoculation in captivity, community structure of *Bd* exposed frogs' microbiomes differed from unexposed counterparts (*see Chapter Four*). Further, *Bd* inoculation has been found to result in reduced *Bd* loads (McMahon et al 2014). We therefore hypothesized that we would find long-term differences between treatment groups after reintroduction.

Finally, our fifth question was how microbiomes and *Bd* infections of reintroduced frogs change over time since captivity relative to natural variation over time. Previous work showed that reintroduced frogs' microbiomes experienced shared changes relative to captivity over the course of months (Kueneman et al., 2022), and we hypothesized we would find similar results over a longer time scale. We predicted that the largest changes would occur in the first year since captivity, and that changes would return to natural levels of variation over time in subsequent years.

Our study is the first to examine differences in amphibian skin microbiomes and *Bd* dynamics among several naturally occurring and restored wild populations across multiple restoration strategies and over a multi-year timescale. This work expands our understanding of the putative impacts of management protocols to microbial dynamics.

Methods

Ethics Statement

Capture and non-invasive sampling of wild *Rana sierrae* from populations in the Desolation Wilderness was conducted with approval from the U.S. Fish and Wildlife Service (permit number TE-40090B) and the California Department of Fish and Wildlife (Memorandum of Understanding). We reserved overnight permits issued by the Lake Tahoe Basin Management Unit of El Dorado National Forest for all field collection trips. Sampling of *Rana sierrae* individuals housed at the San Francisco Zoo was conducted with approval from the San Francisco Zoo Research Review Committee. Finally, all wild and Zoo frog sampling was approved by the UC Davis IACUC (protocol number 18732).

Study Populations and Experimental Design

Management efforts for *Rana sierrae* in the Desolation Wilderness were led by Roland Knapp and the Mountain Lakes Research Group (Sierra Nevada Aquatic Research Laboratory, Mammoth Lakes, CA). Frogs sampled for this study included adult individuals (>40mm snout-to-vent length (SVL)) from five wild populations located at mid-high elevations in the Desolation Wilderness (El Dorado County, California; 2360–2587 m), and one captive population reared at the San Francisco Zoo by Jessie Bushell, Director of Conservation (Figure 5-1). Wild frogs included two naturally occurring populations persisting with *Bd* present (Rivendell Pond and Pyramid Lake) and three restored populations at sites where frogs previously experienced *Bd*-driven extinctions prior to management efforts to repopulate (Tamarack Lake, Lake Lucille, and Lake Jabu).

Adult frogs at restored sites were either directly translocated from naturally occurring source populations, reintroduced from the captive Zoo population, or were descendants of translocated/reintroduced frogs born at restored sites (which we refer to as “new recruits”). Captive frog samples included in this study were analyzed previously (*see Chapter Four*; here we reanalyze frog samples from the final time point of previous work). All captive frogs from the Zoo were part of a *Bd* inoculation experiment conducted by J. Bushell. This involved *Bd* infection and antifungal treatment to clear infection using itraconazole, a triazole-derivative antifungal agent that has a broad spectrum of activity against fungi (Odds, 1993). These inoculations were performed in an attempt to prime host immune systems for *Bd* exposure and lower the odds for deadly levels of infection after reintroduction (*see Chapter Four*). Frogs were divided into two inoculation treatment groups: *Bd*⁺ frogs (exposed to *Bd* prior to itraconazole treatment) and *Bd*⁻ frogs (never exposed to *Bd* but treated with itraconazole). Captive Zoo samples included in this study were collected ten days after the final itraconazole application and three weeks before their release to either Lake Tamarack or Lucille. All captive, translocated, and reintroduced frogs were tagged with 8 mm unique passive integrated transponder (PIT) tags, which allow for differentiation among individuals. New recruit frogs were also tagged at restoration sites by R. Knapp and his team when they were encountered. Some frogs at naturally occurring source populations had been previously tagged, however the majority were not tagged (we did not tag frogs at source populations for this study).

R. Knapp and his team released cohorts of translocated frogs at all three restoration sites each summer in 2014, 2015, 2017, and 2018 (159 frogs translocated overall), and released cohorts of reintroduced frogs from the SF Zoo at two of the three restoration sites (Lake Tamarack and Lake Lucille) each summer in 2014, 2015, and 2016 (196 frogs reintroduced overall). All

reintroduced frogs had been reared to adulthood at the Zoo from egg masses or juveniles originally captured at the Rivendell Pond source population, and all were subjected to the *Bd* inoculation protocol prior to release. Therefore, some reintroduced frogs had been part of the *Bd*- inoculation treatment group (unexposed to *Bd*) while others had been part of the *Bd*+ treatment group (exposed to *Bd*) at the Zoo before release. Here, we analyze data we collected from the captive site from 2016, from the natural sites from two collection trips in the summer of 2017 and two trips in the summer of 2018, and from the three restored sites from two trips in the summer of 2016, three trips in the summer of 2017, and three trips in the summer of 2018.

To address our first question concerning frog-associated microbiomes and the relative importance of host and environmental factors, we examined relationships of frog microbiomes to “site types” (*i.e.*, captive, natural, or restored) and sites (*i.e.*, Zoo, Rivendell, Pyramid, Tamarack, Lucille, and Jabu; Figure 5-1B) and compared to relationships of water microbiomes to site types and sites. We did not focus on Zoo frog or water microbiomes for this question because captive frogs were housed in a small volume of water throughout experimental *Bd* inoculation and sample collection. Therefore, captive frog microbiomes may have impacted their local water microbiomes, while differences in management of frogs at wild sites would likely not affect lake water microbiomes.

To address our second question concerning whether relationships among *Bd* infections, skin microbiomes, and other host factors differed between natural and restored frog populations, we considered all wild-captured frog microbiome samples, and examined associations between *Bd* loads and other metrics by site.

For our third question about impacts of different restoration histories on frogs, their microbiomes, and infections, we focused on restored sites harboring both translocated and

reintroduced frogs in addition to new recruits (Lake Tamarack and Lake Lucille restored sites), and the captive and natural sites from which those restored populations originated (*i.e.*, the captive Zoo and Rivendell source populations; Figure 5-1B). For this subset of sites, we compared microbiomes and *Bd* infections of “restoration types,” including captive, source, translocated, reintroduced, and new recruit frog populations within and across sites (Figure 5-1B).

For our fourth question, we compared treatment groups of frogs from experimental *Bd* inoculation after reintroduction to assess long-term effects on microbiomes and *Bd* infections. We compared these long-term observations to differences between treatment groups in captivity (*i.e.*, comparing long term to short term effects).

Finally, to address our fifth question concerning changes in the microbiomes of reintroduced frogs over time since their release to the wild, we focused on reintroduced frog samples according to years since release. We quantified changes over reintroduced frogs’ first year since release relative to captivity, and then between subsequent years since release at restored sites. To determine whether changes observed in reintroduced frogs over time were greater than natural variation over time, we compared to the magnitude of change in various metrics for the Rivendell natural site source frog samples between collection years.

Sample Collection

We wore nitrile gloves during sample collection. We used sterile synthetic fine tip dry swabs (Medical Wire & Equipment, MW113) to collect frog samples. From each frog, we collected one swab for microbiome analysis (“microbiome swabs”) and one swab for assessing *Bd* presence and infection intensity (“*Bd* swabs”). *Bd* swabs were collected via 30 strokes of ventral surfaces (10 strokes of ventral abdomen, 10 strokes of inner hindlimbs, and 5 strokes on each

hindfoot; “Chytrid Swabbing Protocol,” 2009; Hyatt et al., 2007). Microbiome swabs were collected via 10 strokes on the back of the frog extending from the snout to vent (5 on each side), 5 strokes on the dorsal side of each hind-leg, 10 strokes of the ventral abdomen extending from the drink patch to the mouth (5 on each side), 5 strokes on each inner hind-limb, and 5 strokes of each of the hind-feet (dorsally and ventrally). We chose not to rinse frogs with sterile water prior to collecting microbiome samples based on our previous finding that this practice does not significantly alter the microbial community detected (*see Chapter Three*). For each frog, we recorded the sex, weight (using a Pesola spring scale), snout-to-vent length (SVL; measured using digital calipers), and the individual identity for PIT-tagged individuals (*i.e.*, recording the unique 12-digit tag number). We also collected water filter samples from all sites visited on each sampling trip by filling a 60 mL syringe with tank or lake water, passing the water through a 0.22 μ m Sterivex filter (Millipore), and repeating this process four times (total water filtered = 240 mL per sample) as in Ellison et al. (2019). We recorded air and water temperatures, GPS coordinates, and elevation for each sample (coordinates and elevations determined with a Garmin GPS). For field sampling, we disinfected all sampling equipment and footwear by soaking in a 0.01% solution of quaternary ammonia for a minimum of five minutes in between visits to distinct sites (Figure 5-1). We kept frog swab and water filter samples on dry ice during collection trips and transferred them to a -80 °C freezer for storage upon returning to the lab.

Molecular Methods

***Bd* samples**

We processed *Bd* swabs as described previously (*see Chapter Four*). Briefly, we extracted DNA from swabs using the PrepMan Ultra Sample Preparation Reagent and stored them at -20 °C

or -80 °C prior to standard quantitative PCR (qPCR) assays with *Bd*-specific primers for ITS1 (Boyle et al., 2004; Hyatt et al., 2007). qPCR assays were conducted on DNA extracts in R. Knapp's laboratory at the Sierra Nevada Aquatic Research Laboratory (Mammoth Lakes, California, USA) using plasmid *Bd* standards (Joseph and Knapp, 2018; Longo et al., 2013). We multiplied quantities detected by a dilution factor of 80 (based on dilution during qPCR; *see Chapter Four*) and then divided the resultant quantities of ITS1 copies by 60 to convert to zoospore equivalents (ZE; based on previous findings that *Bd* in the region has an average of 60 ITS1 copies per zoospore; Joseph and Knapp, 2018). We then log transformed ZE quantities (adding one to each quantity and taking the logarithm) to produce units of logZE, our measure of *Bd* load. The *Bd* status of any sample for which we detected logZE greater than zero was considered *Bd* infected.

Microbiome samples

We extracted DNA from microbiome samples as described previously (*see Chapter Two*) using PowerSoil DNA Isolation Kits (MoBio or Qiagen DNeasy kits) with a modified protocol for low-biomass samples. Following DNA extraction, we quantified DNA concentration using a Qubit (Invitrogen, Carlsbad, CA, United States) and the dsDNA High Sensitivity Kit and then stored DNA extracts at -80 °C.

We generated 16S rRNA gene amplicon sequences as described previously (*see Chapter Four*). Briefly, we randomized DNA extracts across 96-well plates and shipped them to the Integrated Microbiome Resource (IMR) at Dalhousie University (Halifax, Nova Scotia, Canada). At IMR, amplicon libraries were generated using a two-step PCR protocol. First, samples were amplified using bacteria-specific primers targeting the hypervariable V3-V4 region of the 16S

rRNA gene (341F and 805R; Klindworth et al., 2013). Next, PCR products were amplified in duplicate using dual indexed “fusion primers” (including adaptors, indexes, and V3-V4 primers), pooled and verified using E-gels (Invitrogen), cleaned and normalized using SequalPrep kits (Invitrogen), and then pooled, Qubit-quantified (dsDNA High-Sensitivity kit; Invitrogen), and sequenced across two runs of an Illumina Miseq (Illumina, Inc., San Diego, CA, USA; using v3 600 cycle chemistry) according to the IMR SOP for amplicon sequencing (Comeau et al., 2017; see published wet-lab protocols at [dx.doi.org/10.17504/protocols.io.4r3l277k3g1y/v1](https://doi.org/10.17504/protocols.io.4r3l277k3g1y/v1)). The resulting sequence data was demultiplexed by IMR.

We obtained sequences for the present study from 403 true samples (including 299 frog swab samples and 104 water filter samples) and 20 controls. Control samples included three dry swab controls (processed in the same way as true samples), nine blank DNA extraction kit controls (for which no sample was added to the PowerBead tube, but otherwise processed in the same way as true samples), and eight PCR negative controls (for which no sample DNA was added to the second PCR step at IMR, and subsequent processing was the same as for true samples).

Microbiome Sequence Processing

Sequence data for this study was generated on the same MiSeq runs as for our previous study (*see Chapter Four*), and was therefore processed together from trimming and filtering through contaminant removal as described previously. Briefly, we used Cutadapt v. 3.5 (Martin, 2011) with Python v. 3.9.10 (Van Rossum and Drake, 2009) to remove primer sequences and used DADA2 v.1.22.0 (Callahan et al., 2016) in R v. 4.2.1 with RStudio v. 1.1.419 (Posit Team, 2022; R Core Team, 2022) to process forward reads. We chose to exclude reverse reads from our analysis because they had lower quality scores than forward reads. We trimmed reads at 250 base pairs,

truncated at the first quality score of 2, and removed those with expected errors greater than 4. We then inferred amplicon sequence variants (ASVs) and constructed sequence tables for each Miseq run separately before merging the two runs. We identified and removed chimeric sequences, assigned taxonomy to the genus level using the Ribosomal Database Project (RDP) naive Bayesian classifier algorithm and the SILVA high quality ribosomal RNA database v. 132 (Quast et al., 2012; Wang et al., 2007; Yilmaz et al., 2014), and assigned taxonomy to species based on exact matching of ASVs to reference strains in the SILVA database. We then assigned unique names to ASVs (*e.g.*, SV1, SV2, etc.).

We used phyloseq v. 1.42.0 (McMurdie and Holmes, 2013) to combine the sequence table, taxa table, and metadata into a phyloseq object. As described previously (*see Chapter Four*), we filtered out ASVs that had a total read count summed across samples of less than 0.1% of the mean sample read depth and then removed non-bacterial ASVs, chloroplasts, mitochondria, and finally, samples with zero reads remaining after filtering steps (which removed four of the PCR negative controls). Then we identified and removed putative contaminants for each set of control samples separately using the prevalence method with Decontam v. 1.14.0, setting a probability threshold of 0.1 and applying the batch argument to identify contaminants from groups of true samples associated with specific negative controls independently (Davis et al., 2018; *see Chapter Four*). We filtered out the resulting list of 166 putative contaminant ASVs, leaving 13,608 unique ASVs across the 403 true samples and 16 remaining control samples in the dataset.

Sample depths were quite variable across true samples for the present study, ranging from 972 to 61,920 read counts. Rarefying samples to an even sampling depth is an effective method to normalize samples with very uneven read count distributions while preserving the integrity of community level comparisons (McKnight et al., 2019; Schloss, 2024; Weiss et al., 2017). We

therefore investigated rarefaction curves to assess possible read depths to rarefy the dataset that would maximize the number of true samples maintained while ensuring that the majority of samples' rarefaction curves had plateaued. We proceeded by rarefying to 2,012 (the minimum sampling depth for a frog microbiome sample; Figure S5-1). This step removed six water filter samples and eight control samples, and resulted in 12,157 ASVs remaining in the dataset.

To generate a phylogeny of ASVs in the dataset, we aligned sequences using DECIPHER v. 2.22.0 (Wright, 2016) and built a maximum likelihood tree using a GTR + $\Gamma(4)$ + I model using phangorn v. 2.8.1 (Schliep et al., 2017; Schliep, 2011) on the UC Davis Bioinformatics Core High Performance Computing Cluster in R v. 4.1.0 (R Core Team, 2022). We used phangorn v. 2.9.0 to midpoint root the resulting tree, and then merged it with our phyloseq object. Finally, we filtered out remaining control samples (n=8) and ASVs that had zero reads across true samples, which left 397 true samples (including 299 frog samples and 98 water filter samples) and 10,417 unique ASVs in the rarefied dataset for downstream analysis.

Data Analysis and Visualization

Host body condition

We calculated the scaled mass index (SMI) for frogs, a metric for host body condition based on host lengths and weights that can be compared among individuals of varying sizes (due to differences, for example, in age or sex). This metric takes into account allometric growth of amphibians (*i.e.*, that the relative proportion of weight and length shift with increasing size) (Brodeur et al., 2020; Landler et al., 2023; Peig and Green, 2009). We followed methods of Peig and Green (2009) to calculate $SMI_i = M_i * (L_0/L_i)^b$, where M_i and L_i are the weight and SVL of individual i , respectively, L_0 is an arbitrary SVL chosen within the range of observations (to

standardize the index to a fixed body size), and b is the slope of a standardized major axis (SMA) regression of $\ln$ -transformed M on L (*i.e.*, the scaling exponent). We fit an SMA regression of $\ln M \sim \ln L$, using the `sma` function from `smatr` v. 3.4.8, specifying the SMA method and the robust line fitting method. The slope of this regression was 3.15 (SMA, $R^2 = 0.90$, $p < 0.001$), which we assigned as the scaling exponent (Figure S5-2). We chose a value for L_0 of 52 based on the mean and median SVL (52.4 mm and 51.6 mm, respectively), and proceeded to calculate the SMI for all frog individuals. Finally, we validated that SMI values were independent of SVL by fitting an SMA regression of $SMI \sim SVL$ to confirm that there was no significant correlation (SMA, $R^2 = 0.01$, $p = 0.08$; Figure S5-2; Brodeur et al., 2020).

Microbial community diversity metrics

To assess microbial community structure between samples (*i.e.*, Beta diversity), we used weighted Unifrac, a phylogenetically informed distance metric that weights the contribution of organisms based on their relative abundances (Lozupone and Knight, 2005; Lozupone et al., 2007). We used the `ordinate` function in `phyloseq` v. 1.42.0 to calculate these distances for different subsets of the data, and visualized ordinations using principal coordinate analysis (PCoA). We used the `betadisper` function in `vegan` v. 2.6.4 to calculate dispersion in community structure (*i.e.*, within-group variance) (Oksanen et al., 2022).

To assess within-sample diversity (*i.e.*, alpha diversity), we examined observed richness, which is the number of unique ASVs per sample, and Shannon diversity, which takes into account relative frequencies of ASVs. We calculated these metrics using the `estimate_richness` function in `phyloseq`.

Anti-Bd predictions

We predicted putative *Bd*-inhibitory function of microbial community members and calculated two metrics to quantify the potential anti-*Bd* function of communities as in Kueneman et al. (2022). Predictions were based on a database of full length 16S rRNA gene sequences from bacteria that were isolated and assayed for their effects on growth of *Batrachochytrium* pathogens (Woodhams et al., 2015). Several previous studies have used this database to predict anti-*Bd* microbial community function, including studies that found biologically relevant associations between *Bd* and these predictions (Bletz et al., 2017; Chen et al., 2022; Jiménez et al., 2022; Kueneman et al., 2022, 2016; Muletz Wolz et al., 2018).

As of the 2023.2 version, the full database includes over 7,900 isolates that have been functionally assayed for *Bd* inhibition. We used the strict inhibitory subset of the database (AmphiBac_InhibitoryStrict_2023.2) which only includes the 2,056 sequenced isolates that showed inhibitory function. We accessed this database and scripts used to assign putative *Bd*-inhibitory function and calculate community inhibitory metrics from the AmphiBac Database Github (see <https://github.com/AmphiBac/AmphiBac-Database/>). First, we identified which ASVs in our dataset clustered at 99% similarity with sequences from the inhibitory database using QIIME2 v. 2020.8.0 (Bolyen et al., 2019) to implement vsearch (Rognes et al., 2016). Then in R, we calculated “anti-*Bd* richness” for each sample as the number of unique ASVs that clustered with inhibitory sequences, and calculated the “anti-*Bd* relative abundance” for each sample as the sum of read counts for putatively inhibitory ASVs divided by the total count of reads per sample (*i.e.*, 2,012 reads per sample in the rarefied dataset).

Relative abundance of bacterial taxa

We visualized the relative abundance of taxa for individual samples and the mean relative abundance of taxa for subsets of samples. We used *microshades* v. 1.11 (Dahl et al., 2022) in R to make stacked bar charts that simultaneously display a higher taxonomic group level (phylum) and a lower taxonomic subgroup level (either family or genus). We transformed rarefied sample counts to relative abundances and then merged ASVs from the same taxonomic subgroup rank using the `tax_glom` function in *phyloseq* (specifying `NArm=FALSE` to maintain unclassified taxa). Next, we used the `create_color_dfs` *microshades* function to assign colors to the five bacterial phyla that together appeared to account for the highest relative abundance across frog samples based on exploratory analysis: Proteobacteria, Bacteroidetes, Actinobacteria, Verrucomicrobia, and Planctomycetes. Within the phylum with highest relative abundance across samples, we chose to display nine subgroups that represented the highest relative abundance within the phylum in descending order, with the remaining taxa present in that phylum falling into an “other” subgroup category (using the `extend_group` *microshades* function and specifying `n_add = 5`). Within the other four displayed phyla, we show only the four highest abundance subgroups, again with the remaining taxa falling into “other” (this is the default). The relative abundances of all other phyla across samples were collapsed into an “other” group category. We then ensured that the phylum and associated subgroup representing the highest relative abundance across samples were displayed at the bottom of stacked bar charts using the `reorder_samples_by` *microshades* function (specifying `sink_abundant_groups = TRUE`). In cases where we show relative abundance by sample, we ordered samples by the most abundant subgroup (specifying the name of the subgroup in the `order` argument of the `reorder_samples_by` function). We created the legend for stacked bar

charts using the `custom_legend` `microshades` function and generated stacked bar charts using the `plot_microshades` function.

Statistical analyses

To assess our power to detect *Bd* infected samples based on sample sizes of subsets of the dataset, we used the `dbinom` function in `stats` v. 4.2.1 to calculate the probability of detecting no positives, “Pr(no *Bd*),” if the true frequency of *Bd* occurrence were 11% (a conservative estimate of endemic *Bd* prevalence based on a long term study in Illinois, USA; Talley et al., 2015). We then calculated the frequency of *Bd* occurrence for subsets of the data by dividing the number of *Bd* samples that tested positive for *Bd* infection by the total number of samples assayed. We calculated 95% binomial confidence intervals for these frequencies (95% CI; `binom.test` function in `stats`). We tested for significant differences in the odds of being *Bd* infected among subsets of the data (based on the number of samples that tested positive for *Bd* infection and the number that tested negative for *Bd* infection) using Fisher’s exact tests of independence (`fisher.test` function in `stats`) and *post hoc* pairwise Fisher’s exact tests (`pairwise_fisher_test` function in `rstatix` v. 0.7.2, specifying the Benjamini-Hochberg procedure to control the false discovery rate, FDR).

To test which discrete and continuous variables explained significant variation in microbial community structure, we first filtered the dataset to only include samples with data available for the variable at hand, and then ran permutational multivariate analysis of variance (PERMANOVA) using the `adonis2` function in `vegan` v. 2.6.4 with 9999 permutations, specifying the metadata variable(s) and specifying `by = “terms”` to implement a sequential model (Anderson, 2001; Oksanen et al., 2022). This function compares the variation between groups to the variation within groups for discrete variables and conducts simple linear regression for continuous variables. For

single categorical factors that rejected the null hypothesis in omnibus PERMANOVA models ($p < 0.05$), we performed *post hoc* pairwise PERMANOVA tests to identify which levels within factors differed significantly, using the `adonis.pair` function in `EcolUtils` v. 0.1 with 9999 permutations (Salazar, 2022). We corrected p-values for multiple comparisons using the Benjamini-Hochberg procedure. For discrete grouping factors evaluated in *post hoc* pairwise PERMANOVAs, we also tested for significant differences in mean community dispersion (*i.e.*, differences in within-group variance) using the `permutest.betadisper` function in `vegan` with 9999 permutations. For significant comparisons ($p < 0.05$), we conducted *post hoc* TukeyHSD tests (`TukeyHSD` function in `stats`) to identify which levels within factors differed significantly in group dispersion. These tests adjust p-values to control for the family-wise error rate (FWER).

To test for significant differences in mean values of several metrics among subsets of samples, we first filtered the dataset to only include samples with data available for the metric at hand, and then tested whether subsets of the dataset were normally distributed with equal variances using Shapiro-Wilk normality tests (`shapiro.test` function in `stats`) and Bartlett's tests (`bartlett.test` function in `stats`), respectively. If $p > 0.05$ for both tests, then to test for significant differences among mean values for subsets of the data we conducted Welch's two sample t-tests (`t.test` function in `stats`) or for three or more groups we conducted analysis of variance (ANOVA; `aov` function in `stats`) followed by *post hoc* Tukey tests of pairwise comparisons (tests adjusted p-values to control for the FWER). If subsets of the data were non-normally distributed and/or did not have equal variances ($p < 0.05$), then we used nonparametric Mann-Whitney tests (*i.e.*, two-sample Wilcoxon rank sum tests; `wilcox.test` function in `stats`) or for three or more groups we conducted nonparametric Kruskal-Wallis ranked sum tests (`kruskal.test` function in `stats`) followed by *post hoc* Dunn tests of pairwise comparisons (`dunnTest` function in `FSA` v. 0.9.3; specifying the

Benjamini-Hochberg procedure to correct for multiple comparisons; Ogle et al., 2022). To assess correlations between *Bd* loads and several other variables, we conducted nonparametric Spearman's correlation analyses when variables were non-normally distributed based on Shapiro-Wilk tests, or parametric Pearson's correlations when variables were normally distributed. We corrected p-values for multiple correlation analyses conducted on the same dataset using the function `p.adjust` (specifying the Benjamini-Hochberg method).

We also tested for significant changes in several metrics across time intervals based on years since release (YSR) for reintroduced frogs and based on collection year for source population frogs. In this analysis we included reintroduced frog samples collected at 1 YSR (n=8), 2 YSR (n=8), and 3 YSR (n=8) across Lucille and Tamarack restored sites. For reintroduced frog samples collected at 1 YSR, we examined changes compared to captive Zoo samples (collected in 2016; n=30). Changes in metrics for source frog samples from the Rivendell natural site were compared between the 2017 (n=41) and 2018 (n=60) field seasons. We examined microbial community displacement (*i.e.*, the weighted Unifrac distances between distinct groups) across time intervals for subsets of the data. To do this, we filtered the dataset to only include weighted Unifrac distances between sample pairs collected across subsequent time periods. We calculated pairwise changes in other metrics (including *Bd* loads, anti-*Bd* richness, and anti-*Bd* relative abundance) across time periods by calculating the difference in each metric for all pairwise combinations of frog samples for which one was collected in the later time period and the other from the previous time period. We conducted statistical tests as described above to determine if there were significant differences in the magnitude of change among time periods and between reintroduced and naturally occurring frog sample comparisons.

Results

Frog Recaptures

While all of the captive, reintroduced, translocated, and most new recruit frogs were PIT-tagged, most source population frogs at natural sites were not tagged (Table S5-1); out of 174 frog captures across natural sites, 40 frogs were tagged (25 frogs at the Rivendell natural site and 15 frogs at the Pyramid natural site; Table S5-1). Of the tagged frogs across natural sites, two individuals were recaptured (*i.e.*, sampled twice) at the Rivendell natural site. We note that we could not determine how many untagged frogs at the natural sites were sampled multiple times. At the three restored sites, we sampled many individuals only once, but we also sampled several of the same individuals more than once (Table S5-1). Though we note that we never captured any unique individuals more than once during the same site collection visit.

One possible concern with the fact that individuals were resampled is that this might have some impact on population level metrics. While we could not assess this completely since we could not determine if non-tagged individuals had been resampled, we did analyze resampled frog individuals to assess whether repeated sampling of the same individuals would significantly affect population level parameters. Based on statistical analyses for samples from eight tagged frogs that were recaptured and sampled more than twice (Table S5-1), we found no significant effect of individual identity on host scaled body mass index, *Bd* load, microbial community alpha diversity, or anti-*Bd* prediction metrics (Table S5-2). Individual identity also did not explain significant variation in microbial community structure after accounting for site differences (Table S5-3). Therefore, variation within individuals was not significantly different from variation among individuals for any metrics tested. Finding no measurable impacts of individual identity suggests that other factors were more important to driving variation. This aligns with our previous finding

that differences among individuals in captivity were primarily associated with distinct tank environments (*see Chapter Two*). Here we found that when and where samples were collected appeared to be more important than which individual they were collected from. We therefore felt comfortable proceeding with population level analyses of samples without controlling for known or unknown resampling of individuals.

Host Body Condition

We measured host body condition using the scaled mass index (SMI). We examined SMI to determine whether this host factor was related to differences in frog microbiomes and *Bd* infections among site types and sites (in service of questions one and two) or among restoration types (in service of question three).

Site types and sites

We found that SMI was significantly different among site types and sites (Table S5-4; Figure S5-3). By site type, the frogs from natural sites had significantly lower SMI than did frogs from restored sites (Table S5-4; natural and restored site frogs both had significantly higher SMI than captive site frogs). By site, the Lucille restored site frogs had significantly higher SMI than both natural sites' frogs, though frogs from the other two restored sites did not differ significantly from natural site frogs in terms of host SMI (Figure S5-3).

Restoration types

SMI was significantly different among restoration types across the Zoo, Rivendell, Tamarack and Lucille (Table S5-5; Figure 5-1B), but pairwise differences were all between

restored site frogs and frogs from other site types. *Post hoc* Dunn tests revealed that there were no significant differences among translocated, reintroduced, and new recruit frogs (Figure S5-3). New recruit frogs had higher SMI than source frogs, and translocated, reintroduced, and new recruit frogs all had significantly higher SMI than captive frogs (Figure S5-3). We also confirmed that restoration types within Lucille and Tamarack restored sites and between Lucille and Tamarack restored sites did not differ significantly in terms of SMI.

Host Infection Status and *Bd* Loads

We compared the frequency of *Bd* infections and *Bd* loads among site types and sites and examined associations with host body mass index (both in service of question two). We then examined these infection metrics among restoration types (in service of question three). Finally, we compared infection metrics between *Bd* inoculation treatment groups in captivity and after reintroduction (in service of question four).

Site type and site comparisons

We found no significant difference in the odds of being *Bd* infected between field site types (natural sites vs. restored sites; Table S5-6; Table S5-7) or among field sites (Table S5-6; Table S5-7). The overall frequency of *Bd* occurrence across all field sites was 84.0% (79.1–88.2 CI; Table S5-6).

Bd loads of *Bd* infected wild-collected samples were significantly higher at restored sites than at natural sites (Table S5-8). When comparing among all field sites (Figure 5-2A), we found that mean *Bd* loads of *Bd* infected samples were significantly higher at the Tamarack and Lucille restored sites than at the Rivendell natural site, and that *Bd* loads were also higher at the Lucille

restored site than at the Pyramid natural site. Mean *Bd* loads among sites ranged from 1.68–2.29 logZE (Figure 5-2A).

Bd load and host body condition among sites

We noticed that patterns of host *Bd* loads among field site types and sites (Figure 5-2) differed in some of the same pairwise comparisons as did host SMI (*see above*; Figure S5-3). We therefore examined whether these variables were correlated. While we found that *Bd* loads of *Bd* infected field-collected samples were positively correlated with SMI across all field site frog samples (Spearman's $\rho=0.17$; Table S5-9), we found no significant correlations within each field site (Table S5-9). We also found that *Bd* loads of all field-collected samples (*i.e.*, those testing positive and negative for *Bd* infection) were positively correlated with SMI across all field sites (Spearman's $\rho=0.24$; Table S5-10), but within sites, we only found similar significant correlations for one natural site and one restored site (the Pyramid natural site with Spearman's $\rho=0.41$ and the Tamarack restored site with Spearman's $\rho=0.42$, respectively; Table S5-10).

Restoration type comparisons

The odds of being *Bd* infected did not differ among restoration types across the Rivendell natural site and the Tamarack and Lucille restored sites (Table S5-11; Figure 5-1B), or among restoration types within each restored site (Tamarack, Lucille, and Jabu restored sites). Only two frog samples assayed had *Bd* loads over 4.0 logZE, which was previously identified as the threshold for host mortality (Figure 5-2; Vredenburg's "10,000 zoospore rule"; Kinney et al., 2011; Vredenburg et al., 2010). These were from two frogs translocated to the Lucille restored site in 2015 that we sampled in 2016, and neither of these individuals were recaptured again.

We found that mean *Bd* loads of frog samples that tested positive for *Bd* infection were significantly higher for translocated, reintroduced, and new recruit frogs than for source frogs (Figure 5-2B; Table S5-12). However, we found no significant differences in *Bd* loads among the restored site restoration types (*i.e.*, no differences among translocated, reintroduced, and new recruits; Figure 5-2B). As these comparisons of restored site restoration types were across two restored sites, we also confirmed that there were no significant differences among restoration types within each restored site or for restoration types by site across the Tamarack and Lucille restored sites.

Long-term effects of *Bd* inoculation treatment

We investigated whether history of *Bd* inoculation treatment group was associated with differences in *Bd* infection across reintroduced frog samples. While in captivity, *Bd*⁺ group frogs experienced *Bd* infection followed by anti-fungal treatment. *Bd*⁻ group frogs were treated with the anti-fungal drug but were never exposed to *Bd* (*see Chapter Four*). For samples from reintroduced frogs, the frequency of *Bd* infected frog samples was similar between *Bd*⁺ and *Bd*⁻ treatment groups: 8 out of 10 *Bd*⁻ group samples (collected from four distinct individuals) tested positive for *Bd* infection, while 14 out of 17 *Bd*⁺ group samples (collected from eight distinct individuals) tested positive. Further, the mean *Bd* load of *Bd* infected reintroduced frog samples from the *Bd*⁻ inoculation treatment group ($2.14 \log_{10} \text{ZE} \pm 0.92 \text{ sd}$) was not significantly different from the mean *Bd* load for *Bd* infected reintroduced frog samples from the *Bd*⁺ inoculation treatment group ($2.25 \log_{10} \text{ZE} \pm 0.55 \text{ sd}$; ANOVA, $p=0.73$).

Drivers of Variation in Microbial Community Structure

We measured microbial community structure using weighted Unifrac distances (*i.e.*, Beta diversity). We examined microbial community structure of frogs and water among site types and sites (in service of question one). We then considered environmental site parameters and microbiome structure (also in service of question one) and examined drivers of microbial community structure for frogs and for water within sites (in service of questions one and two). Next, we focused on frog microbiomes, considering microbial community structure among restoration types (in service of question three), between inoculation status groups (in service of question four), and within reintroduced frogs (in service of question five).

Microbiome structure of frogs and water among site types and sites

Before addressing our first question concerning the relative importance of host and environmental factors to frog microbiomes, we first confirmed that frog and water microbiome structure differed from each other within each of our six sites (Table S5-13). Most sites also harbored higher community dispersion in water microbiomes than in frog microbiomes (Table S5-14). This could indicate that variance in microbial communities alone was different between frog and water samples from those sites, rather than the mean community structure. However, clear separations of clusters observed on PCoA plots suggest that both dispersion and mean location of community structure in ordination space (*i.e.*, group centroids) differed between frogs and water for all field sites (Figure S5-4).

In subsequent analyses we considered frog and water microbial community structure separately in order to examine whether frog microbiome structure exhibited different relationships to site types and sites than did water microbiome structure. Community structure was significantly

different among all three site types for frogs (*i.e.*, captive, natural, and restored; Table S5-15; Table S5-16). We also detected differences in community dispersion among site types, including the finding that natural site frogs harbored significantly lower microbial community dispersion than restored site frogs (Table S5-17; Table S5-18). For water samples, community structure was also significantly different among all three site types (Table S5-19; Table S5-20). However, water sample dispersion did not differ between natural and restored site types (Table S5-21; Table S5-22).

For frog samples and for water samples, when we included site after site type in sequential models (*i.e.*, variation assigned to the first term was removed before assigning variation to the next term), site still explained a significant amount of variation (Table S5-15; Table S5-19). When site was included as the first term in these models, it explained a greater amount of variation, encompassing variation that had been assigned to site type (Table S5-15; Table S5-19). For frog microbiome samples, most site comparisons were significantly different except for the Lucille restored site versus the Tamarack restored site (Figure 5-3A; Table S5-16). Frogs at the Jabu restored site had significantly higher microbial community dispersion than did frogs at the Rivendell natural site, though clustering on PCoA plots suggested that group centroids differed between these sites in addition to dispersion (Table S5-18; Figure 5-3A). For water microbiome samples, most site comparisons were also significantly different except for the Rivendell natural site versus the Pyramid natural site (Figure 5-3C; Table S5-20). There were no significant differences in water-associated microbial community dispersion among sites after p-value correction in *post hoc* tests (Figure 5-3C; Table S5-21; Table S5-22). We also examined whether frog microbiome structure across the Lucille and Tamarack restored sites was significantly closer to microbiomes of frogs from the Rivendell natural site than to frogs from the other natural site

(since frogs from Lucille and Tamarack restored sites all originated from the Rivendell natural site directly, indirectly, or as descendants; Figure 5-1B). We found that mean microbial community displacement between Rivendell natural site frogs and frogs from the Lucille and Tamarack restored sites was significantly lower than between the Pyramid natural site frogs and frogs from these two restored sites (Wilcoxon, $p < 0.001$).

Environmental site parameters and microbiome structure

We found that more variation in community structure was explained by site than any other variable tested among frog samples and among water samples. Differences in air and water temperatures and elevations among site types and sites may contribute to observed differences in community structure (Figure S5-5). For example, mean air temperatures at the Lucille and Tamarack restored sites were $\leq 20^{\circ}\text{C}$ on average, while they were $> 20^{\circ}\text{C}$ on average at the third restored site and at both natural sites (Figure S5-5A). In contrast, mean water temperatures were just below 20°C on average at all three restored sites, but water temperatures were significantly lower on average at both natural sites (Figure S5-5B). There were also qualitative ecological differences observed among field sites on maps generated using the National Map Viewer from the USGS (Figure S5-6; <https://apps.nationalmap.gov/viewer/>). For example, both natural sites and one restored site (Jabu) are surrounded primarily by barren land and shrubland, while vegetation around Lucille and Tamarack restored sites includes evergreen forest (Figure S5-6B; Jon Dewitz, 2019). In summary, several of these differences align with site types (*i.e.*, differences between restored and natural site types), however others align with site differences regardless of site type. We would need additional site replicates to separate the effect of site from the effect of some specific site characteristic.

Drivers of microbiome structure within sites

After accounting for site variation in frog microbiome structure, we found significant effects of frog scaled mass index (SMI) and sample collection year (Table S5-15) on the frog microbiome. We also found significant interactions between site and each of these terms (*i.e.*, SMI and year), indicating that effects of SMI and year on variation in microbiome structure differed depending on the site (Table S5-15). Neither frog sex nor frog snout-to-vent length (SVL) explained significant variation in microbiome structure after accounting for site differences. For water microbiome samples, after accounting for site variation, there was also a significant effect of year in the PERMANOVA model, but there was not a significant interaction with site (Table S5-19).

Next, we examined whether *Bd* infection of frogs explained variation in associated microbiome structure for wild-collected frogs (we chose not to include the captive Zoo samples in this model as their *Bd* loads and infection status had been experimentally manipulated; *see Chapter Four*). After accounting for site variation in the PERMANOVA model, we found significant effects of *Bd* load, SMI, year, and significant interactions among terms (Table S5-23). The interaction between *Bd* load and SMI suggests that the impact of infection intensity on microbiome structure differs depending on host body condition (or *vice versa*). *Bd* status (*i.e.*, testing positive or negative for *Bd* infection) did not explain significant variation in microbiome structure, even when included as the first term in the model.

Microbiome structure among frog restoration types

To investigate the effect of restoration types (*i.e.*, captive, source, translocated, reintroduced, and new recruits) on microbial community structure, we analyzed frog samples from the restored sites that harbored both reintroduced and translocated frogs (*i.e.*, Tamarack and Lucille restored sites) and frog samples from the sites where these populations originated (*i.e.*, the Zoo captive site and the Rivendell natural site used to source frogs; Figure 5-1B). We found that restoration type explained significant variation in community structure across these samples (Table S5-24). However, *post hoc* tests revealed that there were no differences among translocated, reintroduced, and new recruit frogs at restored sites (Table S5-25). Microbial community structure of captive frogs and source frogs differed significantly from frogs of all other restoration types (Table S5-25), which aligned with site differences. Captive frogs harbored significantly higher community dispersion than source frogs (Table S5-26; Table S5-27). However, observed clustering of samples in PCoA visualization suggests that centroids of community structure also differed between captive and source frogs (Figure 5-3B). PCoA visualization also showed the overlap in microbial community structure for the restored site restoration types (*i.e.*, translocated, reintroduced, and new recruit; Figure 5-3B). Further, we confirmed that restoration types within the Lucille restored site and within the Tamarack restored site did not differ significantly in community structure, and that restoration types by site did not differ across these two restored sites.

Microbiome structure of *Bd* inoculation treatment groups

We investigated community structure among treatment groups from experimental *Bd* inoculation (*see Chapter Four*; *Bd*⁺ group exposed to *Bd* vs. *Bd*⁻ group unexposed to *Bd*), and

whether we would detect differences in frog microbiome structure between treatment groups after reintroduction. We found that treatment groups of captive frogs differed significantly in community structure with *Bd*⁺ group frogs having higher community dispersion than *Bd*⁻ group frogs (Table S5-28; Table S5-29; Figure S5-7). However, we did not find a significant difference between inoculation treatment groups after reintroduction (Table S5-28; Figure S5-7), suggesting that present environment outweighs history as a determinant of the microbiome.

Drivers of reintroduced frog microbiome structure

We looked further at reintroduced frog samples to explore factors that may have contributed to their microbial community structure. However, we found no significant effect of site (between Lucille and Tamarack restored sites), sample collection year, years since release (YSR), *Bd* status, or *Bd* load on community structure of reintroduced frog samples, even when variables were included as the first term in sequential PERMANOVA models.

Drivers of Variation in Microbial Community Diversity

We measured within-sample microbial community diversity (*i.e.*, alpha diversity) using two metrics: observed richness and Shannon diversity. We first examined microbiome diversity of frogs and water among site types and sites (in service of question one). We then investigated associations between *Bd* loads and microbiome diversity (in service of question two). Finally, we compared microbiome diversity among restoration types (in service of question three).

Microbiome diversity of frogs and water among site types and sites

Prior to comparing the relationship of frog microbiome diversity and water microbiome diversity to sites and site types (to address question one), we first examined whether frog and water microbiome diversity differed from each other within sites. We found that observed richness was significantly higher for frog microbiomes compared to water microbiomes at the Zoo and at the Jabu restored site, but richness did not differ significantly between frogs and water at the other four sites (Figure S5-8). Shannon diversity was significantly higher in water samples than on frogs at all sites except for the Jabu restored site, where there was no significant difference (Figure S5-8). We considered frog and water samples separately in subsequent analyses because we were interested in whether frog and water microbiomes showed different relationships to site types and sites.

Neither observed richness of frog microbiomes nor of water microbiomes differed significantly between the natural and restored sites (Table S5-4; Table S5-30). They also did not differ significantly in any pairwise comparisons of field sites (Figure 5-4A, C).

We found that microbiomes of restored site frogs harbored significantly higher Shannon diversity than did microbiomes of natural site frogs (Table S5-4). In contrast, water microbiomes from natural sites had significantly higher Shannon diversity than from restored sites (Table S5-30). Broken down by site, we found that frog microbiomes from two restored sites (Lucille and Jabu) had significantly higher Shannon diversity than both natural sites (Rivendell and Pyramid; Figure 5-4D; Table S5-4), while water microbiomes from one natural site (Pyramid) had significantly higher Shannon diversity than one restored site (Jabu; Figure 5-4F; Table S5-30).

Bd loads and microbiome diversity

Restored site frogs harbored higher mean *Bd* loads and higher mean Shannon diversity than natural sites (*see above*; Table S5-4; Table S5-8). For example, Lucille restored site frog microbiomes had significantly higher Shannon diversity and *Bd* loads than both Rivendell and Pyramid natural site frog microbiomes (Figure 5-2A; Figure 5-4D). After observing these shared relationships of microbial Shannon diversity and *Bd* load at the population level, we examined whether these variables were correlated across all field frogs and for frogs within each field site (we chose not to include Zoo samples as their infection status and *Bd* loads had been experimentally manipulated by *Bd* inoculation treatments; *see Chapter Four*).

In opposition to the pattern among site types and sites, we found a significant negative correlation between microbial Shannon diversity and *Bd* load across all field frog samples when we included only samples that tested positive for *Bd* infection (Spearman's $\rho = -0.13$; Table S5-9) and when samples across both *Bd* status groups were included (Spearman's $\rho = -0.14$; Table S5-10). By site, both of these negative correlations were only detected in frog samples from one natural site (Pyramid; Spearman's $\rho = -0.38$ and -0.33 , respectively; Table S5-9; Table S5-10), and we detected a negative correlation between all *Bd* loads and microbial Shannon diversity for frog samples from one restored site (Tamarack; Spearman's $\rho = -0.57$; Table S5-10). The other three sites showed no correlation of Shannon diversity and *Bd* load.

Microbiome diversity among frog restoration types

Observed richness and Shannon diversity were lower in captive frogs than all other frog types (Figure 5-4B, E; Table S5-5). Observed richness did not differ among other frog restoration

types, but Shannon diversity was significantly higher for both translocated and reintroduced frogs than for source frogs (Figure 5-4B; Table S5-5).

These comparisons revealed that there were no significant differences in either diversity metric among translocated, reintroduced, and new recruit frogs (*i.e.*, the restoration types across the two restored sites considered, Tamarack and Lucille; Figure 5-1B; Figure 5-4B, E). We further confirmed that there were no significant differences in either metric among restoration types within each restored site considered separately. However, when we compared restoration types by site across Tamarack and Lucille restored sites, we found that translocated, reintroduced, and new recruit frogs from Lucille harbored significantly higher Shannon diversity than new recruit frogs from Tamarack (Tukey, all with $p < 0.05$), indicating that site had some impact in these comparisons.

***Bd*-inhibitory Predictions and Associations with Metadata**

Out of 10,417 ASVs present in our rarified dataset across frog and water samples, 155 ASVs shared $\geq 99\%$ sequence similarity with bacterial 16S rRNA gene sequences from the *Bd* inhibitory database (Table S5-31; Woodhams et al., 2015). We calculated the anti-*Bd* richness (*i.e.*, the number of ASVs clustering with sequences from the *Bd* inhibitory database) and the anti-*Bd* relative abundance (*i.e.*, the summed relative abundances of all ASVs clustering with inhibitory database sequences) for frog and water microbiome samples.

We compared anti-*Bd* predictions for frog microbiomes and water microbiomes among site types and sites (in service of question one). We next examined associations between frog microbiome anti-*Bd* predictions and *Bd* infections (in service of question two). Then, we compared frog microbiome anti-*Bd* predictions among restoration types (in service of question three) and

between inoculation treatment groups (in service of question four). Finally, we investigated the relationship of the dominant frog-associated putatively inhibitory bacterium to site types and sites (in service of questions one and two). Further, we examined the association between this bacterium and anti-*Bd* relative abundance to elucidate interpretation of this anti-*Bd* metric (in service of all study questions).

Taxonomy of putatively anti-Bd bacteria

The 155 predicted anti-*Bd* community members were distributed across five phyla based on SILVA v.132 taxonomic assignments of our ASVs: Actinobacteria (n=25), Bacteroidetes (n=21), Deinococcus-Thermus (n=1), Firmicutes (n=1), and Proteobacteria (n=107). Within the Proteobacteria, >20 putatively anti-*Bd* ASVs were assigned to each of the following families: Burkholderiaceae (n=23), Enterobacteriaceae (n=28), and Pseudomonadaceae (n=24). The dominant ASV across frog samples in the dataset, SV1 (an undescribed member of the family Burkholderiaceae), had 100% sequence similarity to a bacterial isolate from the *Bd* inhibitory database (AmphiBac_1576 / Ranamuscosa-inhibitory_37; Woodhams et al., 2015). This inhibitory database bacterium was originally isolated from the sister species of *R. sierrae*, *Rana mucosa*.

Anti-Bd predictions for frog and water microbiomes among site types and sites

Anti-*Bd* richness was significantly higher for frog microbiomes than for water microbiomes from both natural sites and from two restored sites (Lucille and Jabu restored sites; Figure S5-9). This was dissimilar from comparisons of overall observed richness for the two natural sites and the Lucille restored site, for which there were no significant differences between frog and water microbiomes (see above; Figure S5-8). We found no significant difference between

anti-*Bd* richness of water and frog samples at the third restored site (Tamarack; Figure S5-9), which mirrored finding no significant difference in observed richness between these sample categories at that site (*see above*; Figure S5-8). Anti-*Bd* relative abundance was significantly higher for frog microbiomes than for water microbiomes across all six sites (Figure S5-10). In subsequent analyses we considered frog and water samples separately to examine whether they exhibited different relationships to site types and sites.

Natural site frog microbiomes harbored significantly higher anti-*Bd* richness than restored site frog microbiomes (Table S5-4). Contrastingly, water microbiome anti-*Bd* richness did not differ significantly between natural and restored sites (Table S5-30). Further, by site, water-associated anti-*Bd* richness did not differ among any field sites (Figure S5-11A), while frog-associated anti-*Bd* richness was significantly higher at one natural site (Rivendell) than the other natural site (Pyramid) and than at two restored sites (Tamarack and Lucille restored sites; Figure 5-5A). Additionally, the pattern of frog-associated anti-*Bd* richness by site contrasts with our finding that overall frog-associated observed richness did not differ among field sites (Figure 5-3A).

Putatively anti-*Bd* microbial relative abundance was significantly higher for both frogs and water from natural and captive sites compared to restored sites (Table S5-4; Table S5-30). By site, frog microbiomes had a greater number of significant differences in the anti-*Bd* relative abundance among field sites than did water microbiomes (Figure 5-5C; Table S5-4; Figure S5-11B; Table S5-30). Both frog and water microbiomes at one natural site (Rivendell) had higher anti-*Bd* relative abundance than at one restored site (Lucille). However, frog microbiomes also had higher anti-*Bd* relative abundance at the other natural site than at that restored site (Lucille), and both natural sites' frog microbiomes had higher anti-*Bd* relative abundance than at a second restored site (Jabu).

Water microbiomes did not differ in putative anti-*Bd* relative abundance among these field sites (Figure S5-11B).

Associations among frog-associated anti-Bd predictions and Bd infections

Bd loads were higher at restored than natural sites while anti-*Bd* prediction metrics were lower at restored sites (*see above*; Table S5-4; Table S5-8). Site comparisons reiterated these differences. For example, while frog-associated anti-*Bd* richness was higher at the Rivendell natural site than at the Tamarack and Lucille restored sites, *Bd* loads at these two restored sites were higher than at the natural site (Figure 5-2A; Figure 5-5A). Additionally, while frog-associated anti-*Bd* relative abundance was higher at both of the natural sites (Rivendell and Pyramid natural sites) compared to the Lucille restored site, this restored site had significantly higher *Bd* loads than both natural sites (Figure 5-2A; Figure 5-5C). Based on these apparent negative associations between each frog-associated anti-*Bd* metric and *Bd* loads of *Bd* infected frog samples at the population level, we examined correlations among these variables across all field frogs and within sites (we did not include captive Zoo samples here as their *Bd* loads had been experimentally manipulated during *Bd* inoculation treatment; *see Chapter Four*).

Putative anti-*Bd* richness was negatively correlated with *Bd* load across all field frog samples that tested positive for *Bd* (Spearman's $\rho = -0.15$), however this correlation was not evident in any analysis within a single site (Table S5-9). Contrastingly, putative anti-*Bd* relative abundance was positively correlated with *Bd* load across all field frog samples and across only those that tested positive for *Bd* (Spearman's $\rho = 0.16$ and 0.15 , respectively), and these positive relationships also occurred within some sites. One natural site showed both positive correlations (Pyramid; Spearman's $\rho = 0.32$ and 0.43 , respectively) and one restored site showed a positive

correlation when samples that tested positive and negative for *Bd* infection were included (Tamarack restored site; Spearman's $\rho=0.62$; Table S5-9; Table S5-10). These positive correlations between putative anti-*Bd* relative abundance and *Bd* load were opposite from the negative population-level association.

Anti-Bd predictions among frog restoration types

By restoration type, we found no significant differences in anti-*Bd* richness or the anti-*Bd* relative abundance of frog microbiomes among translocated, reintroduced, and new recruit populations at restored sites (Figure 5-5B, D). Source frog microbiomes harbored higher anti-*Bd* richness than all three restored site restoration types, but did not differ significantly from captive frog microbiomes in terms of anti-*Bd* richness or anti-*Bd* relative abundance (Figure 5-5B; Table S5-5). Interestingly, captive frog-associated anti-*Bd* richness was only significantly higher than for reintroduced frogs, despite the fact that reintroduced frogs had been released from captivity (Figure 5-5B). Finally, source and captive frog microbiomes harbored significantly higher anti-*Bd* relative abundance than both translocated and reintroduced frog microbiomes (Figure 5-5D; Table S5-5).

Anti-Bd predictions between Bd inoculation treatment groups

We compared anti-*Bd* prediction metrics between frog microbiomes from distinct *Bd* inoculation treatment groups for captive frogs and for reintroduced frogs to assess short- and long-term differences. Neither the anti-*Bd* richness nor the anti-*Bd* relative abundance differed significantly between *Bd* inoculation treatment groups in captivity or after reintroduction (Table S5-32).

Associations between prediction metrics and a putatively *Bd*-inhibitory taxon

We compared relative abundance of the dominant ASV from frog samples, SV1, across sites and restoration types (Figure 5-5E, F). SV1 was putatively *Bd*-inhibitory based on exact matching to the inhibitory database (Woodhams et al., 2015). We found that the trends in SV1 relative abundance resembled those of anti-*Bd* relative abundance among sites and restoration types (Figure 5-5, C–F). Anti-*Bd* and SV1 relative abundances were highly correlated across all frog samples (Spearman's $\rho=0.96$, Figure S5-12). Further, we observed that samples with higher anti-*Bd* richness tended to occur above the regression line of anti-*Bd* relative abundance on SV1 relative abundance (Figure S5-12). This indicates that samples whose anti-*Bd* relative abundance was higher than predicted by SV1 relative abundance tended to have higher anti-*Bd* richness (*i.e.*, other *Bd*-inhibitory ASVs contributed to the relative abundance of anti-*Bd* taxa in those samples).

Relative Abundance of Taxa

Proteobacteria was the phylum with highest mean relative abundance in frog microbiomes across all sites and restoration types (Figure S5-13). Within Proteobacteria, Burkholderiaceae was the family with highest mean relative abundance, and at the genus level, unknown genera (*i.e.*, unclassified taxa at the genus level) were highest in mean relative abundance (Figure S5-13; Figure S5-14), which included the dominant and putatively anti-*Bd* bacterium SV1.

Mean relative abundances of taxa were variable among site types, sites, and restoration types by site (Figure S5-13). We also observed variability in relative abundance of taxa among individual samples from captive and restored populations (Figure 5-6A; Figure S5-15).

Taxa relative abundances for Zoo recaptures after reintroduction

We visualized relative abundances of taxa associated with Zoo recaptures before and after reintroduction in service of our fifth study question. Out of the 30 captive frogs sampled as part of the 2016 Zoo cohort (all of which were subsequently released to Tamarack or Lucille restored sites) we recaptured only four after reintroduction. This small sample size limits our ability to draw conclusions based on these samples, but we describe them here to provide a baseline of information on what we observed. The relative abundances of bacterial phyla and families from before and after reintroduction for these four unique individuals are shown in Figure 5-6B. Two were recaptured two months after their release (*i.e.*, zero years since release, or 0 YSR), and two were recaptured 13 months after their release (*i.e.*, 1 YSR). Taxa relative abundances for these microbiome samples all appeared distinct from each other to varying degrees. All four individuals experienced shifts in the relative abundances of associated taxa between the Zoo and after reintroduction, whether they were recaptured at 0 YSR or at 1 YSR.

Microbial Shifts for Reintroduced Frogs Compared to Natural Variation Over Time

To address our fifth study question, we examined how microbial community displacement, *Bd* loads, and microbial anti-*Bd* prediction metrics changed over time for reintroduced frogs in the years since release (YSR) from captivity. Further, we compared changes across these time intervals to the magnitude of change observed over a year in a naturally occurring population (the Rivendell natural site). For reference, the sample values for each metric in each time period are shown in Figure S5-16.

Mean changes in all four metrics were significantly different for captive–1 YSR than for natural variation over a year (Figure 5-7). Community displacement and change in *Bd* loads were

both significantly higher for captive–1 YSR than for the natural population over time. On average, anti-*Bd* richness did not change between years in the natural population and anti-*Bd* relative abundance increased, while changes in both of these metrics were negative on average for reintroduced frogs between captivity and 1 YSR.

For 1-2 YSR, pairwise community displacement and pairwise change in *Bd* load were not significantly different from natural variation over time, suggesting that these metrics had stabilized for reintroduced frogs. Additionally, during this time interval, pairwise change in anti-*Bd* richness for reintroduced frogs was slightly positive and significantly higher than the natural population over time (Figure 5-7).

For 2-3 YSR, mean community displacement for reintroduced frog microbiomes became significantly greater than for natural variation over time, though it was still significantly lower than the displacement seen between captivity and 1 YSR. The mean pairwise change in *Bd* load was slightly negative for 2-3 YSR, making it significantly lower than natural variation in source frogs over time. At the same time, the mean change in anti-*Bd* richness was slightly negative and significantly lower compared to the previous time period (Figure 5-7).

Discussion

In this study, we examined amphibian skin microbiomes, host body condition, and *Bd* infections among two naturally occurring and three restored wild *Rana sierrae* populations across multiple restoration strategies (*i.e.*, captivity followed by reintroduction versus direct translocation from natural source populations). We provide some evidence consistent with a primary association between frog microbiomes and factors also shaping environmental microbiomes among sites. However, we also provide evidence for features of frog microbiomes that suggest a primary

association with host-related factors. Further, we show that *Bd* loads and frog microbiome metrics putatively associated with host-related factors both generally differed between natural and restored frog populations, suggesting that management may have played a role. Nevertheless, our evidence suggests that specific restoration protocols applied to *R. sierrae* do not result in long-term differences between translocated and reintroduced populations or between *Bd* inoculation treatment groups in terms of their microbiomes or *Bd* infections. Finally, we show that reintroduced frog microbiomes may experience changes greater than natural variation over time for years after their initial release.

***Bd* displayed enzootic dynamics in all populations regardless of management**

Before discussing results related to our initial questions and hypotheses, we first want to emphasize two findings across all wild frog populations: (1) the odds of being infected by *Bd* did not differ among any field sites (Table S5-7), and (2) nearly all frogs sampled across field sites harbored *Bd* loads lower than the threshold associated with mortality (4.0 logZE; Vredenburg's "10,000 zoospore rule"; Kinney et al., 2011; Vredenburg et al., 2010). There are multiple possible explanations for these findings. One is that survivorship bias affected these results. Frogs never recaptured after release could have harbored higher *Bd* loads that resulted in mortality. Further, the two translocated frogs that did harbor lethal infection loads were never captured again (Figure 5-2). Though this evidence is anecdotal, as many frogs were never captured more than once. Another possible explanation relates to host genetics. Previous studies suggested that populations of *R. sierrae* persisting with *Bd* in Yosemite National Park had undergone rapid genetic evolution in genes associated with disease resistance as a response to *Bd* invasion (Knapp et al., 2023). It is therefore possible that most frogs across all populations in our study maintained *Bd* loads below

the mortality threshold because of previous host genetic selection for improved resistance in source populations used to repopulate restored sites. Based on our data, all populations appeared to be in an enzootic state with *Bd*, which is encouraging news for restored populations. However, we still found differences between natural and restored populations, including higher *Bd* loads at restored sites. This suggests that putative natural selection for adaptive disease resistance alleles, while possibly limiting lethal infection loads, cannot be the only determinant of infection load. Microbiomes, other host factors, and environmental site parameters associated with differences in *Bd* loads are discussed in subsequent sections.

Differences in microbiomes among natural and restored sites indicate putative impacts of both environmental and host management related factors

To tease apart possible models for frog-microbiome dynamics and assess the relative importance of environmental and host-related factors, we examined relationships in measures of frog and water microbiomes. This worked in the following way. First, we used statistical tests to examine whether frog microbiome features (including structure, diversity, and putative *Bd*-inhibitory function) were related to site and site type. Then we repeated this analysis with water microbiome features to determine whether they were related to site and site type. Next, we looked at two aspects of the relationships between microbiome features and sites / site types: (1) whether there were significant differences in pairwise comparisons, and if there were then (2) the direction of those differences. If frog microbiome features were primarily associated with factors that also shape environmental microbiomes we would expect that the water and frog microbiomes would show the same general patterns for (1) and/or (2). If instead frog microbiomes were primarily

associated with host related factors (potentially including history of management) we would expect that the water and frog microbiomes would show different patterns for (1) and/or (2).

While frog and local water microbiome structure differed significantly from each other (Figure S5-4), we found that frog microbiome structure and observed richness largely differed in the same pairwise comparisons of site types and sites as did water microbiome structure and richness (Figure 5-3; Figure 5-4). For example, observed microbial richness did not differ among any field sites for frogs or for water, and both frog and water microbiome structure differed significantly between each natural site and each restored site. Local environments and microbiomes are known to play a role in community assembly of frog microbiomes (Estrada et al., 2019; Loudon et al., 2014b; Walke et al., 2014). Further, water microbiomes at field sites are not likely to be impacted by whether frogs at those sites had been relocated, but they are strongly influenced by local environmental factors (Sperlea et al., 2021). Considering that we found the same general patterns for differences in pairwise comparisons of sites, we suggest that frog microbiome structure and richness may be primarily associated with factors also shaping local environmental microbiomes rather than with factors unique to hosts, like management.

There were two exceptions to the shared relationship of frog and water microbiome structure to sites. First, while frog microbiome structure differed significantly between the two natural sites, water microbiome structure did not. Second, while frog microbiome structure did not differ significantly between two of the restored sites (Tamarack and Lucille), water microbiome structure was significantly different in that pairwise comparison. It is unlikely that gene flow occurs among study sites due to distance, geographic barriers, and the presence of fish in intervening lakes. Previous work found that amphibian skin-associated community dissimilarity was associated with genetic distance among hosts (Griffiths et al., 2018) and host population-level

genetic differentiation (Hernández-Gómez et al., 2017). Therefore, even though environmental microbiome structure at the natural sites was similar, frog microbiome structure could have differed due to host genotypes. On the other hand, frogs from the Lucille and Tamarack restored sites all originated from the same naturally occurring source population (moved from the Rivendell natural site directly, via the Zoo, or as descendants). Therefore, frog microbiome structure may not differ significantly between these restored sites because of genetic relatedness between frog populations, which ultimately would be an impact of management. Further, we found that mean microbial community displacement between the Rivendell source frogs and frogs from Lucille and Tamarack restored sites was significantly lower than between these restored site frogs and frogs from the other natural site. Finding that these restored site frog microbiomes have a closer resemblance to frog microbiomes from their source population is what we would expect if host genotypes contributed to frogs' microbiome structure. Genetic analysis of frogs across natural and restored sites in the Desolation Wilderness is needed to confirm whether host genetics are indeed related to observed patterns.

For Shannon diversity, putative anti-*Bd* richness, and in some cases putative anti-*Bd* relative abundance, we found differences among field site types and sites for frog microbiomes that did not match the general patterns observed for lake water microbiomes in the same comparisons. We suggest this may indicate that host-associated factors (including differences in the way hosts and *Bd* interact with the environment) were more important to these metrics than were environmental factors that shape lake water microbiomes. Further, differences in these metrics among field sites were primarily between natural and restored frog populations (discussed below). This supports our hypothesis that features of frog microbiomes putatively associated with

host factors would differ between natural and restored frog populations, which is what we would expect if management played a role.

Natural and restored frogs harbored differences in *Bd* loads, body condition, and microbiome metrics

To examine whether host *Bd* infections and host body condition were associated with management efforts, we examined the relationship of each of these metrics to sites and site types. For cases where we detected differences in hosts and/or their infections among sites, we determined whether these differences were in comparisons of natural and restored frog populations. Next, we examined how frog body condition and frog microbiome features putatively associated with host factors that differed according to management (*see above*) were related to *Bd* infections in population level comparisons and within sites. These microbiome features included Shannon diversity, putative anti-*Bd* richness, and putative anti-*Bd* relative abundance.

While the frequency of *Bd* infection did not differ among wild frog populations, we found that in general, host *Bd* loads were higher at restored sites than natural sites (driven by both the Tamarack and Lucille restored sites; Figure 5-2A). Previous work suggested that lower host densities were associated with lower *Bd* loads (the opposite of what we found here) (Bosch et al., 2020). However, other more recent work found that low density translocated populations showed equivalent or higher *Bd* loads than source populations (Wilber et al., 2022). Therefore, other host-associated factors including how hosts and *Bd* interact with their environment are more likely leading to higher *Bd* loads at restored sites (Wilber et al., 2022). For example, *Bd* prefers lower temperatures, and the two restored sites harboring increased *Bd* loads had lower air temperatures on average (Figure S5-5; Fisher et al., 2009). Further, those two restored sites contain areas of

evergreen forest not present at natural sites (Figure S5-6), which may impede some frogs' ability to behaviorally raise their body temperatures in direct sunlight, a proposed host thermoregulatory response to infection that can reduce *Bd* loads (Karavlan and Venesky, 2016; Waddle et al., 2024). Additionally, reduced host genetic diversity has been associated with higher mean pathogen loads and disease severity in other wildlife-pathogen systems (Smith et al., 2009). The Tamarack and Lucille restored populations that harbored higher *Bd* loads were sourced from a single natural population, and they may represent only a subset of the genetic diversity of frogs from their source. These possible drivers of increased *Bd* loads at two of the restored sites all relate to management efforts (*i.e.*, choice of restoration sites and of animals used to repopulate them) and should be assessed further.

Host scaled mass index (SMI), a measure of body condition, was also generally higher for frogs from restored sites than for frogs from natural sites, but this was driven primarily by frogs from one restored site (Lucille; Figure S5-3). Frogs likely tended to have higher body condition values at the Lucille restored site because lower population densities allowed for greater access to food resources (MacCracken and Stebbings, 2012; Peig and Green, 2009). As discussed above, frogs from the Lucille restored site also harbored higher *Bd* loads than natural sites (Figure 5-2A). While we found no correlation between SMI and *Bd* loads at Lucille, these factors were positively correlated at another restored site and at one natural site (Table S5-10). In other words, frogs with higher body condition tended to become more infected at some locations. Positive relationships between host condition and parasite load in wildlife have been found in many other animal host systems, though they are reported less frequently than negative relationships, possibly because they are less intuitive and thus underreported (Sánchez et al., 2018). Perhaps higher values of frog body condition at the Lucille restored site helped them to better tolerate their enhanced infection

intensities, which has been suggested in other host-pathogen systems previously (Sánchez et al., 2018). However, increased SMI does not conclusively signify improved overall health or fitness (Peig and Green, 2009). For example, in some cases higher values may be related to reduced participation in reproduction (Baumgartner and Gollmann, 2017). It is therefore also possible that restored site frogs experienced negative impacts of enhanced *Bd* loads on their health and fitness despite having higher SMIs.

Higher *Bd* loads were also generally associated with higher microbial Shannon diversity at the population level (Figure 5-2A; Figure 5-4A). Contrastingly, we found that microbial Shannon diversity and all *Bd* loads were negatively correlated at one natural site (Pyramid) and one restored site (Tamarack; Table S5-10). A previous study of *R. sierrae* from the same two natural sites we studied also found these factors were negatively correlated at the Pyramid natural site (Ellison et al., 2018). Further, frog populations experiencing epizootic *Bd* dynamics (e.g., with *Bd* loads at lethal levels) have been shown to harbor lower microbial diversity than those in an enzootic state (Bates et al 2018). However, we did not detect significant correlations between *Bd* loads and microbial Shannon diversity at the other three field sites (Table S5-10). Restored site frog populations harboring greater microbial Shannon diversity than natural sites (i.e., Lucille and Jabu restored sites) and/or restored site populations that harbored higher *Bd* loads than both natural sites (i.e., the Lucille restored site), therefore, did not reiterate a negative relationship between microbiome diversity and *Bd* loads (Figure 5-3D; Figure 5-2A; Table S5-9; Table S5-10). Further, population level comparisons for those sites suggest the opposite relationship. Therefore, while population level comparisons were at least in part pertinent to differentiation of natural and restored sites, the relationship between *Bd* and microbial diversity within sites was more complicated and did not differ according to differences in management alone.

Our finding that frog populations harboring lower anti-*Bd* microbial richness also tended to harbor higher *Bd* loads supports previous findings that increasing the number of *Bd*-inhibitory microbes present may improve host resistance to high levels of infection (Figure 5-2A; Figure 5-5A; Bell et al., 2018). Previous work has shown that combinations of anti-*Bd* bacteria can have additive and even synergistic impacts to *Bd* growth *in vitro* (Loudon et al., 2014a). Further, when anti-*Bd* bacteria were grown in co-culture, they produced emergent antifungal metabolites that enhanced their inhibitory abilities (Loudon et al., 2014a). Therefore, increased anti-*Bd* richness likely increases the odds that effective combinations of inhibitory microbes are present.

Our evidence suggests that the Rivendell natural site frogs may have selected for an increased number of anti-*Bd* members relative to the Lucille and Tamarack restored site frogs (Woodhams et al., 2023). For example, we found differences in frog-associated anti-*Bd* richness between natural and restored sites for which overall frog-associated observed richness and water-associated anti-*Bd* richness did not differ. In other words, reduced anti-*Bd* richness of these restored frog microbiomes did not result from harboring fewer community members, nor was it because they had reduced access to potential *Bd*-inhibitory colonists from their environmental reservoir. It is possible that this could relate to higher host density at the Rivendell natural site allowing for greater horizontal transmission of anti-*Bd* microbes (Baquero et al., 2021; Browne et al., 2017; Shapiro and Turner, 2014; Tarwater and Martin, 2001). It is also possible, as we suggested above, that there is higher host genetic diversity at the Rivendell natural population relative to the Tamarack and Lucille restored populations; if certain host genotypes are less effective at acquiring and/or transmitting anti-*Bd* microbes, then reduced host genetic diversity at the population level could result in fewer types of anti-*Bd* microbes acquired (Baquero et al., 2021; Jamieson, 2011; Parker et al., 2017).

We found general relationships of lower *Bd* loads and increased anti-*Bd* relative abundance in microbiomes from natural site frog populations relative to restored frog populations (Figure 5-2A; Figure 5-5C), which aligns with previous work that showed higher putative anti-*Bd* relative abundance predicted lower *Bd* loads (Chen et al., 2022). In our study, the predicted anti-*Bd* relative abundance of frog microbiomes was highly correlated with the relative abundance of a single putatively *Bd*-inhibitory bacterium that dominated microbiomes on average across all frog populations (Family Burkholderiaceae; Figure S5-12; Figure 5-5E). The observed negative population-level association between *Bd* loads and anti-*Bd* relative abundance, therefore, suggests that this bacterium may play a role in mitigating *Bd* loads. However, positive correlations between *Bd* loads and anti-*Bd* relative abundance within one natural site and one restored site suggest that higher relative abundances of this particular bacterium may not be effective at reducing *Bd* loads (Table S5-9; TableS5-10). Alternatively, since both of those sites also showed significant negative correlations between microbial Shannon diversity and *Bd* load (Table S5-9; Table S5-10), it is possible that increased relative abundance of this putatively anti-*Bd* microbe was observed because of reduced abundances of other *Bd*-inhibitory taxa, and that could have contributed to higher *Bd* loads.

We have found members of the Burkholderiaceae to be widely associated with *Rana sierrae* in this study and in our previous work, including evidence that several members increased significantly in normalized relative abundance during *Bd* infection (see *Chapter Two*, *Chapter Four*). However, without functional information we cannot know whether they are working to inhibit *Bd*. Some members of the Burkholderiaceae have been shown to suppress fungal pathogens while others are themselves pathogenic (Carrión et al., 2018; Coenye, 2014). While exact sequence matching to a known *Bd*-inhibitory bacterium provides some evidence that our dominant

Burkholderiaceae may have similar inhibitory abilities (Woodhams et al., 2015), isolation and functional assays are required to determine its interactions with *Bd* and *Rana sierrae*.

Distinct restoration protocols were not associated with differences in microbial dynamics

We examined whether specific restoration protocols were associated with frog skin microbiomes, *Bd* infections, and other host factors. We found that translocated and reintroduced frogs and their microbiomes did not differ significantly from each other for any metric tested. These findings differed from our original hypothesis. This could be because we compared microbiomes from the skin of frogs, which would likely be more influenced by the present environment than other host-associated microbiomes, like from the gut. For example, one study of Przewalski's horses found that fecal microbiomes from four horses born in captivity collected three years after release to a nature reserve maintained significantly reduced alpha diversity and distinct community structure relative to co-occurring horses born at the reserve or translocated there from another reserve (Metcalf et al., 2017). This suggests that horse fecal microbiomes were associated with captive versus wild birth location, while frog skin microbiomes in the present study were not.

As we expected, microbial community structure associated with the relocated frog populations (*i.e.*, reintroduced and translocated) each differed significantly from captive and source frog microbiomes. Frog skin microbiomes across restored site restoration types tended to harbor higher diversity, lower anti-*Bd* richness, and lower anti-*Bd* relative abundance compared to captive and source frogs. These anti-*Bd* predictions contrast with previous findings that anti-*Bd* richness and relative abundance increased over time in frog microbiomes after release from captivity to mesocosms (Kueneman et al., 2022). Our finding that translocated and reintroduced

frogs and their microbiomes never differed significantly from new recruits, however, agrees with recent evidence from that study and others that frog microbiomes shift to resemble local wild frog microbiomes after release from captivity (Estrada et al., 2022; Korpita et al., 2023; Kueneman et al., 2022) or after translocation (Bletz et al., 2016). Further, finding no difference between translocated and new recruit frogs agrees with previous findings that fecal microbiomes from Przewalski's horses born at a nature reserve did not differ significantly from horses translocated there from another reserve (Metcalf et al., 2017).

Together, our evidence indicates that restored frog skin microbiomes were not associated with history of specific restoration protocol. As ours is the first study comparing microbiomes of reintroduced and translocated frogs, further research is needed to confirm our observations.

Distinct *Bd* inoculation treatment groups were not associated with long term differences in microbial dynamics

Experimental *Bd* inoculations performed at the Zoo included a *Bd*⁺ treatment group (*i.e.*, exposed to *Bd*), and a *Bd*⁻ treatment group (*i.e.*, unexposed). Our previous work showed that ten days after treatment concluded, frog microbiome structure was significantly different between treatment groups, with *Bd*⁺ group frogs having higher community dispersion than *Bd*⁻ group frogs (*see Chapter Four*). We confirmed these same differences here using a different metric for community structure (Figure S5-7). However, after reintroduction, we found no difference in community structure or dispersion between treatment groups (Figure S5-7). This suggests that while there were short-term effects of treatment group on microbiomes, there were not long-term effects. Further work is needed to confirm these findings, as no previous studies have examined long-term differences in microbiomes associated with distinct *Bd* inoculation treatment groups.

Interestingly, after reintroduction we also found no difference in the frequency of *Bd* occurrence or mean *Bd* loads between inoculation treatment groups. *Bd* inoculation treatment groups have been shown to result in differences in resultant *Bd* loads previously, supporting the idea that *Bd*⁺ treatment improves host outcomes (McMahon et al 2014). However, there has also been evidence that *Bd* loads did not differ between inoculation treatment groups after release of *Rana draytonii* individuals to the wild (Adams, Bushell, and Grasso, 2022). We do not suggest that there is no long-term impact of *Bd* inoculation, as we do not have the data or sample sizes needed to assess differences in success. It is possible that survivorship bias could skew our findings as there may have been greater differences for *Bd*⁻ animals that succumbed to disease and were therefore never captured. Alternatively, it is possible that the efficacy of *Bd* inoculations was lost over time, a phenomenon found for vaccinations in many wildlife systems (Barnett and Civitello, 2020). Reintroduced frogs were sampled months to years after *Bd* inoculation in captivity, and our comparisons of treatment groups were averaged across time of sampling. Another possible explanation could relate to the fact that none of these reintroduced frogs experienced infection levels above the mortality threshold (Vredenburg et al 2010). As discussed above, all reintroduced animals may have already had what they needed to maintain low-moderate infections after release if their original source population previously underwent selection in genes associated with improved resistance (Knapp et al., 2023). We therefore suggest that studies of host genetics in these wild populations and an increased number of studies on long-term efficacy of *Bd* inoculation in multiple amphibian systems would elucidate our results.

Microbial dynamics for reintroduced frogs differed from natural variation over time

We observed that year of collection had significant effects on microbiome structure for all frogs, which aligns with previous evidence that frog microbiomes vary temporally (Ellison et al., 2021; Estrada et al., 2019). Therefore, to determine whether changes in microbiomes of reintroduced frogs after release were magnified due to disturbance caused by captivity, treatment, and relocation, we compared to changes in a natural population over an equivalent time frame (Shade et al 2012).

Reintroduced frogs experienced changes in microbial community displacement, *Bd* loads, and anti-*Bd* prediction metrics across years since their release that were significantly different from changes over time for naturally occurring frogs (Figure 5-7). As expected, most reintroduced frogs become *Bd* infected in the first year after release from captivity while natural site frogs had relatively stable *Bd* loads on average across years. Microbial community displacement was also greatest for reintroduced frogs in the first year after captivity, which aligns with our finding that microbiome structure was quite different in captivity compared to any wild collected frogs (Figure 5-3). Captivity has been shown to greatly impact microbiomes in many animal systems previously, and recently it has been shown that frog microbiomes shift rapidly after release from captivity (Bataille et al., 2016; Dallas and Warne, 2023; Estrada et al., 2022; Korpita et al., 2023; Kueneman et al., 2022, 2016). However, we found that this change in community structure was associated with reductions in anti-*Bd* richness and the anti-*Bd* relative abundance of reintroduced frogs' microbiomes one year after release relative to captivity. As discussed above, these changes were inconsistent with previous findings that these anti-*Bd* prediction metrics increased in reintroduced frogs (Kueneman et al., 2022). However, that study was methodologically different from ours in several ways. They studied a different species (*Atelopus varius*), their frogs had not undergone *Bd*

inoculation and antifungal treatment in captivity, they resampled each frog individual over a shorter time frame, and they soft-released frogs to mesocosms (*i.e.*, our wild released frogs could interact with more diverse substrates and other local frogs). This does not explain why we observed that frogs lost some of their anti-*Bd* function in the first year after reintroduction, but it does shed light on the fact that differences in study approach and associated outcomes require further investigation.

Over the second year after release, we found that community displacement and changes in *Bd* load on average had stabilized to natural levels for reintroduced frogs. At the same time, anti-*Bd* richness increased slightly on average and this increase was significantly greater than the change in anti-*Bd* richness observed for the natural population. This could indicate that after reintroduced frogs were infected, selection for anti-*Bd* microbes to prevent high levels of infection occurred (Bell et al., 2018; Woodhams et al., 2023). Interestingly, reintroduced frogs' microbial community displacement deviated from natural variation again over their third year at restored sites and was significantly greater than the change observed in the natural population. This could indicate that reintroduced frogs and their microbiomes may take years to fully adjust to their new site.

Next, we focused on the four reintroduced frog individuals that had been sampled at the Zoo to examine whether these frogs experienced similar changes in relative abundance of taxa relative to captivity (Figure 5-6B). However, these four individuals were already quite distinct in terms of taxonomic composition at the Zoo, and changes in relative abundances of taxa appeared to be unique for each individual. This result contrasts with a previous study that found microbiomes of four *Anaxyrus boreas boreas* experienced very similar transitions over the first two months after release from captivity (Korpita et al 2023). Those four toads were similar to each other in terms of

taxonomic composition in captivity, which may relate to the fact that they did not undergo *Bd* inoculation (*i.e.*, inoculation may have contributed to greater microbiome variability in the four *R. sierrae* here). Further, while taxonomic composition for the *A. boreas boreas* became more dissimilar to captivity over time, we did not observe that taxonomic composition of reintroduced *R. sierrae* appeared more dissimilar from captivity after one year at restored sites than after two months. It is possible that qualitative changes in taxonomic composition plateau two months post-release. Though in our population-level pairwise comparisons above, reintroduced frogs experienced community displacement greater than natural variation years after release. It is also important to note that we were only able to recapture four individuals that had been sampled at the Zoo and each was only recaptured once after release. Therefore, small sample size greatly limits our ability to draw conclusions. Research on transitions from captivity to the wild for a larger number of individuals and over a finer timescale is needed for *R. sierrae* to better understand the microbial community succession that occurs.

Limitations of the study

One limitation of our study was that, for the most part, we were unable to differentiate frog individuals captured from naturally occurring populations as we did not have permission to invasively insert unique PIT-tags for this highly endangered species. While uniquely tagged frogs at restored sites were never recaptured multiple times during the same visit, we did recapture a subset of those individuals multiple times. We found that individual identity did not explain variation in any metrics for frogs recaptured three or more times, likely because other factors were more important drivers of variation in host microbiomes and *Bd* loads at field sites. We therefore decided to conduct population-level analyses, treating resampling events as independent samples.

One benefit of this approach was that we did not treat restored population samples differently from natural population samples for which resampling was unknown.

Another limitation relates to our anti-*Bd* predictions. These predictions are based on sequence similarity to the 16S rRNA gene of bacterial isolates shown to have *Bd*-inhibitory function (Woodhams et al., 2015). While similarity in the 16S rRNA gene denotes close phylogenetic relationships among bacteria, which can indicate shared function (Langille et al., 2013), this does not necessarily mean that they will have the same inhibitory genes or functions (Becker et al., 2015; Jaspers and Overmann, 2004; Sevigny et al., 2019). In addition, there could be other *Bd*-inhibitory bacteria present on frogs that have yet to be isolated and functionally assayed, and thus would not have been considered in our anti-*Bd* predictions. Despite these caveats, the inhibitory database is still a useful tool to predict patterns in *Bd*-inhibitory function with amplicon data, which has been applied in several previous studies (Bletz et al., 2017; Chen et al., 2022; Jiménez et al., 2022; Kueneman et al., 2022, 2016; Muletz Wolz et al., 2018).

Conclusions

Amidst the current mass extinction crisis, efforts to restore populations and species have come to the forefront of conservation biology (Novacek and Cleland, 2001; Svenning, 2020). Recent evidence has elucidated the importance of host-associated microbial communities to host health across many systems, and accordingly, has brought attention to the importance of taking microbiomes into account when planning and executing restoration efforts (Redford et al., 2012; Trevelline et al., 2019). However, as of yet, restoration work has largely failed to incorporate microbiome research (Treveline et al., 2019). In this study, we identified some features of frog microbiomes that appeared to be reflective of the environmental factors also shaping relationships

in lake water microbiomes among site types and sites. Other differences among site types and sites, however, were unique to amphibian microbiomes, suggesting a primary association with host related factors. These microbiome metrics, *Bd* loads, and host body condition may be associated with the presence of management (*i.e.*, differences were primarily between restored and natural site types). This is likely related to a combination of factors, which we hypothesize to include restoration site choice (*i.e.*, influences of site parameters that affect host-pathogen-microbiome interactions), choice of restored frog individuals (*i.e.*, potential for reduced diversity of adaptive resistance genotypes at restored sites), and reduced host density at restored sites (*i.e.*, potentially reducing horizontal transmission of beneficial microbes). We suggest genotyping of natural and restored populations in the Desolation Wilderness would be beneficial, as some of our results are consistent with the hypothesis that restored populations sourced from a single natural site harbored reduced host genetic diversity, which could impede their success long term. Additionally, continued work on characterizing *Bd* inhibitory function of amphibian-associated bacteria will improve our ability to accurately estimate the anti-*Bd* function of microbiomes. Further work in this area is also needed to determine the role of the putatively anti-*Bd* Burkholderiaceae bacterium dominant on frogs across captive and wild populations of *R. sierrae* described here. Finally, the fact that we were unable to detect differences among microbiomes or *Bd* infections of hosts that underwent distinct inoculation treatments (*Bd*⁺ group exposed to *Bd* versus unexposed *Bd*⁻ group) or distinct restoration protocols (translocation versus reintroduction) suggests that they are difficult to manipulate long term. These results align with previous findings that frogs treated with probiotics did not harbor detectable levels of introduced microbes in the long term (Knapp et al., 2022; Woodhams et al., 2020, 2012), and could make it difficult to successfully manipulate microbiomes for the benefit of the host. Fortunately for *R. sierrae*, all wild populations examined

appeared to exhibit enzootic *Bd* dynamics despite the fact that *Bd* loads were higher for restored frogs than for naturally occurring frogs. However, these results may be unique to species for which animals used in restoration efforts are sourced from populations that have survived previous pathogen invasions.

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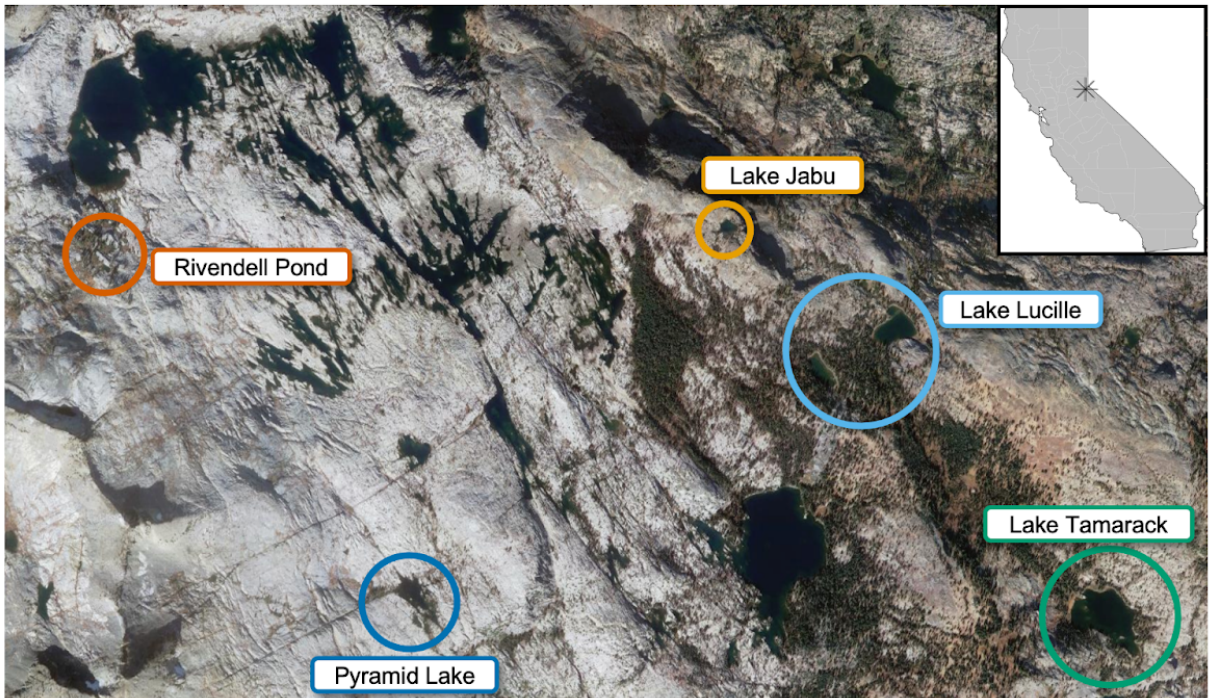
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Figures

Figure 5-1. Study design. (A) Field sites in the Desolation Wilderness (GoogleEarth; accessed January 2, 2024); inset map of California indicates the location of the study area; samples from each site were collected from the main lake body as well as stream inlets/outlets to the main lake; the Lake Lucille site area includes both Lake Lucille and Lake Margery as well as streams connecting these lakes; (B) Sample scheme across site types including natural, captive, and restored, across sites including the Rivendell Pond and Pyramid Lake natural sites, the captive Zoo site, and the Lake Tamarack, Lake Lucille, and Lake Jabu restored sites, and across restoration types including source frogs (adult frogs from natural sites that were used as a source for frogs at captive and restored sites), captive frogs (adult frogs reared at the SF Zoo), translocated frogs (adult frogs moved directly from source sites to restored sites), reintroduced frogs (frogs reared at the SF Zoo from egg masses or juveniles captured at one natural site and released to two of the restored sites as adults), and new recruit frogs (adult frogs born at restored sites that were descendants of translocated or reintroduced frogs); the number of samples for each frog restoration type are shown by site, as are the number of water samples per site (only samples in the rarefied dataset are included here); arrows indicate the management strategies taking place across sites; grey hashed box indicates sites/samples included in analyses of the impacts of restoration type: for these analyses we examine only two of the restored sites that harbored both translocated and reintroduced frogs in addition to new recruits (*i.e.*, Tamarack and Lucille restored sites), the captive Zoo site, and only one of the natural sites (Rivendell) that acted as the source for all captive-reared frogs and for frogs translocated to those two restored sites.

A



B

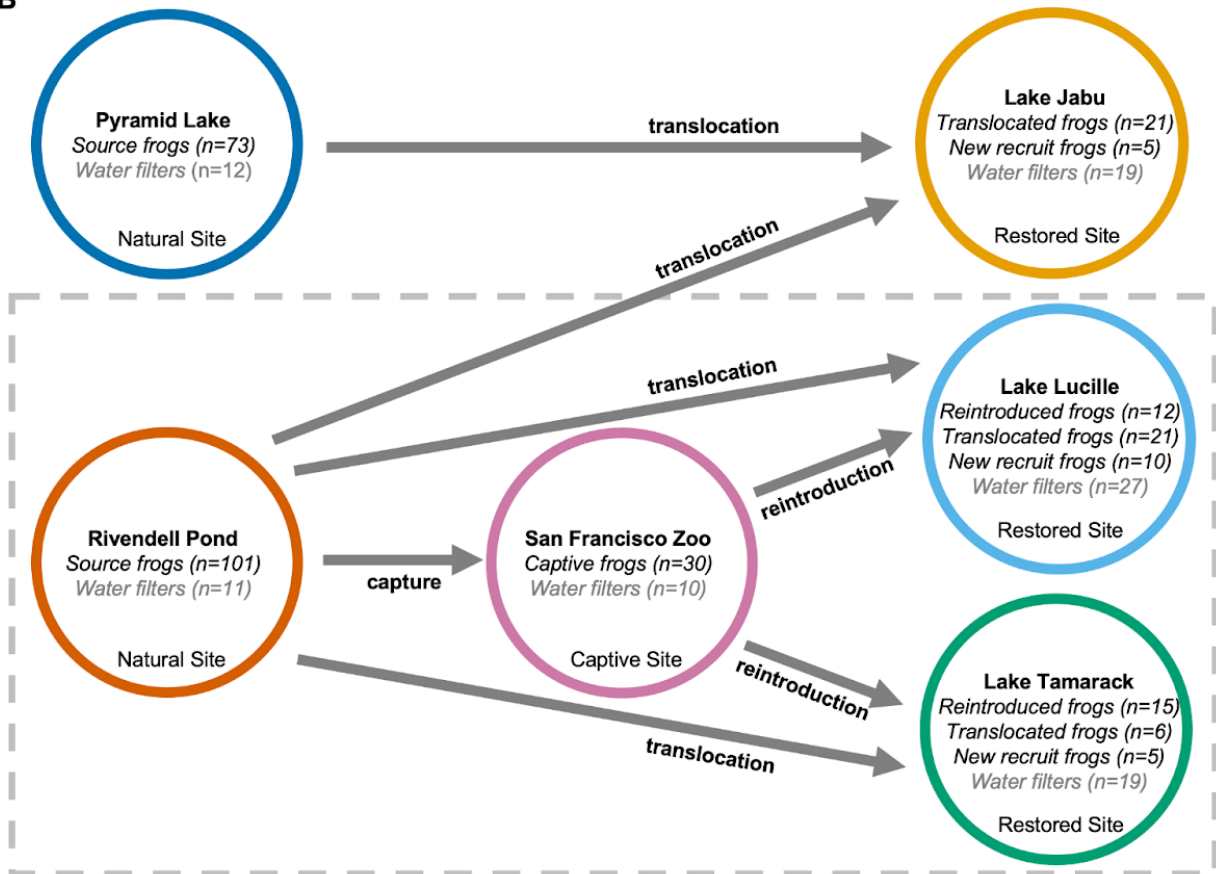


Figure 5-2. Mean *Bd* loads of wild-captured frog samples across sites and restoration types.

(A) *Bd* loads (*i.e.*, logZE) of samples that tested positive for *Bd* infection by field site (*i.e.*, Rivendell, Pyramid, Tamarack, Lucille, and Jabu) across site types (*i.e.*, captive, naturally occurring, and restored); boxplots and points are grouped and colored by site and are shaped by site type; Results of ANOVA and *post hoc* Tukey tests are shown; Mean *Bd* loads by site: Rivendell = $1.68 \text{ logZE} \pm 0.74 \text{ sd}$, Pyramid = $1.83 \text{ logZE} \pm 0.75 \text{ sd}$, Tamarack = $2.35 \text{ logZE} \pm 0.87 \text{ sd}$, Lucille = $2.29 \text{ logZE} \pm 1.01 \text{ sd}$, Jabu = $1.79 \text{ logZE} \pm 0.72 \text{ sd}$; **(B)** *Bd* loads of samples that tested positive for *Bd* infection by field restoration type (*i.e.*, source, translocated, reintroduced, and new recruits) across Rivendell, Tamarack, and Lucille; Boxplots and points are grouped and colored by restoration type and are shaped by site; Results of Kruskal-Wallis and *post hoc* Dunn tests are shown; Mean *Bd* loads by restored site restoration type: translocated = $2.39 \text{ logZE} \pm 1.2 \text{ sd}$, reintroduced = $2.21 \text{ logZE} \pm 0.69 \text{ sd}$, new recruit = $2.34 \text{ logZE} \pm 0.91 \text{ sd}$; **(A-B)** Level of significance of *post hoc* tests: “*” = $p < 0.05$; “**” = $p < 0.01$; “***” = $p < 0.001$.

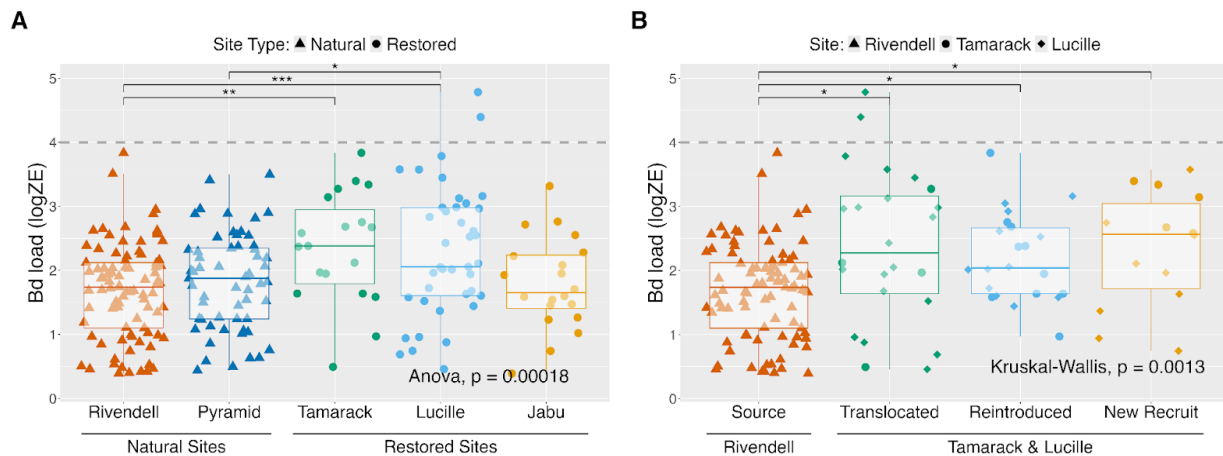


Figure 5-3. Microbiome structure across sites and restoration types. PCoAs of weighted Unifrac distance ordinations; ellipses represent 95% confidence intervals; **(A)** community structure of all frog samples colored and grouped by site and shaped by site type (*i.e.*, captive, naturally occurring, and restored); frog sample community structure differed significantly by site (PERMANOVA, $R^2=0.157$, $p<0.001$; Table S5-15) for most site comparisons (pairwise PERMANOVAs, $p<0.05$; Table S5-16) except for the Lucille restored site versus the Tamarack restored site (pairwise PERMANOVA, $p>0.05$; Table S5-16), and both the Jabu restored site and the Zoo had significantly higher community dispersion than the Rivendell natural site (TukeyHSD, $p<0.01$; Table S5-18) **(B)** community structure of frog samples grouped and colored by restoration type (*i.e.*, captive, source, translocated, reintroduced, and new recruit) and shaped by site; frog sample community structure was significantly different among restoration types (PERMANOVA, $R^2=0.142$, $p<0.001$; Table S5-24), with captive and source frogs differing significantly from all other restoration types (pairwise PERMANOVAs, $p<0.001$; Table S5-25), however there were no significant differences among translocated, reintroduced, and new recruit frog microbiomes (pairwise PERMANOVAs, $p>0.05$; Table S5-25); captive frogs harbored higher community dispersion than source frogs (TukeyHSD, $p<0.01$), with no other differences in dispersion among restoration types (Table S5-27; **(C)** community structure of all water samples colored and grouped by site and shaped by site type; community structure for water samples differed significantly by site (PERMANOVA, $R^2=0.308$, $p<0.001$; Table S5-19) for most site comparisons (pairwise PERMANOVAs, $p<0.05$; Table S5-20) except for the Rivendell natural site versus the Pyramid natural site (pairwise PERMANOVA, $p>0.05$; Table S5-20), with no differences in community dispersion among sites after p-value correction in *post hoc* tests (TukeyHSD, $p>0.05$; Table S5-22).

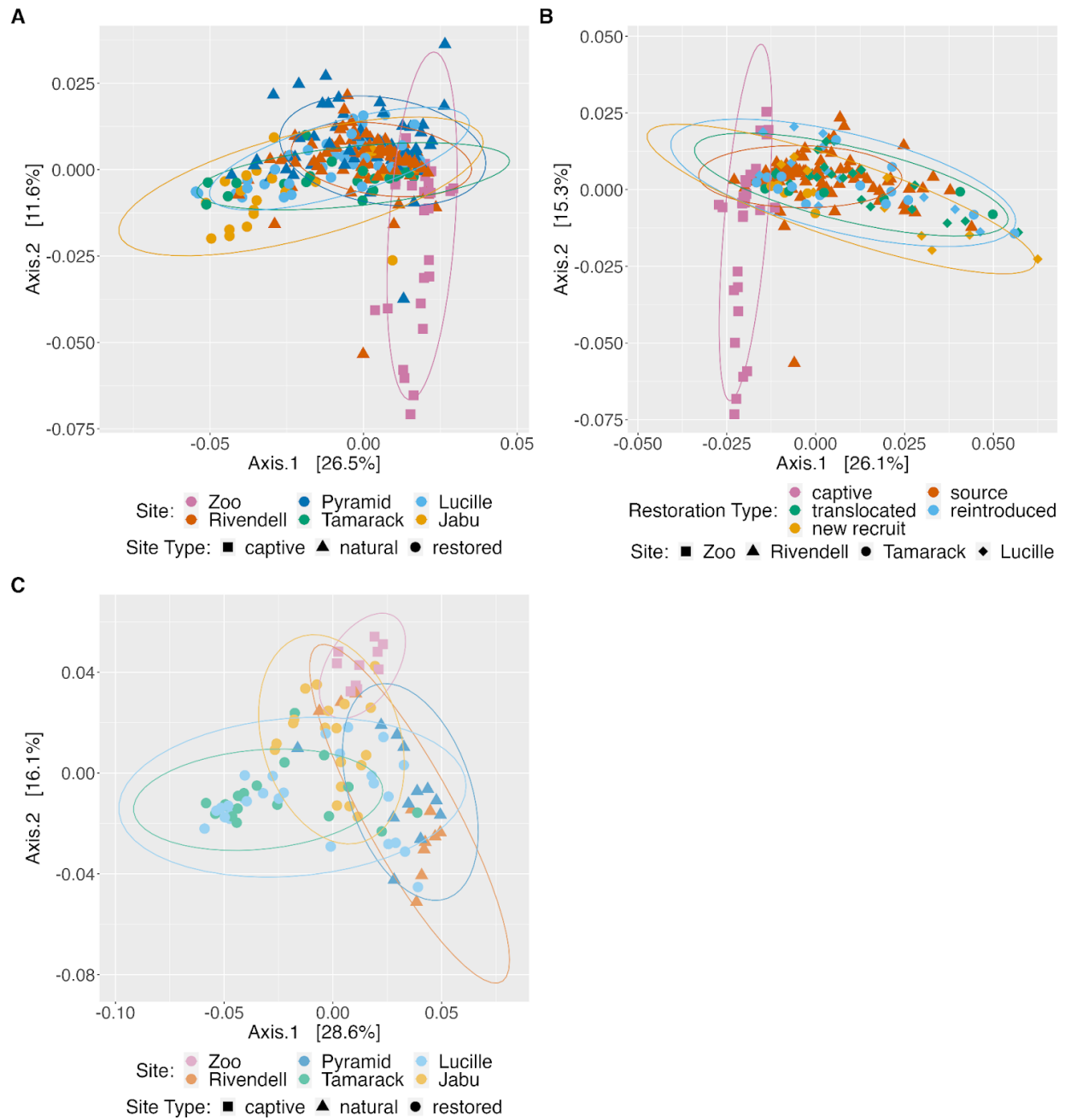


Figure 5-4. Microbiome diversity across sites and restoration types. Within-sample diversity in terms of observed richness (**A–C**) and Shannon diversity (**D–F**); (**A, D**) Boxplots and points for all frog samples are colored and grouped by site and shaped by site type (*i.e.*, captive, natural, or restored); (**B, E**) Boxplots and points for frog samples from the Zoo captive site, Rivendell natural site, and Tamarack and Lucille restored sites are colored and grouped by restoration type (*i.e.*, captive, source, translocated, reintroduced, and new recruits) and shaped by site; (**C, F**) Boxplots and points for all water samples are colored and grouped by site and shaped by site type; (**A–E**) Results of Kruskal-Wallis and *post hoc* Dunn tests are shown; (**F**) Results of ANOVA and *post hoc* Tukey tests are shown; (**A–F**) Level of significance of *post hoc* tests: “*” = $p < 0.05$; “**” = $p < 0.01$; “***” = $p < 0.001$.



Figure 5-5. Frog-associated anti-*Bd* predictions across sites and restoration types. Anti-*Bd* predictions are based on >99% sequence identity match to sequences from a database of bacteria shown to inhibit *Bd* growth (Woodhams et al., 2015); Here we show three metrics of anti-*Bd* function across frog samples; **(A–B)** Anti-*Bd* richness (*i.e.*, the observed number of putatively *Bd*-inhibitory microbes); **(C–D)** Anti-*Bd* relative abundance (*i.e.*, the summed relative abundances of putatively *Bd*-inhibitory bacteria); **(E–F)** Relative abundance of SV1, an unclassified member of the Burkholderiaceae with 100% sequence identity match to a *Bd*-inhibitory taxon (Woodhams et al., 2015); **(A,C,E)** Boxplots and points are grouped and colored by site and shaped by site type (*i.e.*, captive, naturally occurring, and restored); **(B,D,F)** Boxplots and points are grouped and colored by restoration type (*i.e.*, captive, source, translocated, reintroduced, and new recruits) and shaped by site; **(A–F)** Results of Kruskal-Wallis and *post hoc* Dunn tests are shown (“*” = $p < 0.05$; “**” = $p < 0.01$; “***” = $p < 0.001$).

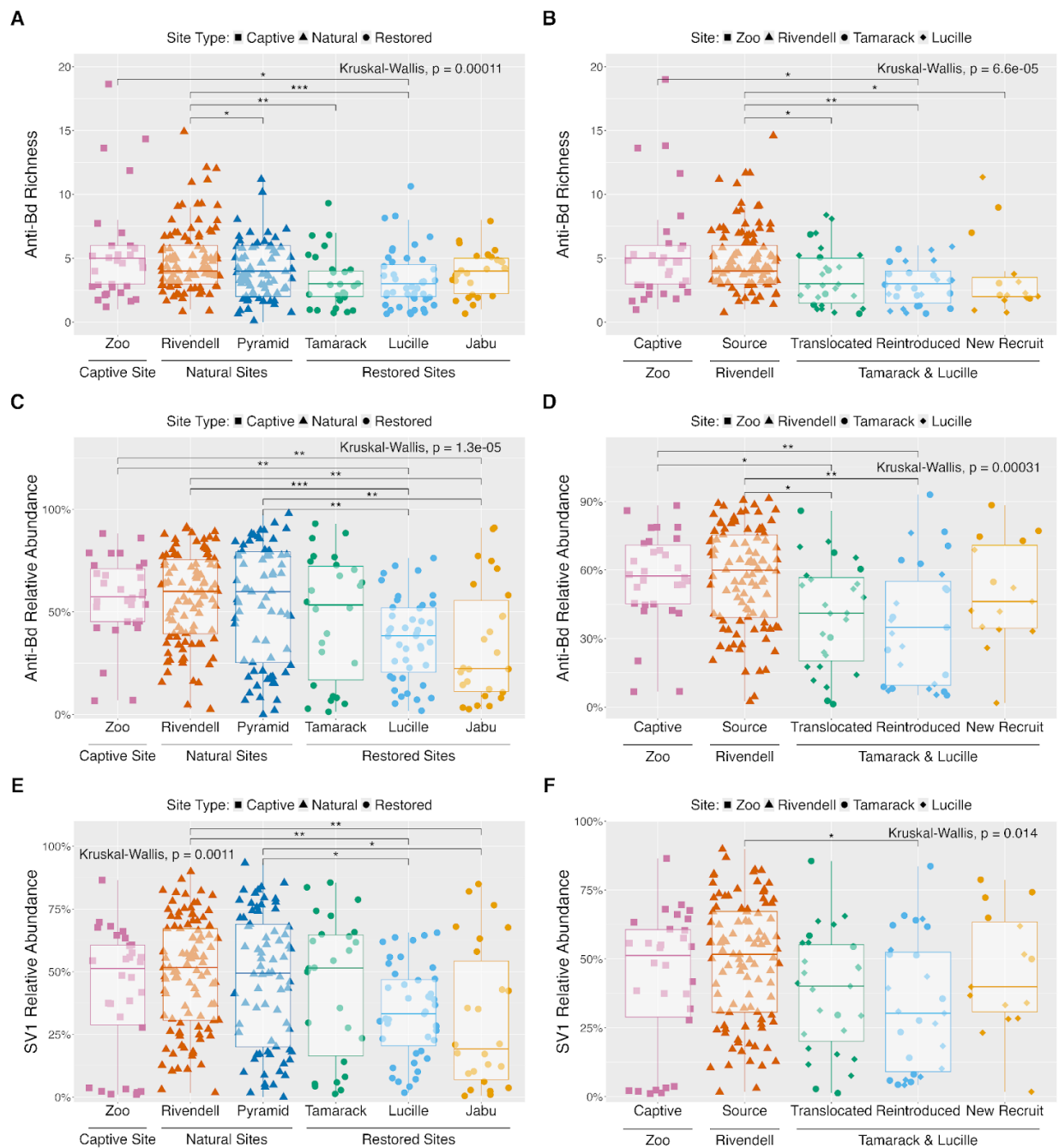


Figure 5-6. Relative abundance of bacterial phyla and families across captive, reintroduced, and new recruit frog samples. (A) Sample relative abundance bar charts faceted by restoration type and site for captive frog samples from the Zoo and for reintroduced and new recruit frog samples from Tamarack and Lucille restored sites; (B) Sample relative abundance bar charts faceted by frog individual and site for four unique frog individuals sampled at the Zoo that were recaptured and sampled after reintroduction at Tamarack or Lucille restored sites; Reintroduced frogs were either recaptured zero years since release (0 YSR; recaptured ~2 months after release) or one year since release (1 YSR; recaptured ~13 months after release); Recapture 1 was part of the *Bd*- treatment group from experimental inoculation at the Zoo (*i.e.*, unexposed to *Bd*; *see Chapter Four*), and tested negative for *Bd* infection at both timepoints; Recaptures 2, 3, and 4 were part of the *Bd*+ inoculation treatment group (*i.e.*, exposed to *Bd*); Recapture 2 had a *Bd* load of 0.60 logZE at the Zoo but tested negative for *Bd* infection after reintroduction; Recapture 3 tested negative for *Bd* infection at both timepoints; Recapture 4 tested negative for *Bd* infection at the Zoo and had a *Bd* load of 1.58 logZE after reintroduction; (A–B) Stacked bar charts were generated using microshades v. 1.11(Dahl et al., 2022); Taxa are colored by phylum, and families within each phylum are colored with unique shades of the associated phylum color; Within each stacked bar chart, taxa are ordered first by mean relative abundance of phyla and then by mean relative abundance of families within each phylum (*i.e.*, taxa with higher relative abundance appear lower in each stacked bar); Colors associated with each phylum are the same across figure panels, but family color assignments within each phylum may vary between panels as the order is based on mean relative abundance within each subset of samples.

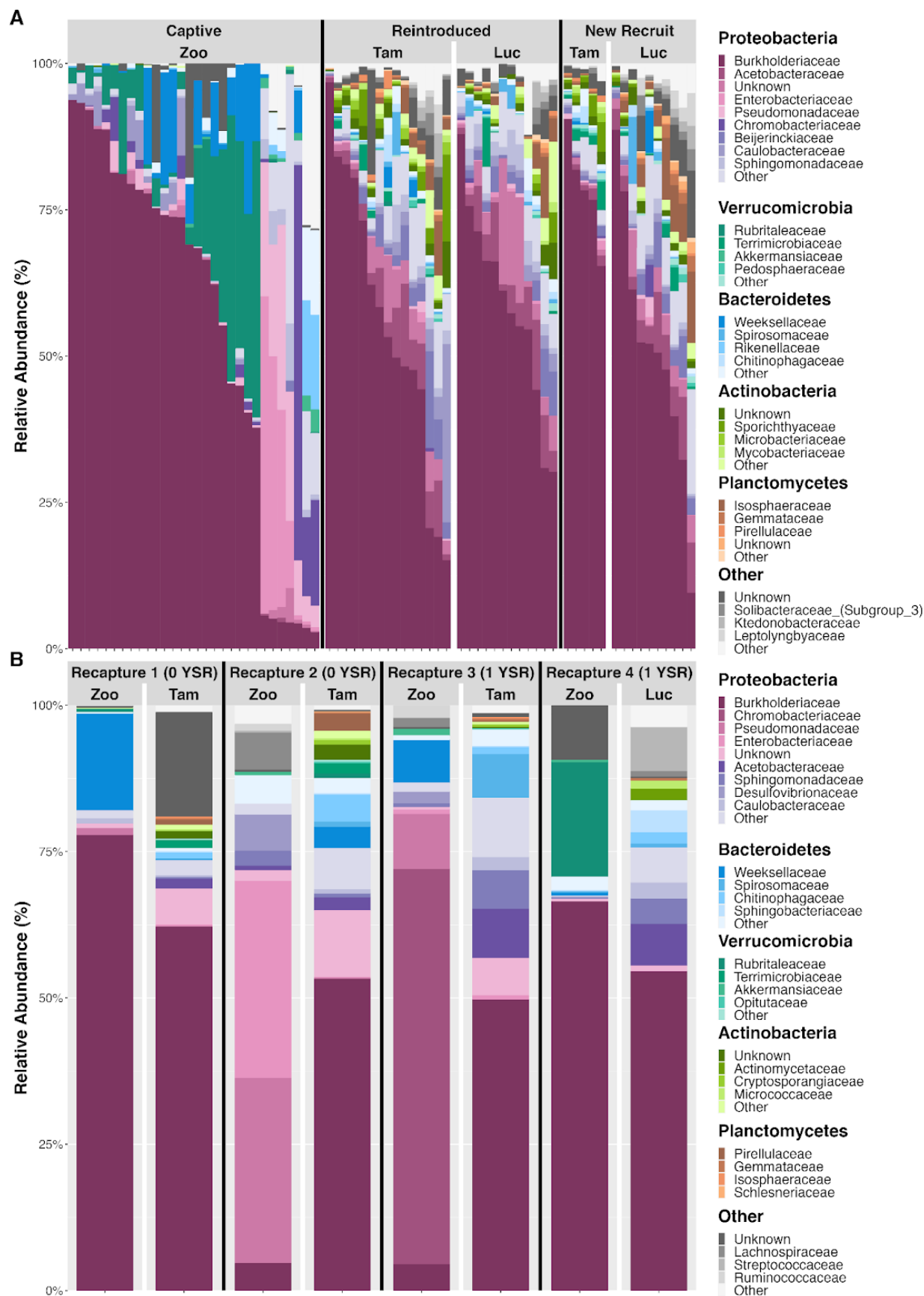
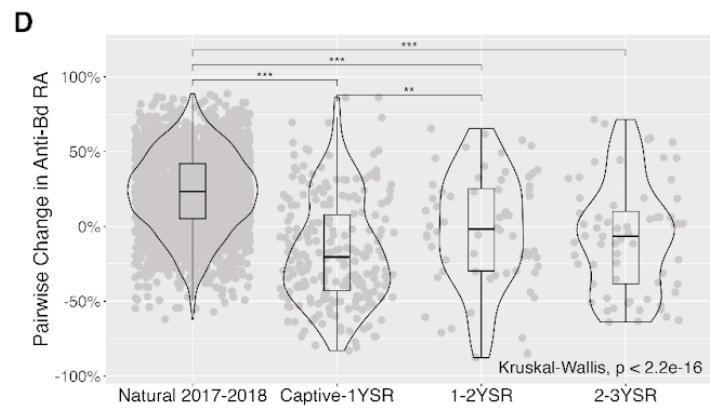
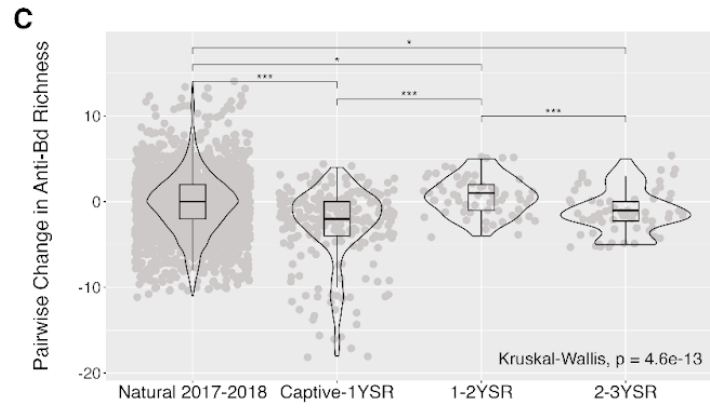
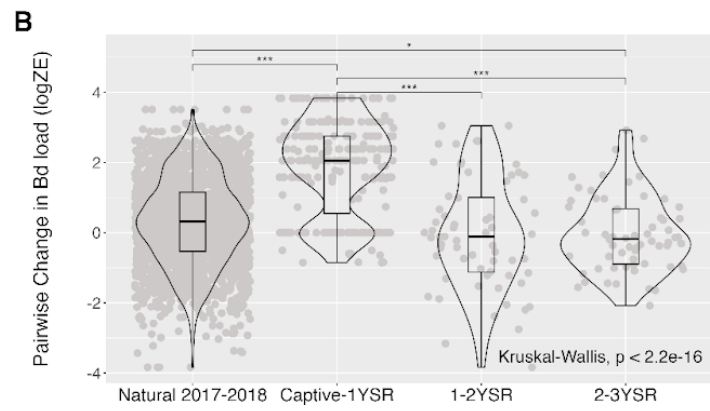
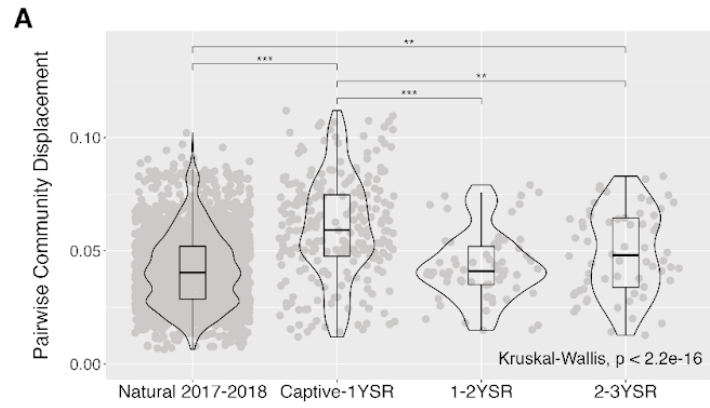


Figure 5-7. Changes in microbial communities over time for reintroduced frogs relative to natural variation. Here we compare the change in **(A)** community displacement (*i.e.*, pairwise weighted Unifrac distances), **(B)** *Bd* loads (*i.e.*, logZE), **(C)** putative anti-*Bd* richness, and **(D)** putative anti-*Bd* relative abundance across years since release (YSR) time intervals for reintroduced frogs at restored sites and across collection years for source frogs at one natural site (Rivendell, the source population for captive populations that were reintroduced); Points represent pairwise distance metrics **(A)** or pairwise differences in metrics **(B–D)** for frog samples collected from distinct subsequent time intervals; Results of Kruskal-Wallis and *post hoc* Dunn tests are shown (“*” = $p < 0.05$; “**” = $p < 0.01$; “***” = $p < 0.001$); Frog sample values for *Bd* load, anti-*Bd* richness, and anti-*Bd* relative abundance within each time period are shown in Figure S5-15.



Supplementary Material

Table S5-1. Wild frog captures and sample counts. Table includes all wild-captured frog samples; The total number of frog samples includes the number of samples from untagged frogs and the number of samples from tagged frogs; The number of unique tagged frogs is based on the number of unique PIT-tag IDs; We show the number of unique tagged frogs captured more than once and more than twice; Two reintroduced frogs from the Tamarack restored site were sampled four and five times, respectively; Three reintroduced frogs from the Lucille restored site were sampled three, three, and four times, respectively; Three translocated frogs from the Jabu restored site were sampled three, three, and four times, respectively; We note that we could not determine whether untagged frogs had been sampled multiple times.

Site Type	Site	Restoration type	No. frog samples	No. samples from untagged frogs	No. samples from tagged frogs	No. unique tagged frogs	No. frogs sampled more than once	No. frogs sampled more than twice
Natural	Rivendell	source	101	74	27	25	2	0
	Pyramid	source	73	58	15	15	0	NA
Restored	Tamarack	translocated	6	0	6	6	0	NA
		reintroduced	15	0	15	7	3	2
		new recruit	5	0	5	3	2	0
	Lucille	translocated	21	0	21	18	3	0
		reintroduced	12	0	12	5	3	3
		new recruit	10	3	7	6	1	0
	Jabu	translocated	21	0	21	10	7	3
		new recruit	5	2	3	3	0	0
Overall			269	137	132	98	21	8

Table S5-2. Several metrics do not differ significantly among unique frog individuals.

Samples from eight unique PIT-tagged frog individuals that were each sampled more than twice (n=29) are included for analyses of all metrics except for logZE (*i.e.*, *Bd* load) for which we only examined samples that tested positive for *Bd* infection from seven of those unique individuals that had more than two *Bd* infected samples (n=23); Host SMI is the scaled mass index of frogs, a measure of host body condition; Observed richness, Shannon diversity, anti-*Bd* richness, and anti-*Bd* relative abundance are all metrics describing frog-associated microbiomes; All p-values were >0.05 and thus were not significant (“ns”).

Metric	Statistical test	Test statistic	df	p-value
Host SMI	Kruskal-Wallis	8.9191	7	0.2585 ns
logZE	Kruskal-Wallis	12.143	6	0.05885 ns
Observed richness	Kruskal-Wallis	5.7753	7	0.5662 ns
Shannon diversity	ANOVA	1.528	7	0.212 ns
Anti- <i>Bd</i> richness	Kruskal-Wallis	9.2716	7	0.2337 ns
Anti- <i>Bd</i> relative abundance	Kruskal-Wallis	10.991	7	0.139 ns

Table S5-3. Frog-associated microbiome structure does not differ among unique frog individuals. Results of sequential PERMANOVA test (9999 permutations) on weighted Unifrac dissimilarities for samples from eight unique PIT-tagged frog individuals that were each sampled more than twice (n=29) are shown; The level of significance for each p-values is indicated (“ns” = $p>0.05$; “*” = $p<0.05$; “**” = $p<0.01$; “***” = $p<0.001$). After controlling for site differences, there was no significant effect of individual identity on community structure.

Terms	df	R ²	F	p-value
Site	2	0.19868	3.5213	0.0006 ***
PIT.Tag	5	0.20887	1.4808	0.0612 ns
Residual	21	0.59244		
Total	28	1		

Table S5-4. Differences in host-associated metrics among site types and sites. All frog samples were included in analyses; Kruskal-wallis test statistic is chi-squared; Dunn test statistic is Z; p-values associated with multiple comparisons were corrected using the BH method; The level of significance for each p-values is indicated (“ns” = $p > 0.05$; “*” = $p < 0.05$; “**” = $p < 0.01$; “***” = $p < 0.001$); *post hoc* Dunn tests for site pairwise comparisons shown in Figure S5-3 (SMI), Figure 5-4 (observed richness and Shannon), and Figure 5-5 (anti-*Bd* richness, anti-*Bd* relative abundance, and SV1 relative abundance).

Metric	Group Factor	Comparison	Statistical test	Test statistic	df	p-value
Host SMI	Site Type	all	Kruskal-Wallis	16.1510	2	0.0003 ***
		captive - natural	post hoc Dunn	-2.1130		0.03456 *
		captive - restored	post hoc Dunn	-3.7300		0.0006 ***
		natural - restored	post hoc Dunn	-2.8440		0.0067 ***
Observed microbial richness	Site	all	Kruskal-Wallis	18.0470	5	0.0029 **
		captive - natural	post hoc Dunn	63.4400	2	1.675e-14 ***
		captive - restored	post hoc Dunn	-7.3528		2.91e-13 ***
		natural - restored	post hoc Dunn	-7.7362		3.07e-14 ***
Microbial Shannon diversity	Site type	all	Kruskal-Wallis	-1.3062		0.1915 ns
		captive - natural	post hoc Dunn	63.8660	5	1.926e-12 ***
		captive - restored	post hoc Dunn	31.4560	2	1.477e-07 ***
		natural - restored	post hoc Dunn	-3.1564		0.0016 **
Microbial anti- <i>Bd</i> richness	Site type	captive - restored	post hoc Dunn	-5.2910		3.65e-07 ***
		natural - restored	post hoc Dunn	-3.7948		0.0002 ***
		all	Kruskal-Wallis	35.1000	5	1.437e-06 ***
		captive - natural	post hoc Dunn	16.8260	2	0.0002 ***
Microbial anti- <i>Bd</i> relative abundance	Site type	captive - natural	post hoc Dunn	0.2781		0.7810 ns
		captive - restored	post hoc Dunn	2.6511		0.0120 *
		natural - restored	post hoc Dunn	3.9215		0.0003 ***
		all	Kruskal-Wallis	25.4700	5	0.0001 ***
SV1 relative abundance	Site type	all	Kruskal-Wallis	26.2070	2	2.038e-06 ***
		captive - natural	post hoc Dunn	0.0675		0.9460 ns
		captive - restored	post hoc Dunn	3.0903		0.0030 **
		natural - restored	post hoc Dunn	4.9687		2.02e-06 ***
SV1 relative abundance	Site	all	Kruskal-Wallis	30.2730	5	1.303e-05 ***
		captive - natural	post hoc Dunn	15.7080	2	0.0004 ***
		captive - restored	post hoc Dunn	-1.2517		0.3160 ns
		natural - restored	post hoc Dunn	1.2225		0.2215 ns
SV1 relative abundance	Site	natural - restored	post hoc Dunn	3.9468		0.0002 ***
		all	Kruskal-Wallis	20.3350	5	0.0011 **

Table S5-5. Differences in host-associated metrics among restoration types. Includes frog samples from the Zoo captive site (captive frogs), the Rivendell natural site (source frogs), and the Tamarack and Lucille restored sites (translocated, reintroduced, and new recruit frogs); Test statistic for Kruskal-Wallis is chi-squared; The level of significance for each p-values is indicated (“ns” = $p > 0.05$; “*” = $p < 0.05$; “**” = $p < 0.01$; “***” = $p < 0.001$); *Post hoc* Dunn test results for restoration types shown in Figure S5-3 (SMI), Figure 5-4 (observed richness and Shannon), and Figure 5-5 (anti-*Bd* richness, anti-*Bd* relative abundance, and SV1 relative abundance).

Metric	Group Factor	Statistical test	Test statistic	df	p-value
Host SMI	Restoration type	Kruskal-Wallis	18.24	4	0.0011 **
Observed microbial richness	Restoration type	Kruskal-Wallis	60.02	4	2.873e-12 ***
Microbial Shannon diversity	Restoration type	Kruskal-Wallis	28.476	4	9.989e-06 ***
Microbial anti- <i>Bd</i> richness	Restoration type	Kruskal-Wallis	24.422	4	6.573e-05 ***
Microbial anti- <i>Bd</i> rel. abundance	Restoration type	Kruskal-Wallis	21.081	4	0.0003 ***
SV1 relative abundance	Restoration type	Kruskal-Wallis	12.553	4	0.0137 *

Table S5-6. Frequency of *Bd* occurrence across wild-collected frog samples. Summary of *Bd* status data across field site types, field sites, and restoration types within field sites; Pr(no *Bd*) is the probability of detecting no *Bd* positive samples if the true prevalence were 11% (a conservative estimate of endemic *Bd* prevalence (Talley et al 2015)) given the sample size, which represents our power to detect *Bd* (level of significance is indicated: “ns” = not significant, with $p \geq 0.05$; “*” = $p < 0.05$; “**” = $p < 0.01$; “***” = $p < 0.001$); Pr(no *Bd*) was not significant for restoration types within the restored sites due to low sample sizes, thus estimates for frequency of *Bd* occurrence for these populations may be inaccurate.

Site Type	Site	Restoration Type	No. Positive	No. Negative	Total	Percent infected (95% CI)	Pr(no <i>Bd</i>)
Natural			146	28	174	83.91% (77.59–89.03 CI)	1.56E-09 ***
	Rivendell	Source	89	12	101	88.12% (80.17–93.71 CI)	7.73E-06 ***
	Pyramid	Source	57	16	73	78.08% (66.86–86.92 CI)	2.02E-04 ***
Restored			80	15	95	84.21% (75.30–90.88 CI)	1.56E-05 ***
	Tamarack		19	7	26	73.08% (52.21–88.43 CI)	4.83E-02 *
		Translocated	4	2	6	66.67% (22.28–95.67)	4.97E-01 ns
		Reintroduced	10	5	15	66.67% (38.38–88.18 CI)	1.74E-01 ns
		New Recruit	5	0	5	100.00% (47.82–100.00 CI)	5.58E-01 ns
	Lucille		41	2	43	95.35% (84.19–99.43 CI)	6.66E-03 **
		Translocated	20	1	21	95.24% (76.18–99.88 CI)	8.65E-02 ns
		Reintroduced	12	0	12	100.00% (73.54–100.00 CI)	2.47E-01 ns
		New Recruit	9	1	10	90.00% (55.50–99.75 CI)	3.12E-01 ns
	Jabu		20	6	26	76.92% (56.35–91.03 CI)	4.83E-02 *
		Translocated	18	3	21	85.71% (63.66–96.95 CI)	8.65E-02 ns
		New Recruit	2	3	5	40.00% (5.27–85.34 CI)	5.58E-01 ns
Overall			226	43	269	84.01% (79.08–88.18 CI)	2.43E-14 ***

Table S5-7. *Bd* status does not differ among field site types and sites. *Bd* status is a categorical variable with two levels: *Bd* infected and uninfected; Analyses included *Bd* sample data from all wild-collected frog samples (Table S5-6); The level of significance for each p-value is indicated (“ns” = $p > 0.05$; “*” = $p < 0.05$).

Metric	Group Factor	Comparison	Statistical test	Odds ratio	95% CI	p-value
<i>Bd</i> status	Site type	all	Fisher's Exact Test	0.977759	0.457-2.0242	1.000 ns
	Site	all	Fisher's Exact Test	NA	NA	0.021 *
		Pyramid-Rivendell	Pairwise Fisher Test	0.4824251	0.193-1.177	0.190 ns
		Pyramid-Jabu	Pairwise Fisher Test	1.0680244	0.300-3.399	1.000 ns
		Pyramid-Lucille	Pairwise Fisher Test	0.1758739	0.020-0.814	0.108 ns
		Pyramid-Tamarack	Pairwise Fisher Test	1.3088116	0.394-4.024	0.749 ns
		Rivendell-Jabu	Pairwise Fisher Test	2.2086493	0.605-7.341	0.330 ns
		Rivendell-Lucille	Pairwise Fisher Test	0.3638886	0.038-1.750	0.330 ns
		Rivendell-Tamarack	Pairwise Fisher Test	2.7063043	0.794-8.694	0.169 ns
		Jabu-Lucille	Pairwise Fisher Test	0.1672472	0.015-1.042	0.153 ns
		Jabu-Tamarack	Pairwise Fisher Test	1.2232211	0.291-5.300	1.000 ns
		Lucille-Tamarack	Pairwise Fisher Test	7.3178337	1.239-78.593	0.108 ns

Table S5-8. *Bd* loads differ by field site type and site. *Bd* load was measured in units of logZE; Analyses included samples that tested positive for *Bd* infection from all wild populations; The level of significance for each p-values is indicated (“ns” = $p > 0.05$; “*” = $p < 0.05$; “**” = $p < 0.01$; “***” = $p < 0.001$); *Post hoc* Tukey tests for site comparisons are shown in Figure 5-2.

Metric	Group Factor	Comparison	Statistical test	Test statistic	df	p-value
logZE	Site type	all	Kruskal-Wallis	11.128	1	0.0009 ***
	Site	all	ANOVA	5.804	4	0.0002 ***

Table S5-9. Correlation of wild-captured frog *Bd* loads with other factors. Only samples that tested positive for *Bd* infection from wild-captured frogs were included in analyses; SMI is host scaled body mass index, a measure of host body condition; Shannon diversity, anti-*Bd* richness, and anti-*Bd* relative abundance are all metrics describing frog-associated microbiomes; Corr. Test indicates the correlation test employed (either Spearman or Pearson); Corr. Coef. indicates the correlation coefficient of the respective correlation test (Spearman's rho or Pearson's r); P-values were corrected for multiple comparisons of *Bd* loads within each data subset ("all" or by site) using the BH method; The level of significance for each p-values is indicated ("ns" = $p > 0.05$; "*" = $p < 0.05$; "***" = $p < 0.01$; "****" = $p < 0.001$).

Metric	Site	Corr. Test	Corr. Coef.	p-value
SMI	All	Spearman	0.1717	0.0377 *
	Rivendell	Pearson	0.0456	0.6717 ns
	Pyramid	Pearson	0.2009	0.1377 ns
	Tamarack	Pearson	0.3405	0.3074 ns
	Lucille	Pearson	0.1097	0.6596 ns
	Jabu	Pearson	-0.0867	0.7162 ns
Shannon	All	Spearman	-0.1332	0.0454 *
	Rivendell	Spearman	-0.0631	0.6717 ns
	Pyramid	Spearman	-0.3793	0.0072 **
	Tamarack	Spearman	-0.4088	0.3074 ns
	Lucille	Spearman	-0.0497	0.7572 ns
	Jabu	Spearman	-0.1429	0.7162 ns
Anti-<i>Bd</i> Richness	All	Spearman	-0.1511	0.0377 *
	Rivendell	Spearman	-0.1156	0.6717 ns
	Pyramid	Spearman	-0.2149	0.1377 ns
	Tamarack	Pearson	-0.1027	0.6758 ns
	Lucille	Spearman	0.3496	0.0905 ns
	Jabu	Pearson	-0.1379	0.7162 ns
Anti-<i>Bd</i> Relative Abundance	All	Spearman	0.146	0.0377 *
	Rivendell	Spearman	0.0854	0.6717 ns
	Pyramid	Spearman	0.4251	0.0039 **
	Tamarack	Pearson	0.2499	0.4029 ns
	Lucille	Pearson	0.3144	0.0905 ns
	Jabu	Spearman	0.2677	0.7162 ns

Table S5-10. Correlation of wild-captured frog *Bd* loads (across *Bd* statuses) with other factors. Both samples that tested positive and negative for *Bd* infection from wild-captured frogs were included in analyses; SMI is host scaled body mass index, a measure of host body condition; Shannon diversity, anti-*Bd* richness, and anti-*Bd* relative abundance are all metrics describing frog-associated microbiomes (*see Methods*); Corr. Test indicates the correlation test employed (either Spearman or Pearson); Corr. Coef. indicates the correlation coefficient of the respective correlation test (Spearman's rho or Pearson's r); P-values were corrected for multiple comparisons of *Bd* loads within each data subset ("all" or by site) using the BH method; The level of significance for each p-values is indicated ("ns" = $p > 0.05$; "*" = $p < 0.05$; "***" = $p < 0.01$; "****" = $p < 0.001$).

Metric	Site	Corr. Test	Corr. Coef.	p-value
SMI	All	Spearman	0.2403	0.0003 ***
	Rivendell	Spearman	0.0834	0.5636 ns
	Pyramid	Spearman	0.412	0.0013 **
	Tamarack	Spearman	0.4243	0.0410 *
	Lucille	Pearson	0.0966	0.6438 ns
	Jabu	Pearson	-0.037	0.8575 ns
Shannon	All	Spearman	-0.1402	0.0286 *
	Rivendell	Spearman	-0.0806	0.5636 ns
	Pyramid	Spearman	-0.3261	0.0074 **
	Tamarack	Spearman	-0.5734	0.0044 **
	Lucille	Spearman	-0.0726	0.6438 ns
	Jabu	Spearman	0.0447	0.8575 ns
Anti-<i>Bd</i> Richness	All	Spearman	-0.0367	0.5489 ns
	Rivendell	Spearman	-0.0919	0.5636 ns
	Pyramid	Spearman	0.0286	0.8102 ns
	Tamarack	Spearman	0.1414	0.4909 ns
	Lucille	Spearman	0.2925	0.1153 ns
	Jabu	Pearson	0.0687	0.8575 ns
Anti-<i>Bd</i> Relative Abundance	All	Spearman	0.1644	0.0137 *
	Rivendell	Spearman	0.0572	0.5697 ns
	Pyramid	Spearman	0.3215	0.0074 **
	Tamarack	Spearman	0.6217	0.0028 **
	Lucille	Pearson	0.2918	0.1153 ns
	Jabu	Spearman	0.1018	0.8595 ns

Table S5-11. *Bd* status does not differ by frog restoration type. *Bd* status is a categorical variable with two levels: *Bd* infected and uninfected; Analysis included *Bd* sample data from the Rivendell natural site (source frogs), and from the Tamarack and Lucille restored sites (translocated, reintroduced, and new recruit frogs; Table S5-6); The test result was not significant (“ns”; $p > 0.05$).

Metric	Group Factor	Comparison	Statistical test	p-value
<i>Bd</i> status	Restoration type	all	Fisher's Exact Test	0.7456 ns

Table S5-12. *Bd* loads differ by frog restoration type. *Bd* load is measured in units of logZE; Analysis included samples that tested positive for *Bd* infection from the Rivendell natural site (source frogs), and from the Tamarack and Lucille restored sites (translocated, reintroduced, and new recruit frogs); The level of significance based on the p-value is indicated (“ns” = $p > 0.05$; “*” = $p < 0.05$; “**” = $p < 0.01$; “***” = $p < 0.001$); Sample logZE values and results of *post hoc* Dunn tests are shown in Figure 5-2.

Metric	Group Factor	Comparison	Statistical test	Chi-squared	df	p-value
logZE	Restoration type	all	Kruskal-Wallis	15.786	3	0.0013 **

Table S5-13. Microbiome structure differs between frogs and water for all sites. Results of PERMANOVA tests (9999 permutations) on weighted Unifrac dissimilarities by sample category (*i.e.*, frogs versus water) are shown; Analyses included all frog and water samples; The level of significance for each p-values is indicated (“ns” = $p > 0.05$; “*” = $p < 0.05$; “**” = $p < 0.01$; “***” = $p < 0.001$).

Site	df	R ²	F	p-value
Zoo	1	0.08658	3.6021	0.0086 **
Rivendell	1	0.22214	31.413	0.0001 ***
Pyramid	1	0.22097	23.543	0.0001 ***
Tamarack	1	0.34081	22.232	0.0001 ***
Lucille	1	0.27772	26.147	0.0001 ***
Jabu	1	0.13667	6.8072	0.0001 ***

Table S5-14. Microbial community dispersion differs between frogs and water for most sites.

Results of permutests (9999 permutations) on dispersion in weighted Unifrac dissimilarities by sample category (*i.e.*, frogs versus water) are shown; Analyses included all frog and water samples; The level of significance for each p-values is indicated (“ns” = $p > 0.05$; “*” = $p < 0.05$; “***” = $p < 0.01$; “****” = $p < 0.001$).

Site	df	F	p-value
Zoo	1	4.625	0.0347 *
Rivendell	1	17.153	0.0004 ***
Pyramid	1	3.3649	0.0682 ns
Tamarack	1	4.816	0.0324 *
Lucille	1	34.07	0.0001 ***
Jabu	1	6.331	0.0152 *

Table S5-15. PERMANOVA models for frog microbiome structure. Microbial community structure was measured using weighted Unifrac distances; Analyses included all frog samples; Sequential model were run with 9999 permutations; The level of significance for each p-values is indicated (“ns” = $p>0.05$; “*” = $p<0.05$; “**” = $p<0.01$; “***” = $p<0.001$).

Model	Term	df	R ²	F	p-value
1	Site type	2	0.1135	19.7204	0.0001 ***
	Site	3	0.0435	5.0408	0.0001 ***
	Residual	293	0.843		
	Total	298	1		
2	Site	5	0.157	11.6368	0.0001 ***
	SMI	1	0.0146	5.3914	0.0004 ***
	Year	2	0.0244	4.5132	0.0001 ***
	Site:SMI	5	0.0204	1.5136	0.0272 *
	Site:Year	6	0.0334	2.0602	0.0007 ***
	Residual	278	0.7503		
	Total	297	1		

Table S5-16. Frog microbiome structure differs in most pairwise comparisons of site types and sites. Results of pairwise PERMANOVAs are shown; Microbial community structure was measured using weighted Unifrac distances; Analyses included all frog samples; Tests were run with 9999 permutations; P-values were corrected using the BH method; The level of significance for each p-values is indicated (“ns” = $p > 0.05$; “*” = $p < 0.05$; “**” = $p < 0.01$; “***” = $p < 0.001$).

Group	Comparison	F	R ²	p-value
Site type	captive - natural	20.51	0.0922	0.0001 ***
	captive - restored	20.48	0.1427	0.0001 ***
	natural - restored	16.75	0.059	0.0001 ***
Site	Jabu - Lucille	8.034	0.1071	0.0001 ***
	Jabu - Pyramid	16.45	0.145	0.0001 ***
	Jabu - Rivendell	18.04	0.1261	0.0001 ***
	Jabu - Tamarack	4.55	0.0835	0.0032 **
	Jabu - Zoo	14.95	0.2168	0.0001 ***
	Lucille - Pyramid	7.61	0.0625	0.0001 ***
	Lucille - Rivendell	8.7	0.0577	0.0001 ***
	Lucille - Tamarack	2.19	0.0316	0.0502 ns
	Lucille - Zoo	16.77	0.191	0.0001 ***
	Pyramid - Rivendell	4.97	0.0281	0.0001 ***

Table S5-17. Frog microbiome dispersion differs by site type and site. Results of permutests are shown; Dispersion in weighted Unifrac distances by group; Analyses included all frog samples; Tests were run with 9999 permutations; The level of significance for each p-values is indicated (“ns” = $p > 0.05$; “*” = $p < 0.05$; “**” = $p < 0.01$; “***” = $p < 0.001$).

Group	df	F	p-value
Site Type	2	9.273	0.0005 ***
Site	5	4.3827	0.0011 **

Table S5-18. Frog microbiome dispersion differs in few pairwise comparisons of site types and sites. *Post hoc* Tukey tests for pairwise comparisons of dispersion in weighted Unifrac distances among site types and sites are shown; All frog samples were included in analyses; p-values were corrected using the FWER method; The level of significance for each p-values is indicated (“ns” = $p > 0.05$; “*” = $p < 0.05$; “**” = $p < 0.01$; “***” = $p < 0.001$).

Group	Comparison	diff	lwr	upr	p-value
Site type	natural-captive	-0.0112	-0.0194	-0.0031	0.0037 **
	restored-captive	-0.0034	-0.0121	0.0052	0.6140 ns
	restored-natural	0.0078	0.0025	0.0130	0.0016 **
Site	Lucille-Jabu	-0.0068	-0.0190	0.0054	0.6043 ns
	Pyramid-Jabu	-0.0103	-0.0215	0.0010	0.0948 ns
	Rivendell-Jabu	-0.0132	-0.0239	-0.0024	0.0072 **
	Tamarack-Jabu	-0.0090	-0.0226	0.0046	0.4049 ns
	Zoo-Jabu	-0.0003	-0.0135	0.0128	1.0000 ns
	Pyramid-Lucille	-0.0035	-0.0129	0.0060	0.8969 ns
	Rivendell-Lucille	-0.0064	-0.0153	0.0026	0.3180 ns
	Tamarack-Lucille	-0.0022	-0.0144	0.0100	0.9950 ns
	Zoo-Lucille	0.0065	-0.0052	0.0181	0.6087 ns
	Rivendell-Pyramid	-0.0029	-0.0104	0.0046	0.8805 ns
	Tamarack-Pyramid	0.0012	-0.0100	0.0125	0.9996 ns
	Zoo-Pyramid	0.0099	-0.0007	0.0206	0.0827 ns
	Tamarack-Rivendell	0.0041	-0.0067	0.0149	0.8813 ns
	Zoo-Rivendell	0.0128	0.0026	0.0230	0.0048 **
	Zoo-Tamarack	0.0087	-0.0045	0.0219	0.4061 ns

Table S5-19. PERMANOVA models for water-associated microbiome structure. Microbial community structure was measured using weighted Unifrac distances; Analyses included all water samples; Sequential models were run with 9999 permutations; The level of significance for each p-values is indicated (“ns” = $p>0.05$; “*” = $p<0.05$; “**” = $p<0.01$; “***” = $p<0.001$).

Model	Term	df	R ²	F	p-value
1	Site type	2	0.2283	15.171	0.0001 ***
	Site	3	0.0796	3.525	0.0001 ***
	Residual	92	0.69216		
	Total	97	1		
2	Site	5	0.3078	11.6368	0.0001 ***
	Year	1	0.02	2.7136	0.0105 *
	Residual	91	0.6721		
	Total	97	1		

Table S5-20. Water-associated microbiome structure differs in most pairwise comparisons of site types and sites. Results of pairwise PERMANOVAs for water microbiome structure by site types and sites are shown; Microbial community structure was measured using weighted Unifrac distances; Analyses included all water samples; Models were run with 9999 permutations. P-values were corrected using the BH method; The level of significance for each p-values is indicated (“ns” = $p > 0.05$; “*” = $p < 0.05$; “**” = $p < 0.01$; “****” = $p < 0.001$).

Group	Comparison	F	R ²	p-value
Site type	captive - natural	12.5871	0.2888	0.0001 ***
	captive - restored	13.5771	0.1568	0.0001 ***
	natural - restored	15.0589	0.1490	0.0001 ***
Site	Jabu - Lucille	5.4679	0.1105	0.0003 ***
	Jabu - Pyramid	7.5438	0.2064	0.0002 ***
	Jabu - Rivendell	7.2546	0.2058	0.0003 ***
	Jabu - Tamarack	7.9065	0.1801	0.0002 ***
	Jabu - Zoo	9.0818	0.2517	0.0002 ***
	Lucille - Pyramid	7.6794	0.1719	0.0002 ***
	Lucille - Rivendell	6.7545	0.1580	0.0003 ***
	Lucille - Tamarack	2.6118	0.0560	0.0324 *
	Lucille - Zoo	11.6445	0.2496	0.0002 ***
	Pyramid - Rivendell	0.9187	0.0419	0.4202 ns

Table S5-21. Water-associated microbial community dispersion differs by site type and site.

Results of permutests are shown; Dispersion in weighted Unifrac dissimilarities by group; All water samples were included in analysis; Tests were run with 9999 permutations; The level of significance for each p-values is indicated (“ns” = $p > 0.05$; “*” = $p < 0.05$; “**” = $p < 0.01$; “***” = $p < 0.001$).

Group	df	F	p-value
Site Type	2	4.433	0.0157 *
Site	5	2.5598	0.0321 *

Table S5-22. Water-associated microbial community dispersion largely does not differ in pairwise comparisons of site types and sites. *Post hoc* Tukey tests for dispersion in weighted Unifrac dissimilarities are shown; All water samples were included in analyses; p-values were corrected using the FWER method; The level of significance for each p-values is indicated (“ns” = $p > 0.05$; “*” = $p < 0.05$; “**” = $p < 0.01$; “***” = $p < 0.001$).

Group	Comparison	diff	lwr	upr	p-value
Site type	natural-captive	-0.0135	-0.0253	-0.0018	0.0199 *
	restored-captive	-0.0062	-0.0167	0.0044	0.3462 ns
	restored-natural	0.0073	-0.0002	0.0149	0.0576 ns
Site	Lucille-Jabu	0.0064	-0.0067	0.0196	0.7124 ns
	Pyramid-Jabu	-0.0056	-0.0219	0.0106	0.9126 ns
	Rivendell-Jabu	0.0005	-0.0161	0.0172	1.0000 ns
	Tamarack-Jabu	-0.0033	-0.0176	0.0109	0.9839 ns
	Zoo-Jabu	0.0123	-0.0049	0.0295	0.3073 ns
	Pyramid-Lucille	-0.0121	-0.0274	0.0032	0.2025 ns
	Rivendell-Lucille	-0.0059	-0.0217	0.0098	0.8816 ns
	Tamarack-Lucille	-0.0098	-0.0229	0.0034	0.2671 ns
	Zoo-Lucille	0.0058	-0.0105	0.0221	0.9023 ns
	Rivendell-Pyramid	0.0062	-0.0122	0.0245	0.9242 ns
	Tamarack-Pyramid	0.0023	-0.0139	0.0185	0.9984 ns
	Zoo-Pyramid	0.0179	-0.0009	0.0368	0.0718 ns
	Tamarack-Rivendell	-0.0038	-0.0205	0.0128	0.9847 ns
	Zoo-Rivendell	0.0118	-0.0075	0.0310	0.4832 ns
	Zoo-Tamarack	0.0156	-0.0016	0.0328	0.0975 ns

Table S5-23. PERMANOVA model for wild-captured frog microbiome structure.

Community structure was measured in weighted Unifrac distances; Analysis included all wild-collected frog samples from field sites; Sequential model was run with 9999 permutations; p-values were corrected using the BH method; The level of significance for each p-values is indicated (“ns” = $p > 0.05$; “*” = $p < 0.05$; “**” = $p < 0.01$; “***” = $p < 0.001$).

Term	df	R ²	F	p-value
Site	4	0.1144	9.2716	0.0001 ***
logZE	1	0.0084	2.7228	0.0107 *
SMI	1	0.0159	5.1449	0.0003 ***
Year	2	0.0283	4.5877	0.0001 ***
Site:SMI	4	0.0194	1.5699	0.0364 *
Site:Year	6	0.0424	2.2896	0.0002 ***
logZE:SMI	1	0.0061	1.9773	0.0479 *
Residual	248	0.7651		
Total	267	1.0000		

Table S5-24. Frog microbiome structure differs by restoration type. Results of PERMANOVA model are shown; Microbial community structure was measured using weighted Unifrac distances; Frog samples from the Zoo captive site (captive frogs), the Rivendell natural site (source frogs), and the Tamarack and Lucille restored sites (translocated, reintroduced, and new recruit frogs) are included; Model was run with 9999 permutations; The level of significance indicated (“ns” = $p > 0.05$; “*” = $p < 0.05$; “**” = $p < 0.01$; “****” = $p < 0.001$).

Term	df	R ²	F	p-value
Restoration type	4	0.1422	8.0834	0.0001 ***
Residual	195	0.8578		
Total	199	1.0000		

Table S5-25. Differences in frog microbiome structure in pairwise comparisons of restoration types. Results of *post hoc* pairwise PERMANOVAs are shown; Microbial community structure was measured using weighted Unifrac distances; Frog samples from the Zoo captive site (captive frogs), the Rivendell natural site (source frogs), and the Tamarack and Lucille restored sites (translocated, reintroduced, and new recruit frogs) were included; Tests were run with 9999 permutations; P-values were corrected using the BH method; The level of significance for each p-values is indicated (“ns” = $p > 0.05$; “*” = $p < 0.05$; “**” = $p < 0.01$; “***” = $p < 0.001$).

Group	Comparison	F	R ²	p-value
Restoration type	captive - new recruit	7.4238	0.1472	0.0002 ***
	captive - reintroduced	10.8446	0.1647	0.0002 ***
	captive - source	17.2819	0.1181	0.0002 ***
	captive - translocated	12.7190	0.1878	0.0002 ***
	new recruit - reintroduced	1.5879	0.0382	0.1700 ns
	new recruit - source	5.2189	0.0438	0.0007 ***
	new recruit - translocated	1.1381	0.0277	0.3067 ns
	reintroduced - source	4.8478	0.0370	0.0005 ***
	reintroduced - translocated	0.7194	0.0136	0.6423 ns
	source - translocated	6.3815	0.0482	0.0002 ***

Table S5-26. Frog microbiome dispersion differs by restoration type. Results of permutest for community dispersion are shown; Microbiome dispersion was the dispersion (*i.e.*, variance) in weighted Unifrac distances; Frog samples from the Zoo captive site (captive frogs), the Rivendell natural site (source frogs), and the Tamarack and Lucille restored sites (translocated, reintroduced, and new recruit frogs) are included; Test run with 9999 permutations. The level of significance is indicated (“ns” = $p > 0.05$; “*” = $p < 0.05$; “**” = $p < 0.01$; “***” = $p < 0.001$).

Group	df	F	p-value
Restoration type	4	3.9128	0.0046 **

Table S5-27. Frog microbiome dispersion largely does not differ in pairwise comparisons of restoration types. *Post hoc* Tukey test results are shown; Microbial community dispersion was the dispersion (*i.e.*, variance) in weighted Unifrac distances; Frog samples from the Zoo captive site (captive frogs), the Rivendell natural site (source frogs), and the Tamarack and Lucille restored sites (translocated, reintroduced, and new recruit frogs) were included; p-values were corrected using the FWER method; The level of significance for each p-values is indicated (“ns” = $p > 0.05$; “*” = $p < 0.05$; “**” = $p < 0.01$; “***” = $p < 0.001$).

Group	Comparison	diff	lwr	upr	p-value
Restoration type	new recruit-captive	-0.0078	-0.0223	0.0068	0.5816 ns
	reintroduced-captive	-0.0052	-0.0174	0.0069	0.7614 ns
	source-captive	-0.0129	-0.0224	-0.0033	0.0025 **
	translocated-captive	-0.0082	-0.0204	0.0040	0.3473 ns
	reintroduced-new recruit	0.0025	-0.0122	0.0173	0.9898 ns
	source-new recruit	-0.0051	-0.0178	0.0076	0.8048 ns
	translocated-new recruit	-0.0004	-0.0152	0.0144	1.0000 ns
	source-reintroduced	-0.0076	-0.0176	0.0023	0.2199 ns
	translocated-reintroduced	-0.0030	-0.0155	0.0095	0.9661 ns
	translocated-source	0.0047	-0.0053	0.0146	0.6970 ns

Table S5-28. Frog microbiome structure differs by *Bd* inoculation treatment group in captivity but not after reintroduction. PERMANOVA model results are shown; Microbial community structure was measured using weighted Unifrac distances; Captive frog samples were collected at the Zoo ($n_{Bd+}=17$, $n_{Bd-}=13$); Reintroduced frog samples were collected from frogs after release to Tamarack or Lucille restored sites ($n_{Bd+}=17$, $n_{Bd-}=10$); Models were run with 9999 permutations; The level of significance for each p-values is indicated (“ns” = $p>0.05$; “*” = $p<0.05$; “**” = $p<0.01$; “***” = $p<0.001$).

Samples	Term	df	R ²	F	p-value
captive	<i>Bd</i> inoculation treatment group	1	0.0875	2.6848	0.0363 *
	Residual	28	0.9125		
	Total	29	1.0000		
reintroduced	<i>Bd</i> inoculation treatment group	1	0.0707	1.9017	0.0758 ns
	Residual	25	0.9293		
	Total	26	1.0000		

Table S5-29. Captive frog microbiome dispersion differs between *Bd* inoculation treatment groups. Results of permutest are shown; Community dispersion was measured as dispersion in weighted Unifrac distances; Captive frog samples were collected at the Zoo ($n_{Bd+}=17$, $n_{Bd-}=13$); Test was run with 9999 permutations; The level of significance is indicated (“ns” = $p>0.05$; “*” = $p<0.05$; “**” = $p<0.01$; “***” = $p<0.001$).

Samples	Group	df	F	p-value
captive	<i>Bd</i> inoculation treatment group	1	4.6955	0.0395 *

Table S5-30. Differences in water-associated microbial metrics among site types and sites.

All water samples included; Kruskal-wallis test statistic is chi-squared; Dunn test statistic is Z; ANOVA test statistic is F; P-values associated with multiple comparisons were corrected using the BH method; The level of significance for each p-values is indicated (“ns” = $p > 0.05$; “*” = $p < 0.05$; “**” = $p < 0.01$; “***” = $p < 0.001$); Results of *post hoc* tests for site pairwise comparisons shown in Figure 5-4 (Observed richness and Shannon) and Figure S5-11 (anti-*Bd* richness, anti-*Bd* relative abundance, and SV1 relative abundance).

Metric	Group Factor	Comparison	Statistical test	Test statistic	df	p-value
Observed richness	Site type	all	Kruskal-Wallis	11.5720	2	0.0031 **
		captive - natural	post hoc Dunn	-3.3871		0.0021 **
		captive - restored	post hoc Dunn	-2.4337		0.0224 *
		natural - restored	post hoc Dunn	1.8806		0.0600 ns
Shannon diversity	Site type	all	Kruskal-Wallis	13.6930	5	0.0177 *
		captive - natural	post hoc Dunn	-4.0025		0.0002 ***
		captive - restored	post hoc Dunn	-2.4793		0.0132 *
		natural - restored	post hoc Dunn	2.7777		0.0082 **
Anti- <i>Bd</i> richness	Site type	all	ANOVA	4.7680	5	0.0006 ***
		captive - natural	Kruskal-Wallis	22.4250	2	1.35e-05 ***
		captive - restored	post hoc Dunn	4.0800		6.75e-05 ***
		natural - restored	post hoc Dunn	4.6836		8.46e-06 ***
Anti- <i>Bd</i> relative abundance	Site type	all	Kruskal-Wallis	0.1875	5	0.8512 ns
		captive - natural	Kruskal-Wallis	24.5370	2	0.0002 ***
		captive - restored	post hoc Dunn	34.2490		3.65e-08 ***
		natural - restored	post hoc Dunn	3.0083		0.0039 **
SV1 relative abundance	Site type	all	post hoc Dunn	5.4969		1.16e-07 ***
		captive - natural	post hoc Dunn	2.9993		0.0027 **
		captive - restored	Kruskal-Wallis	35.5280	5	1.18e-06 ***
		natural - restored	Kruskal-Wallis	10.8090	2	0.0045 **
	Site type	all	post hoc Dunn	2.1410		0.0484 *
		captive - natural	post hoc Dunn	3.2340		0.0037 **
		captive - restored	post hoc Dunn	1.1852		0.2360 ns
		natural - restored	Kruskal-Wallis	11.5390	5	0.0417 *

Table S5-31. Taxonomy of predicted anti-*Bd* community members. Assigned taxonomy (SILVA v. 132) for all 155 ASVs that clustered at 99% similarity with sequences from a database of bacterial isolates shown to inhibit *Batrachochytrium* pathogens (Woodhams et al., 2015); Predicted anti-*Bd* community members were distributed across five phyla: Actinobacteria (n=25), Bacteroidetes (n=21), Deinococcus-Thermus (n=1), Firmicutes (n=1), Proteobacteria (n=107; with >20 unique ASVs in each of the following families: Burkholderiaceae (n=23), Enterobacteriaceae (n=28), and Pseudomonadaceae (n=24)).

Phylum	Family	Genus	Species	ASV(s)
Actinobacteria	Nocardiaceae	Gordonia	Unclassified	SV1996
		Rhodococcus	Unclassified	SV592
	Cellulomonadaceae	Cellulomonas	Unclassified	SV6204
	Dermabacteraceae	Brachybacterium	muris	SV1415
	Intrasporangiaceae	Unclassified	Unclassified	SV7578
	Microbacteriaceae	Agreia	Unclassified	SV3602
		Cryobacterium	Unclassified	SV4981
		Fronidhabitans	Unclassified	SV5896
		Galbitalea	Unclassified	SV187, SV322
		Microbacterium	Unclassified	SV5463
		Unclassified	Unclassified	SV107, SV638, SV2676, SV8195
	Micrococcaceae	Arthrobacter	citreus	SV7223
			Unclassified	SV4693
		Citricoccus	Unclassified	SV8553
		Kocuria	Unclassified	SV1437
		Micrococcus	Unclassified	SV5144
		Paeniglutamicibacter	Unclassified	SV1349
		Pseudarthrobacter	Unclassified	SV993, SV1359, SV13926
	Streptomycetaceae	Streptacidiphilus	Unclassified	SV7567
Bacteroidetes	Chitinophagaceae	Chitinophaga	arvensicola	SV1226
	Spirosomaceae	Flectobacillus	roseus	SV1798
	Flavobacteriaceae	Flavobacterium	Unclassified	SV1322, SV1510, SV13586
			greenlandense	SV111
			solis	SV12592
	Weeksellaceae	Chryseobacterium	Unclassified	SV29, SV232, SV394, SV748, SV1597, SV2164, SV2377, SV3620
			cryoconitis	SV1848
			himalayensis	SV5740

			Unclassified	SV2325, SV2718, SV7219
		Sphingobacterium	Unclassified	SV6603
Deinococcus- Thermus	Deinococcaceae	Deinococcus	radiodurans	SV7000
Firmicutes	Bacillaceae	Bacillus	Unclassified	SV7927
Proteobacteria	Caulobacteraceae	Brevundimonas	Unclassified	SV1733, SV4147
	Beijerinckiaceae	Bosea	massiliensis	SV2142
	Kaistiaceae	Kaistia	Unclassified	SV5392
	Rhizobiaceae	Allorhizobium- Neorhizobium- Pararhizobium- Rhizobium	Unclassified	SV2483, SV5539
		Unclassified	Unclassified	SV1111
	Sphingomonadaceae	Novosphingobium	Unclassified	SV147
		Sphingomonas	Unclassified	SV150, SV486, SV1049
		Unclassified	Unclassified	SV750
	Aeromonadaceae	Aeromonas	lacus popoffii	SV2210 SV9448
			Unclassified	SV434, SV1632, SV5953
	Burkholderiaceae	Burkholderia- Caballeronia- Paraburkholderia	Unclassified	SV669, SV3430
		Comamonas	testosteroni	SV5005
		Delftia	Unclassified	SV1366
		Duganella	Unclassified	SV2081, SV11718, SV13795
		Herbaspirillum	Unclassified	SV1413
		Janthinobacterium	lividum	SV3150
			Unclassified	SV860, SV955
		Massilia	alkalitolerans oculi	SV2171 SV11730
			Unclassified	SV3944, SV4600, SV7756
		Paucibacter	Unclassified	SV865
		Ralstonia	Unclassified	SV1763, SV7876
		Unclassified	Unclassified	SV1, SV79, SV5963
		Variovorax	Unclassified	SV2392
	Chitinibacteraceae	Chitinibacter	Unclassified	SV2608
		Iodobacter	fluviatilis	SV3489
	Chromobacteriaceae	Aquitalea	Unclassified	SV2054
	Enterobacteriaceae	Citrobacter	Unclassified	SV880, SV1232
		Enterobacter	Unclassified	SV569, SV2009
		Erwinia	Unclassified	SV1624, SV1965
		Hafnia- Obesumbacterium	Unclassified	SV516, SV610, SV1414
		Klebsiella	Unclassified	SV446, SV1310, SV1369
		Pantoea	Unclassified	SV2720

	Rahnella	aquaticus	SV1135, SV2025
		Unclassified	SV6500
	Raoultella	Unclassified	SV173, SV345, SV5888
	Serratia	fonticola	SV654
		Unclassified	SV597, SV1361, SV1403, SV3166
	Unclassified	Unclassified	SV560, SV963, SV3052, SV3740
Moraxellaceae	Acinetobacter	Unclassified	SV121, SV174, SV469, SV8409, SV10527
Pseudomonadaceae	Pseudomonas	endophytica	SV71
		pseudoalcaligenes	SV4970
		putida	SV4493
		Unclassified	SV145, SV206, SV477, SV556, SV876, SV902, SV928, SV937, SV1253, SV1293, SV1525, SV1684, SV2196, SV3187, SV3963, SV4968, SV5597, SV6415, SV8231, SV11579, SV13283
Rhodanobacteraceae	Luteibacter	rhizovicius	SV6238
Xanthomonadaceae	Stenotrophomonas	maltophilia	SV7398
		Unclassified	SV662, SV2626, SV5236, SV9663, SV12667

Table S5-32. Anti-*Bd* prediction metrics do not differ between *Bd* inoculation treatment groups in captivity or after reintroduction. Captive frog samples were collected at the Zoo ($n_{Bd+}=17$, $n_{Bd-}=13$); Reintroduced frog samples were collected from frogs after release to Tamarack or Lucille restored sites ($n_{Bd+}=17$, $n_{Bd-}=10$); Test statistic for Wilcoxon rank sum exact test is W; Test statistic for Welch's T-test is t; All test results were not significant (“ns”; $p>0.05$).

Group Factor	Samples	Metric	Statistical test	Test statistic	p-value
Inoculation treatment group	captive	Anti- <i>Bd</i> richness	Wilcoxon	123.5	0.5960 ns
		Ant- <i>Bd</i> relative abundance	Wilcoxon	103	0.7729 ns
	reintroduced	Anti- <i>Bd</i> richness	T-test	1.558	0.1345 ns
		Anti- <i>Bd</i> relative abundance	T-test	-0.817	0.4225 ns

Figure S5-1. Rarefaction curves across sample categories. Rarefaction curves for all microbiome samples are colored by sample categories including control samples, environmental samples (*i.e.*, water filter samples), and frog samples; Vertical dashed line shows cut-off of 2012 reads used to rarefy to an even sampling depth; 2012 reads was the chosen threshold to maximize the preservation of frog samples while ensuring most samples' ASV richness had plateaued.

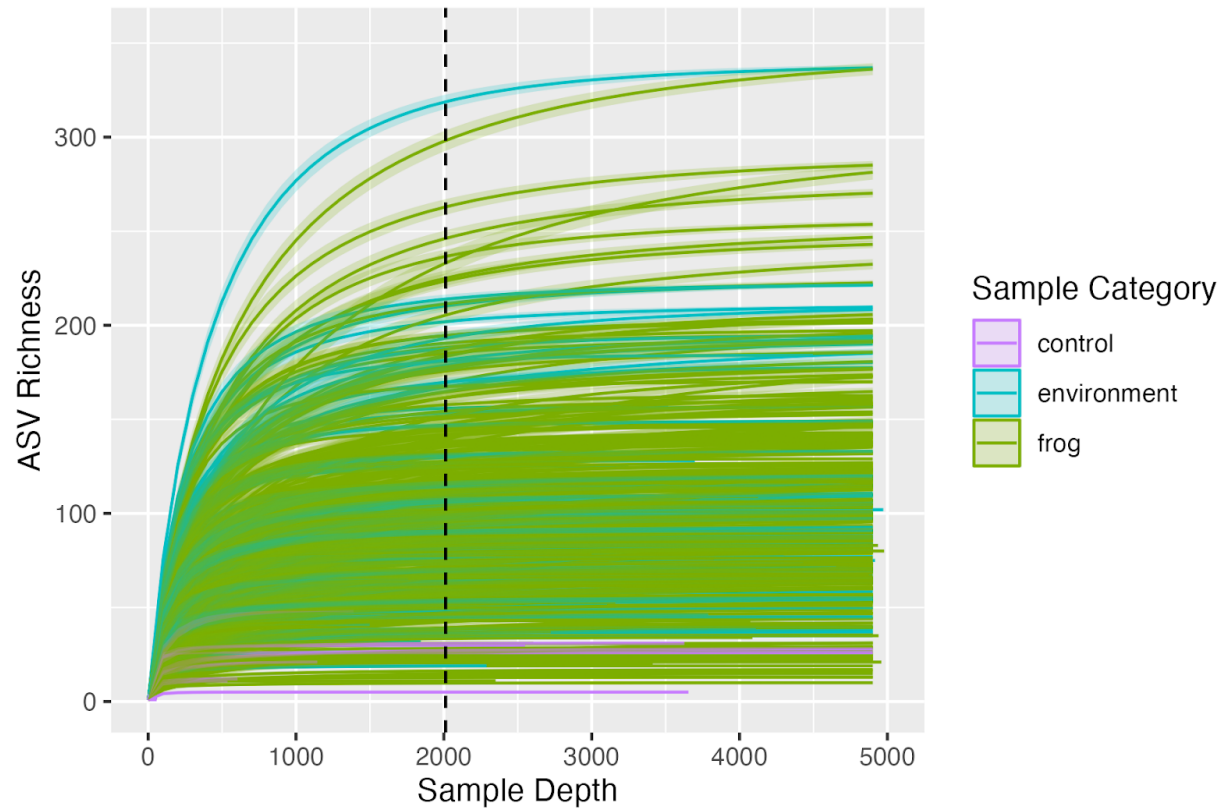


Figure S5-2. Generating scaled mass indices (SMIs). Weight is measured in grams and snout-to-vent length (SVL) is measured in millimeters; Scatterplots include regression lines in dark gray and 95% confidence intervals in light gray; **(A)** plot of weight by SVL shows that these variables do not scale linearly and that there are no major outliers; **(B)** plot of $\ln(\text{weight})$ by $\ln(\text{SVL})$ shows a significant linear relationship with a slope of 3.148898 determined by SMA regression (SMA regression output is shown), which was used as the scaling exponent to calculate SMI for frog individuals; **(C)** plot of SMI by SVL shows that these variables are not correlated (SMA regression output is shown).

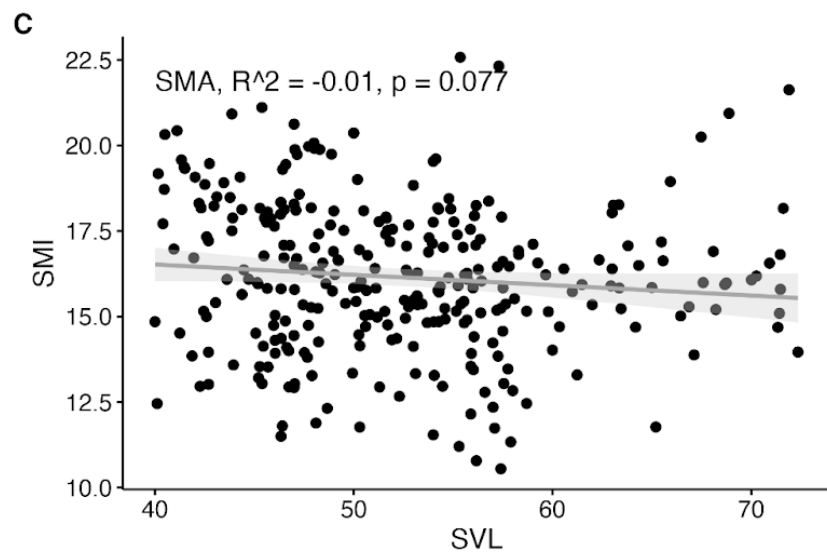
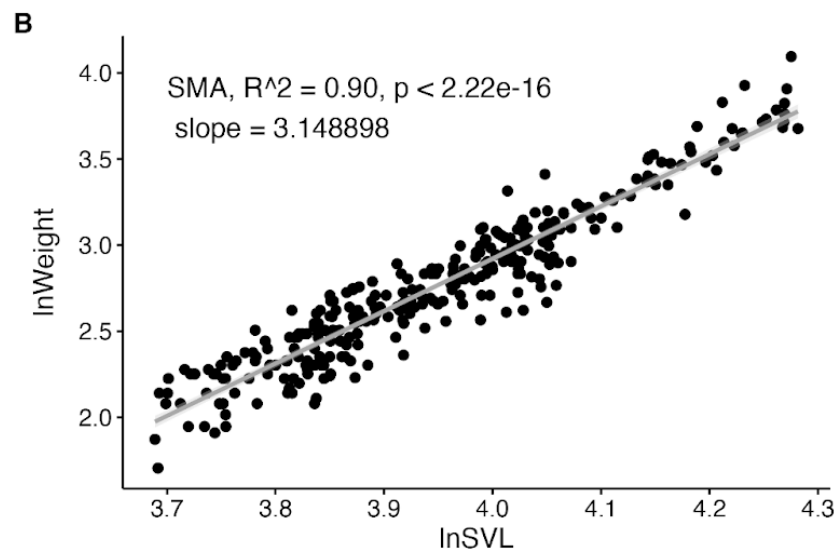
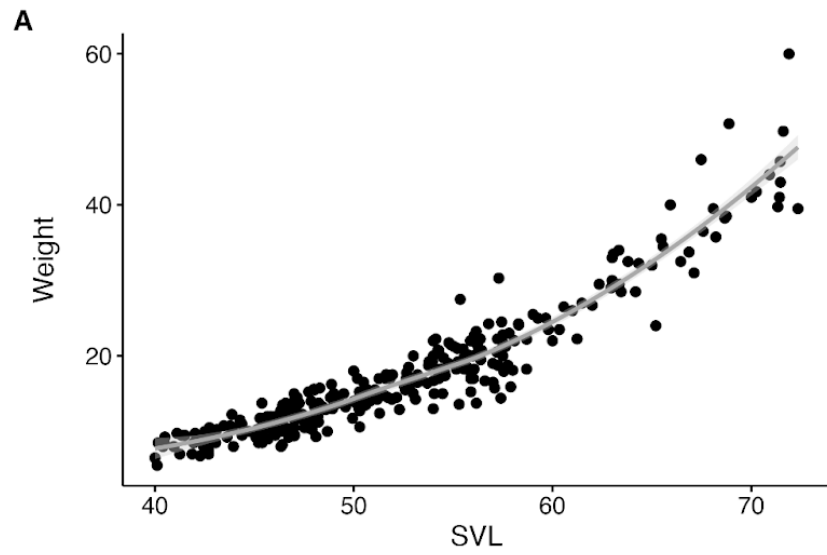


Figure S5-3. Scaled body mass index (SMI) across sites and restoration types. (A) Boxplots and points are grouped and colored by site and shaped by site type (*i.e.*, captive, naturally occurring, and restored); **(B)** Boxplots and points are grouped and colored by restoration type (*i.e.*, captive, source, translocated, reintroduced, and new recruits) and shaped by site type for the Zoo captive frogs, Rivendell source frogs, and Tamarack and Lucille restored frogs; **(A–B)** Results of Kruskal-Wallis and *post hoc* Dunn tests are shown (“*” = $p < 0.05$; “**” = $p < 0.01$; “***” = $p < 0.001$).

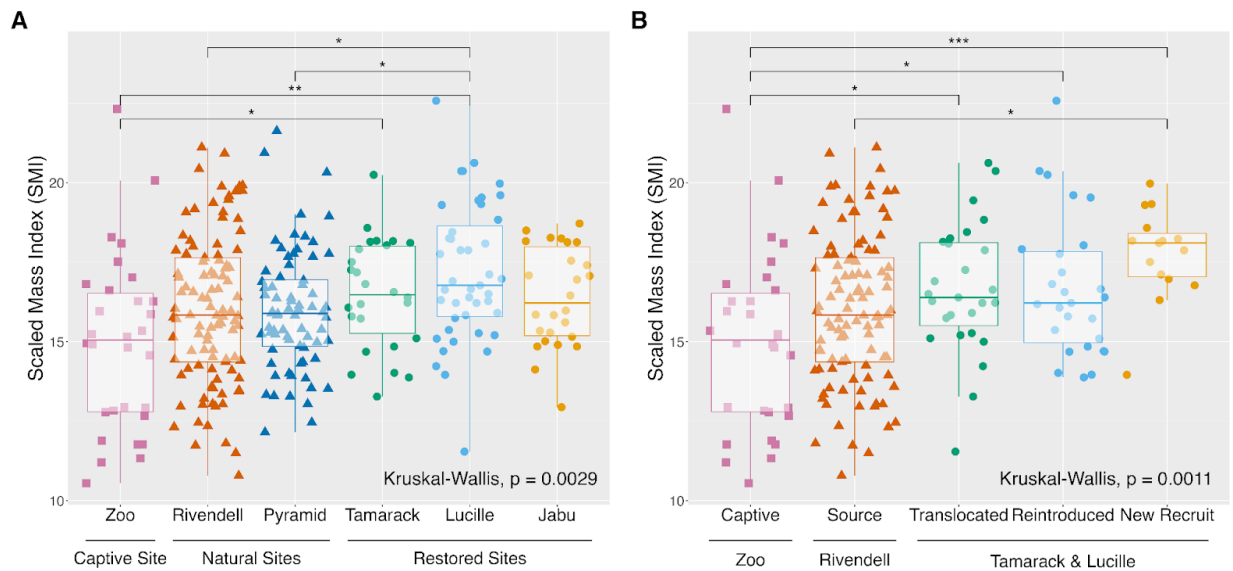


Figure S5-4. Microbial community structure of sample categories (frog vs. water) by site.

Panels show PCoAs of weighted Unifrac distances; Points are shaped and colored by sample category within sites, with water samples colored in a lighter shade of the frog sample color for each site; ellipses represent 95% confidence intervals; Community structure differed significantly between frog and water samples for **(A)** the Zoo captive site (PERMANOVA, $R^2=0.09$, $p<0.01$), **(B)** the Rivendell natural site (PERMANOVA, $R^2=0.22$, $p<0.001$), **(C)** the Pyramid natural site (PERMANOVA, $R^2=0.22$, $p<0.001$), **(D)** the Tamarack restored site (PERMANOVA, $R^2=0.34$, $p<0.001$), **(E)** the Lucille restored site (PERMANOVA, $R^2=0.28$, $p<0.001$), and **(F)** the Jabu restored site (PERMANOVA, $R^2=0.14$, $p<0.001$); **(A,B,D,E,F)** water samples had significantly higher community dispersion than frog samples at the Zoo captive site (permutest, $p<0.05$), the Rivendell natural site (permutest, $p<0.001$), the Tamarack restored site (permutest, $p<0.05$), the Lucille restored site (permutest, $p<0.001$) and the Jabu restored site (permutest, $p<0.05$); **(C)** Water and frog samples at the Pyramid natural site did not differ in community dispersion (permutest, $p>0.05$).

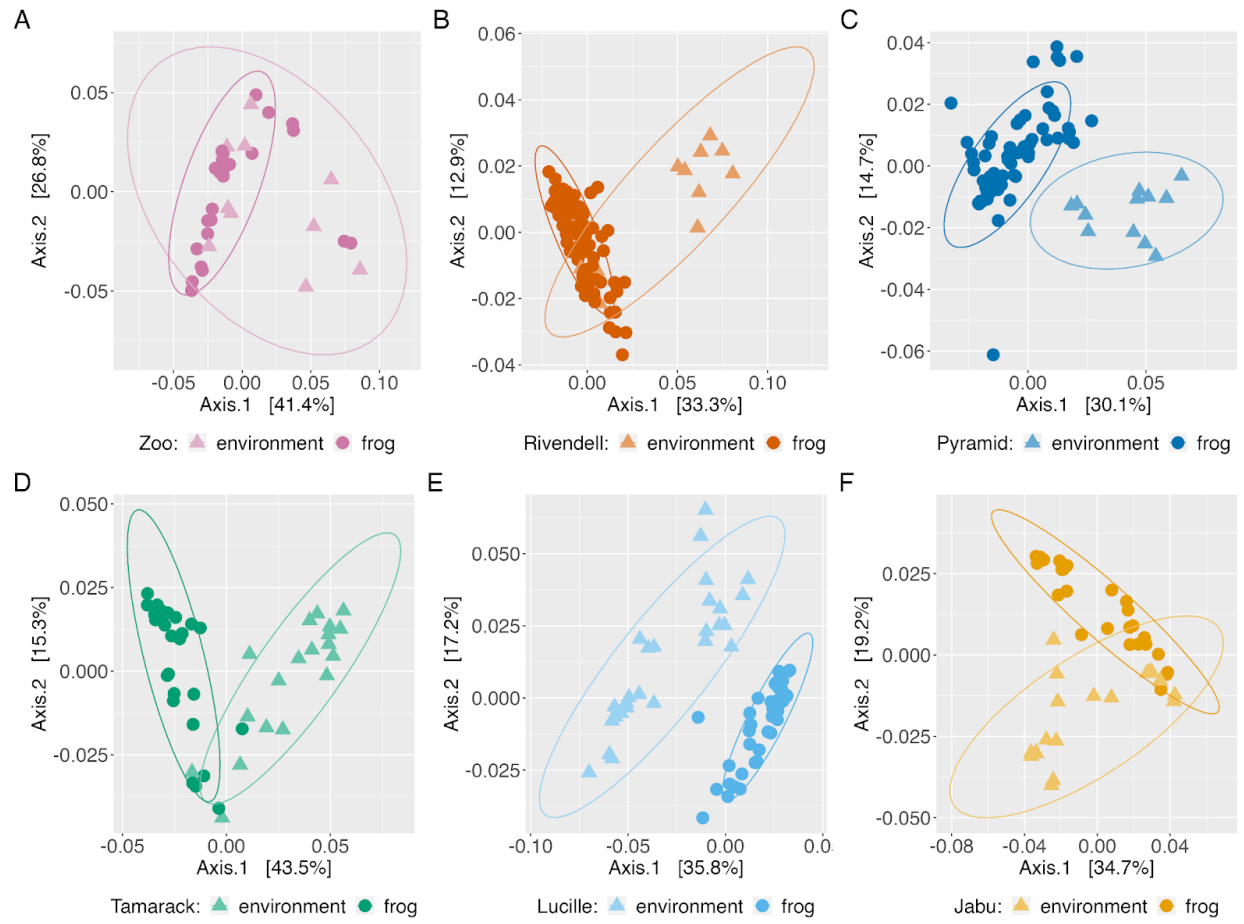


Figure S5-5. Environmental data by site types and sites. (A) Air temperatures (°C), (B) water temperatures (°C), and (C) elevations (meters) associated with each frog and water sample are shown; (A–C) Boxplots and points are colored by site and shaped by site type (*i.e.*, captive, naturally occurring, or restored); (A–B) Results of Kruskal-Wallis and *post hoc* Dunn tests are shown (“*” = $p < 0.05$; “**” = $p < 0.01$; “***” = $p < 0.001$); (C) Results of Kruskal-Wallis test are shown and *post hoc* Dunn tests revealed that elevations were significantly different among all sites in pairwise comparisons (Dunn, $p < 0.001$).

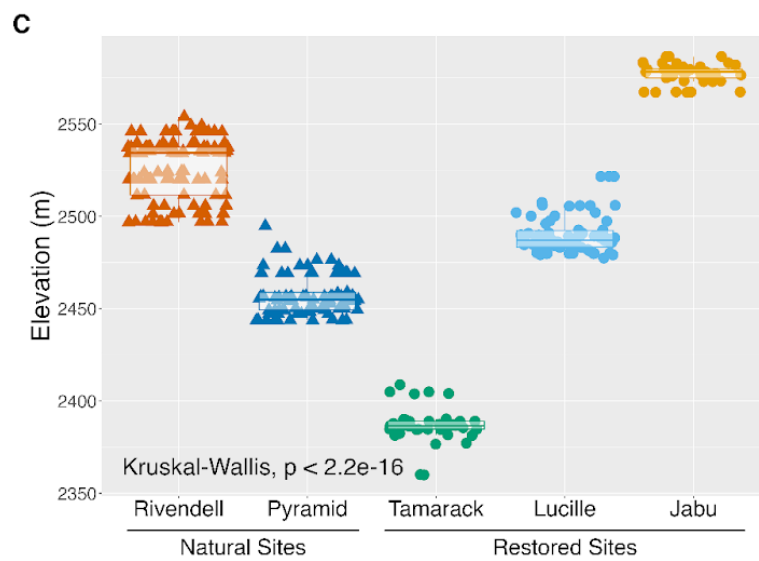
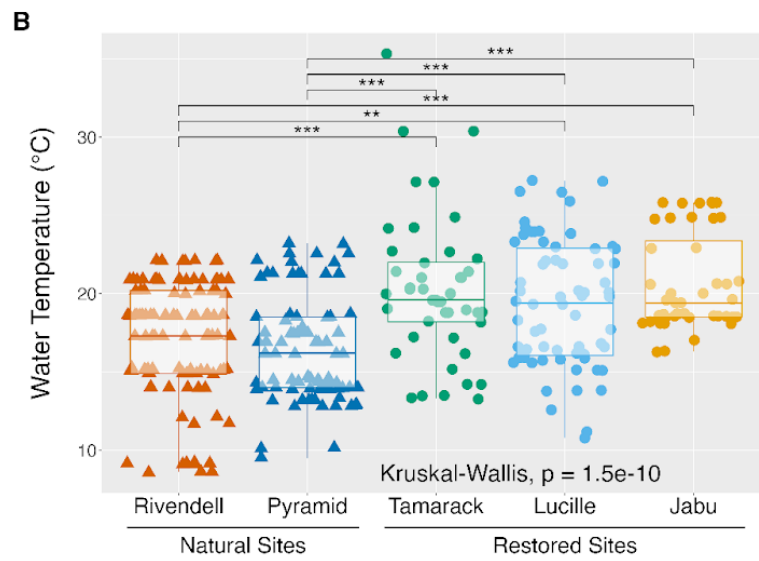
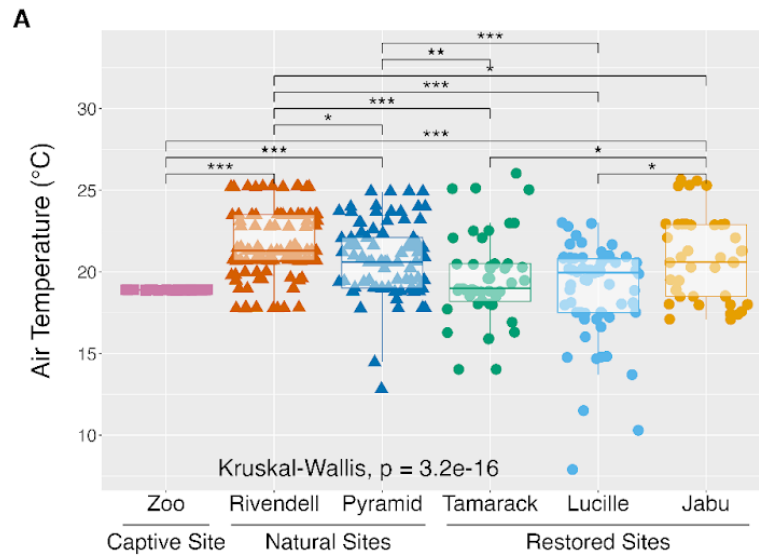
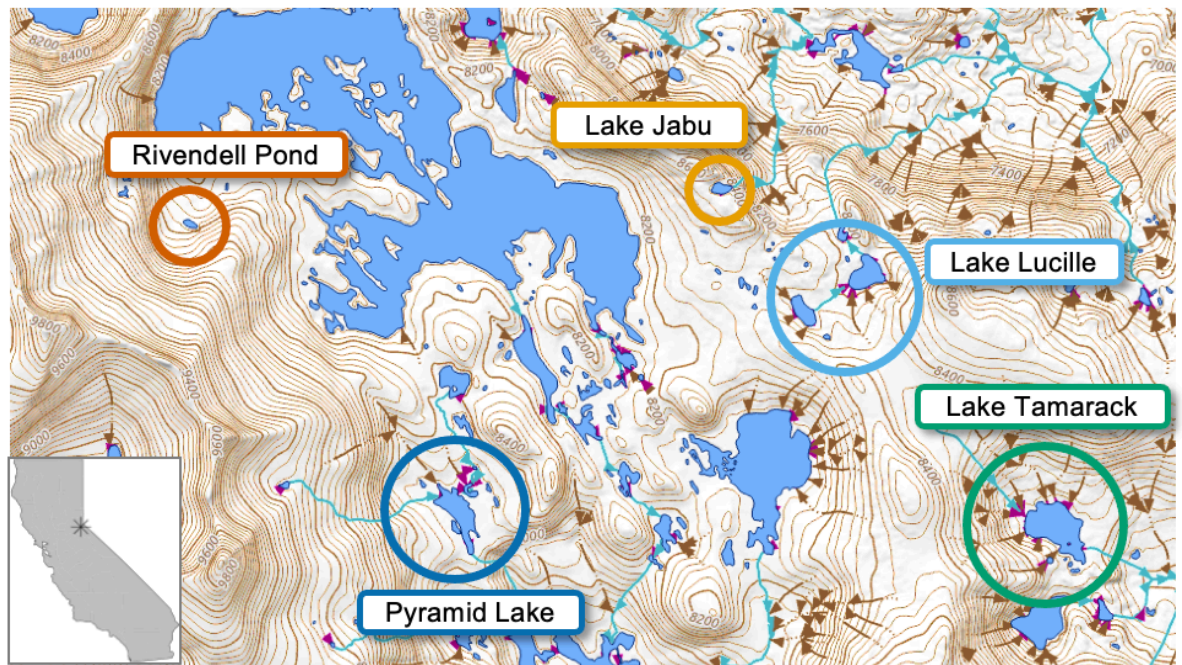


Figure S5-6. National maps of study sites in the Desolation Wilderness. (A) Map includes elevation contours and water flow direction: turquoise flow lines/arrows indicate streams, brown flow lines/arrows indicate ephemeral streams, and magenta flow lines/arrows indicate water flow via artificial paths; inset map of California indicates the location of the Desolation Wilderness; **(B)** Map shows land cover from the National Land Cover Database (Jon Dewitz, 2019): blue areas indicate water, gray areas indicate barren land (*i.e.*, areas of rock/clay/sand; vegetation less than 15% of total cover), light brown areas indicate shrub/scrubland (*i.e.*, dominated by shrubs less than 5 meters tall with shrub canopy, including true shrubs, young trees in an early successional stage or trees stunted from environmental conditions, making up greater than 20% of total vegetation), and dark green areas indicate evergreen forest (*i.e.*, areas dominated by trees generally greater than 5 meters tall and greater than 20% of total vegetation cover, with greater than 75% of tree species maintaining their leaves all year); **(A, B)** Maps built using the National Map Viewer from the USGS (<https://apps.nationalmap.gov/viewer/>) accessed on January 4, 2024; USGS TNM – National Hydrology Dataset Plus High Resolution (NHDPlus HR; data refreshed October, 2022); USGS TNM / NGTOC – 3D National Hydrographic Program (3DHP; data refreshed September 2023); USGS The National Map: 3D Elevation Program; USGS Earth Resources Observation & Science (EROS) Center: GMTED2010 (data refreshed March, 2021); USGS The National Map: 3D Elevation Program (data Refreshed January, 2024).

A



B

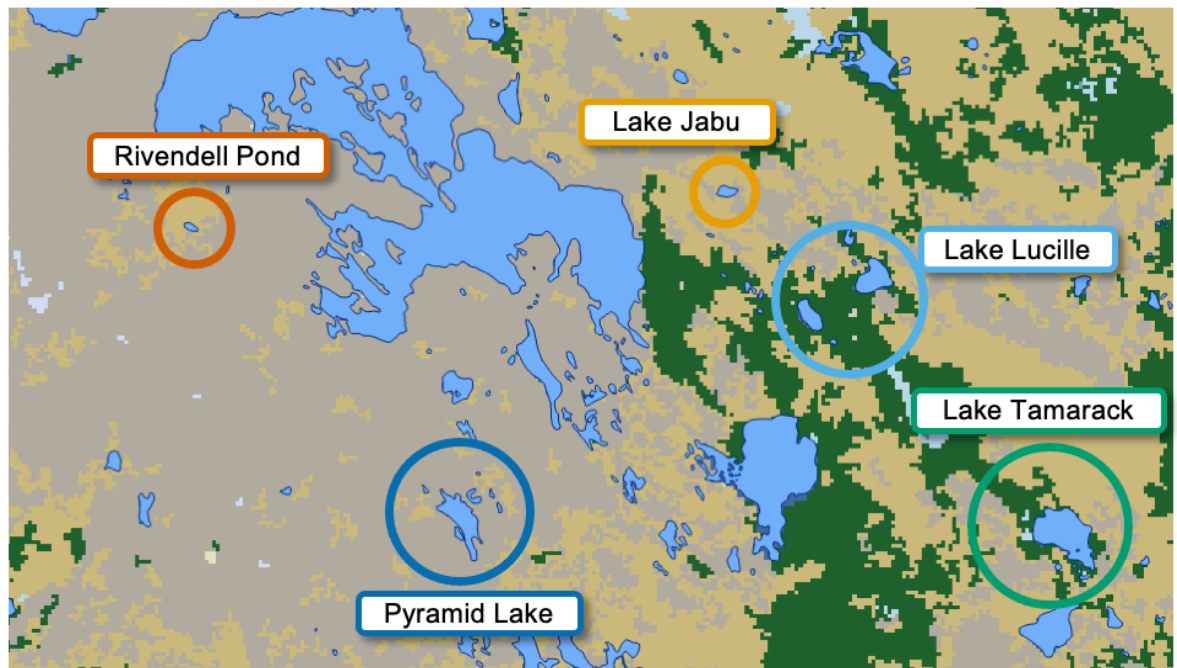


Figure S5-7. Microbiome structure of experimental *Bd* inoculation treatment groups for captive and reintroduced frogs. PCoAs of weighted UniFrac distance ordinations comparing *Bd* inoculation treatment group (*Bd*⁺ and *Bd*⁻; see Chapter Four); ellipses represent 95% confidence intervals; **(A)** For captive Zoo frog samples, community structure (*i.e.*, weighted UniFrac) and dispersion were both significantly different between inoculation treatment groups (PERMANOVA, $p < 0.05$; betadisperion permutest, $p < 0.05$; Table S5-28; Table S5-29); points are colored by inoculation treatment group; **(B)** For reintroduced frogs across Tamarack and Lucille, inoculation treatment group did not explain significant variation in community structure (PERMANOVA, $p = 0.08$; Table S5-28); points are colored by inoculation treatment group and shaped by site.

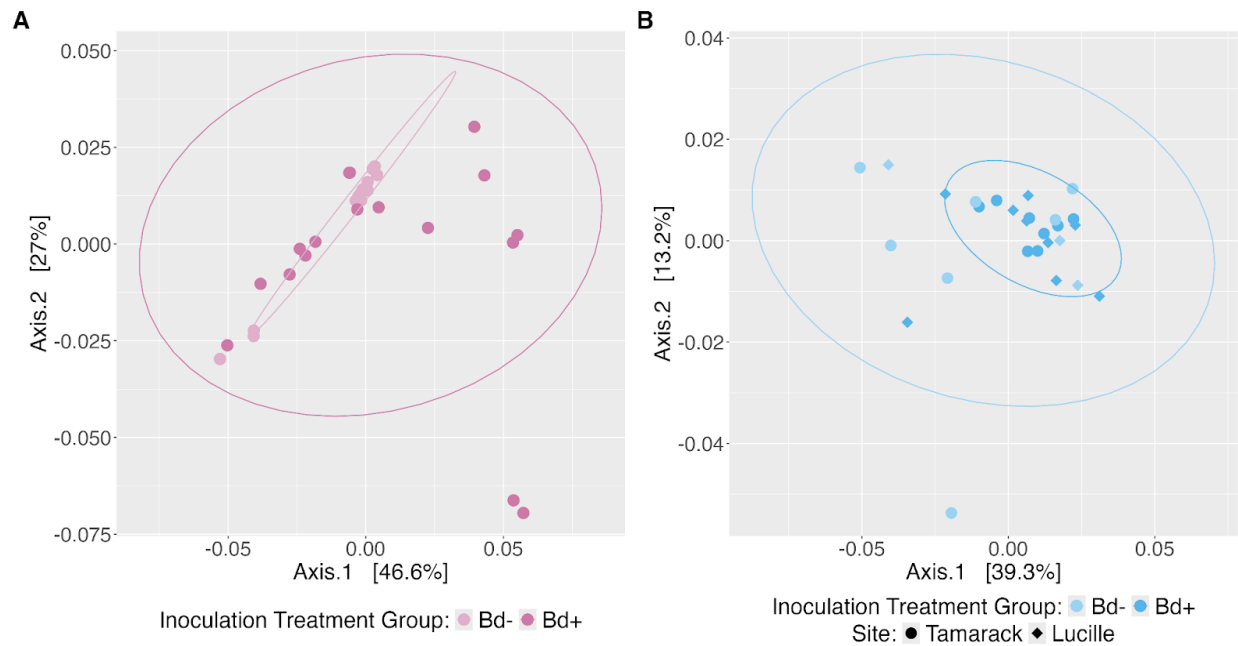


Figure S5-8. Within-sample diversity of sample categories (frog vs. water) by site. (A, C, E, G, I, K) Observed Richness and (B, D, F, H, J, L) Shannon Diversity are shown by sample category for (A-B) Zoo captive site samples, (C-D) Rivendell natural site samples, (E-F) Pyramid natural site samples, (G-H) Tamarack restored site samples, (I-J) Lucille restored site samples, and (K-L) Jabu restored site samples; results of Wilcoxon tests (A-G, I, K, L) or T-tests (H, J) are indicated (“NS” = not significant; “*” = $p < 0.05$; “” = $p < 0.01$; “***” = $p < 0.001$).**

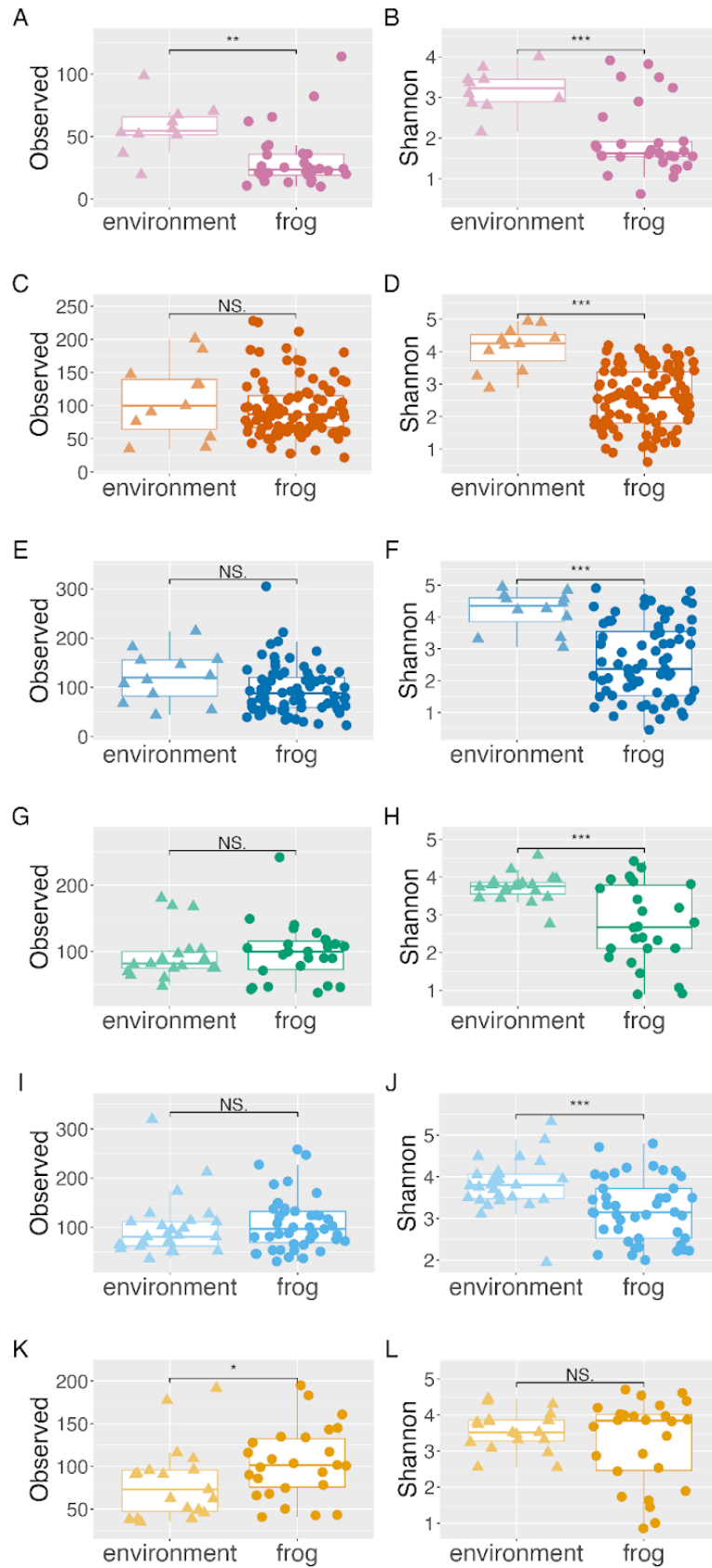


Figure S5-9. Putative anti-*Bd* richness compared between sample categories (frog vs. water) by site; Observed number of microbial community members with >99% sequence identity match to members of a *Bd*-inhibitory database (microbes showing inhibition of *Bd*; Woodhams et al., 2015) for **(A)** Zoo captive site samples, **(B)** Rivendell natural site samples, **(C)** Pyramid natural site samples, **(D)** Tamarack restored site samples, **(E)** Lucille restored site samples, and **(F)** Jabu restored site samples; **(A–F)** Results of Wilcoxon tests are indicated (“NS” = not significant; “*” = $p < 0.05$; “***” = $p < 0.01$; “****” = $p < 0.001$).

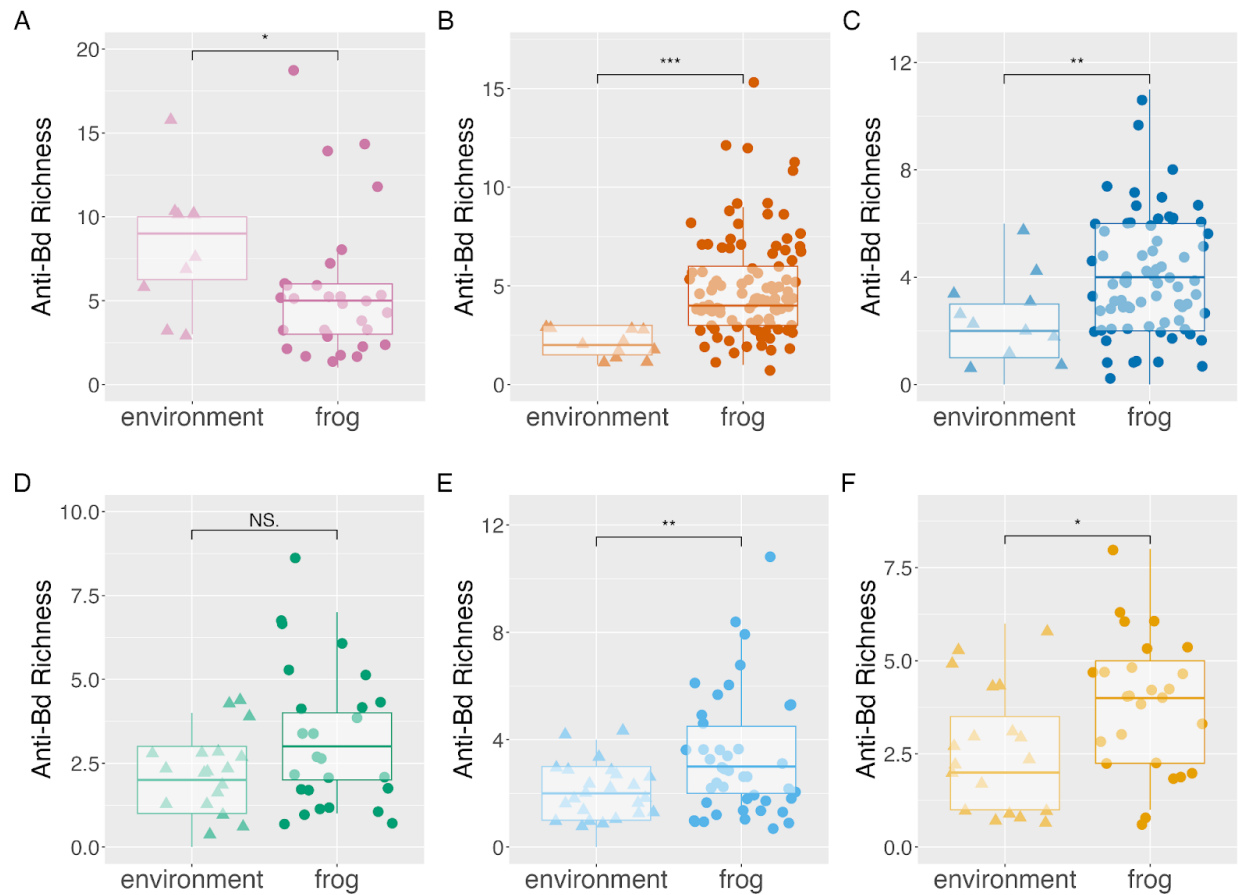


Figure S5-10. Putative anti-*Bd* relative abundance compared between sample categories (frog vs. water) by site. Proportion of the microbial community with >99% sequence identity match to members of a *Bd*-inhibitory database (microbes previously found to inhibit *Bd* growth; Woodhams et al., 2015) for **(A)** Zoo captive site samples, **(B)** Rivendell natural site samples, **(C)** Pyramid natural site samples, **(D)** Tamarack restored site samples, **(E)** Lucille restored site samples, and **(F)** Jabu restored site samples; Results of Welch's T-test **(A)** or Wilcoxon tests **(B, C, D, E, F)** are indicated ("NS" = not significant; "*" = $p < 0.05$; "***" = $p < 0.01$; "****" = $p < 0.001$).

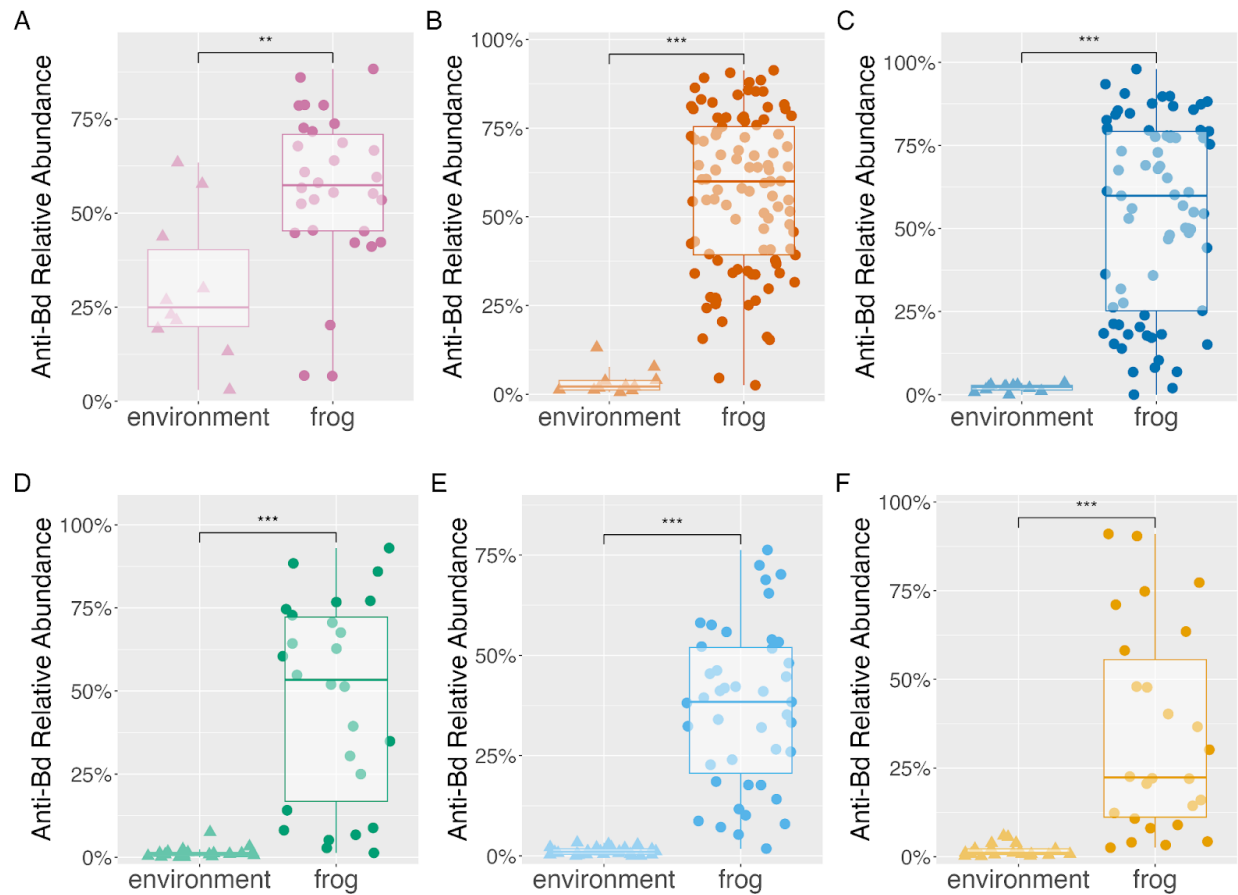


Figure S5-11. Water-associated anti-*Bd* predictions among sites. Anti-*Bd* predictions are based on >99% sequence identity match to sequences from a *Bd*-inhibitory database of bacteria shown to inhibit *Bd* growth (Woodhams et al., 2015); Here we show three metrics of putative anti-*Bd* function across water samples; **(A)** Anti-*Bd* richness (*i.e.*, the observed number of putatively inhibitory microbes); **(B)** Anti-*Bd* relative abundance (*i.e.*, the summed relative abundances of putatively inhibitory microbes); **(C)** Relative abundance of SV1, an unclassified member of the Burkholderiaceae with 100% sequence identity match to a *Bd*-inhibitory taxon (Woodhams et al., 2015); **(A–C)** Boxplots and points are grouped and colored by site and shaped by site type (*i.e.*, captive, naturally occurring, and restored); Results of Kruskal-Wallis and *post hoc* Dunn tests are shown (“*” = $p < 0.05$; “**” = $p < 0.01$; “***” = $p < 0.001$).

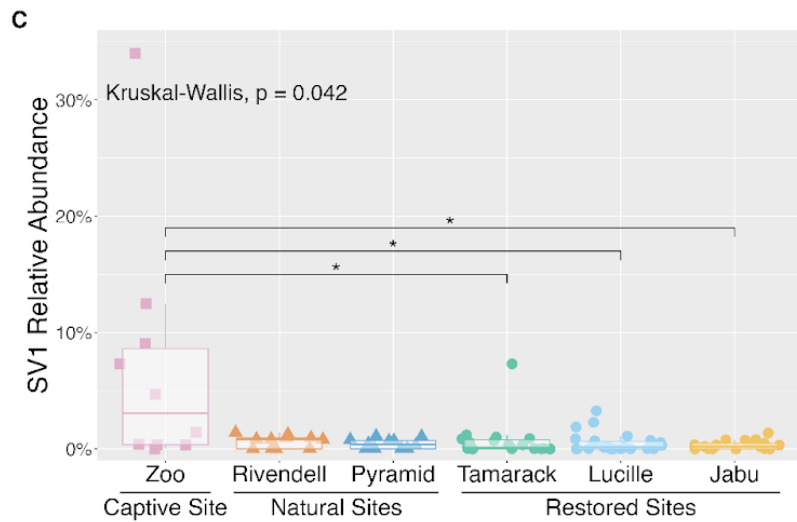
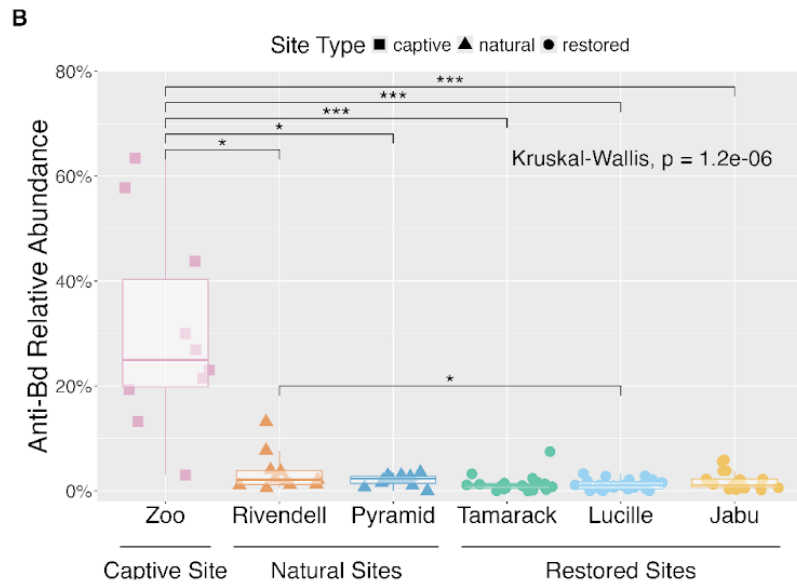
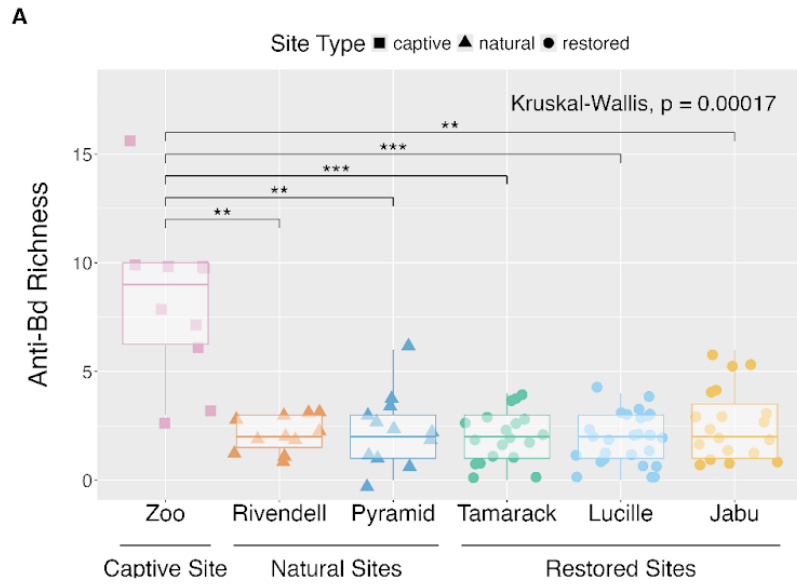


Figure S5-12. Correlation of frog-associated anti-*Bd* and SV1 relative abundances.

Scatterplot of frog sample values includes a regression line in dark gray with the 95% confidence interval in light gray; Points are colored by putative anti-*Bd* richness; Spearman correlation statistics are shown; Samples with higher anti-*Bd* richness primarily occur above the regression line.

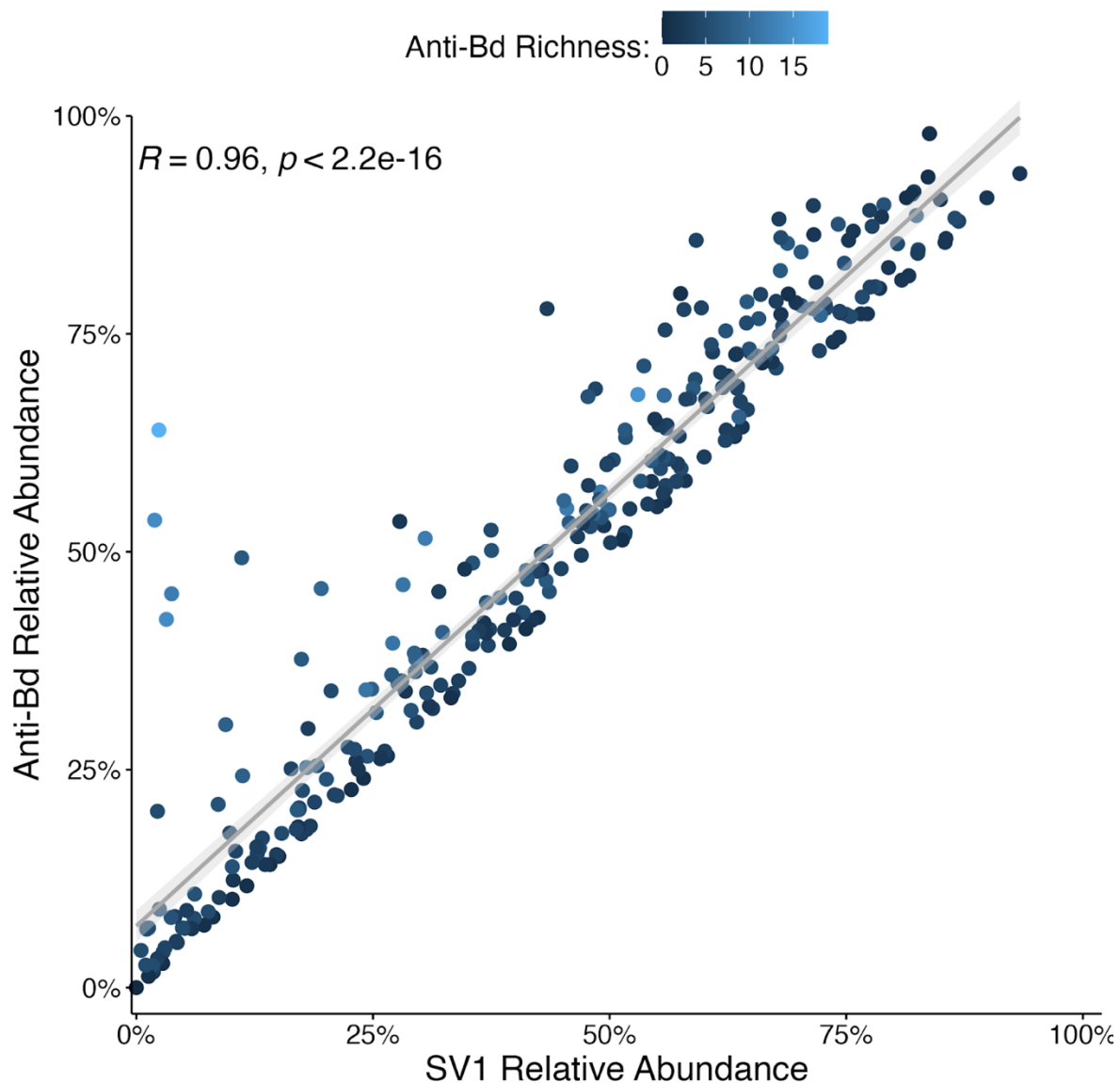


Figure S5-13. Mean relative abundance of bacterial phyla and families across site types, sites, and restoration types. (A) Mean relative abundance bar chart faceted by site type and site for all frog samples; **(B)** Mean relative abundance bar chart faceted by restoration type and site for frog samples from the Tamarack and Lucille restored sites; **(A–B)** Stacked bar charts were generated using microshades v. 1.11 (Dahl et al., 2022); Taxa are colored by phylum, and families within each phylum are colored with unique shades of the associated phylum color; Within each stacked bar chart, taxa are ordered first by mean relative abundance of phyla and then by mean relative abundance of families within each phylum so that taxa with higher relative abundance appear lower in each stacked bar; Colors associated with each phylum are the same across figure panels, but family color assignments within each phylum may vary between panels as the order is based on mean relative abundance within each subset of samples.

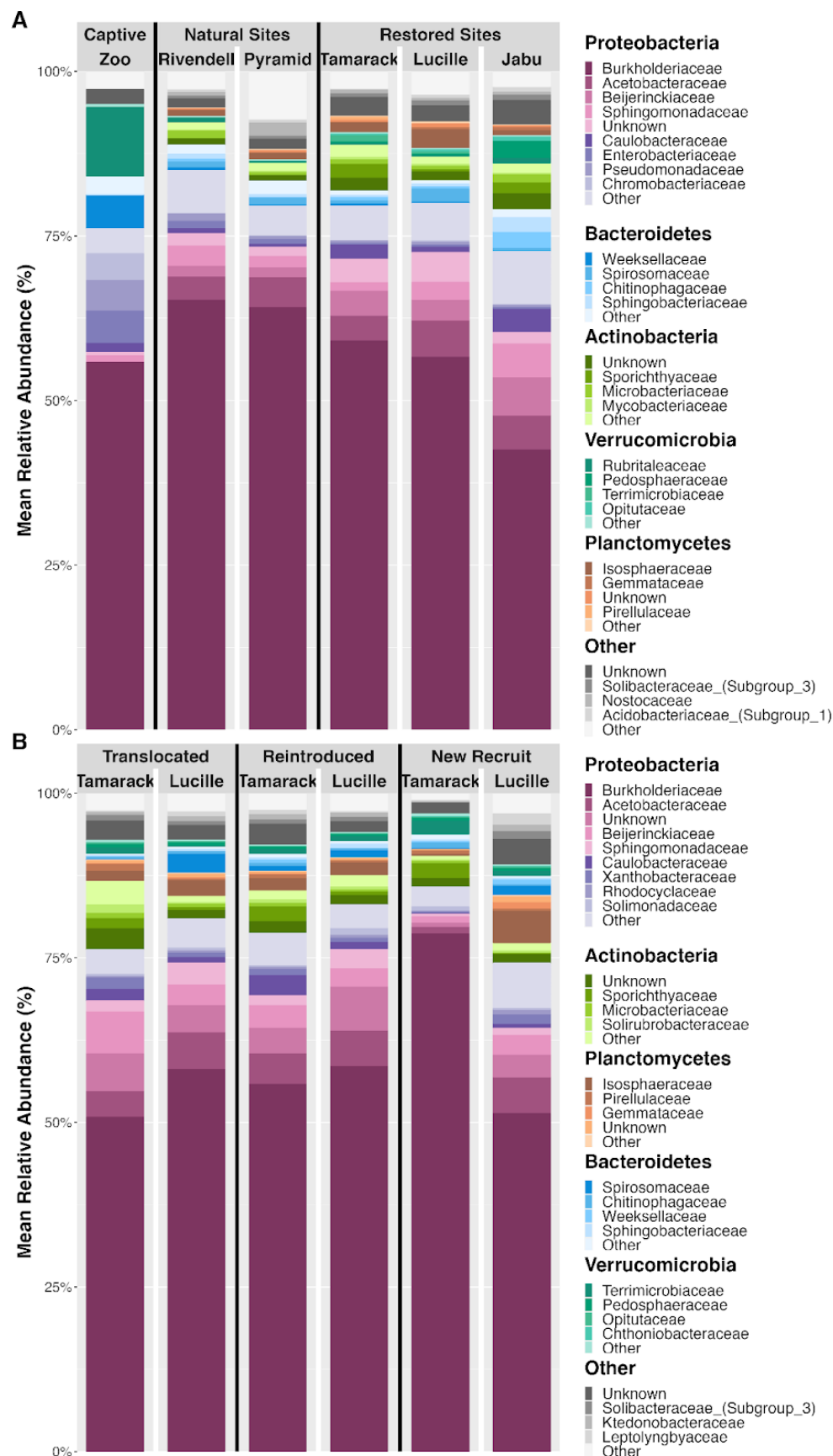


Figure S5-14. Mean relative abundance of bacterial phyla and genera across site types, sites, and restoration types. (A) Mean relative abundance bar chart faceted by site type and site for all frog samples; **(B)** Mean relative abundance bar chart faceted by restoration type and site for frog samples from the Tamarack and Lucille restored sites; **(A–B)** Stacked bar charts were generated using microshades v. 1.11 (Dahl et al., 2022); Taxa are colored by phylum, and genera within each phylum are colored with unique shades of the associated phylum color; Within each stacked bar chart, taxa are ordered first by mean relative abundance of phyla and then by mean relative abundance of genera within each phylum so that taxa with higher relative abundance appear lower in each stacked bar; Colors associated with each phylum are the same across figure panels, but genus color assignments within each phylum may vary between panels as the order is based on mean relative abundance within each subset of samples.

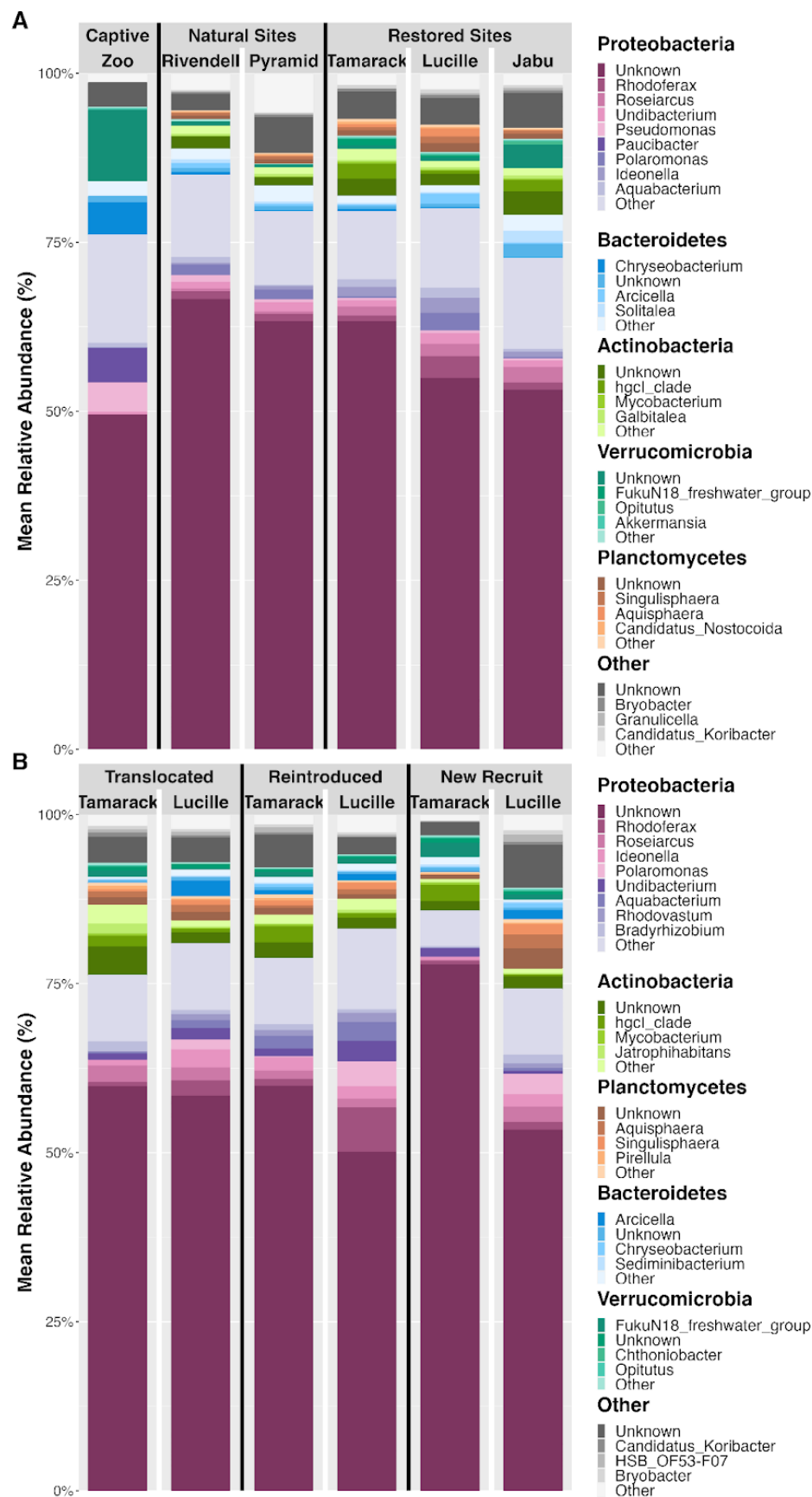


Figure S5-15. Relative abundance of bacterial phyla and families across experimental *Bd* inoculation treatment groups. Sample relative abundance barcharts are faceted by inoculation treatment group (*Bd*⁺ or *Bd*⁻; see *Chapter Four*) and *Bd* status (*i.e.*, testing negative or positive for *Bd* infection) for **(A)** captive frog samples from the Zoo, and **(B)** reintroduced frog samples from Tamarack and Lucille restored sites; **(A–B)** Stacked barcharts were generated using microshades v. 1.11(Dahl et al., 2022); Taxa are colored by phylum, and families within each phylum are colored with unique shades of the associated phylum color; Within each stacked barchart, taxa are ordered first by mean relative abundance of phyla and then by mean relative abundance of families within each phylum so that taxa with higher relative abundance appear lower in each stacked bar; Colors associated with each phylum are the same across figure panels, but family color assignments within each phylum may vary between panels as the order is based on mean relative abundance within each subset of samples.

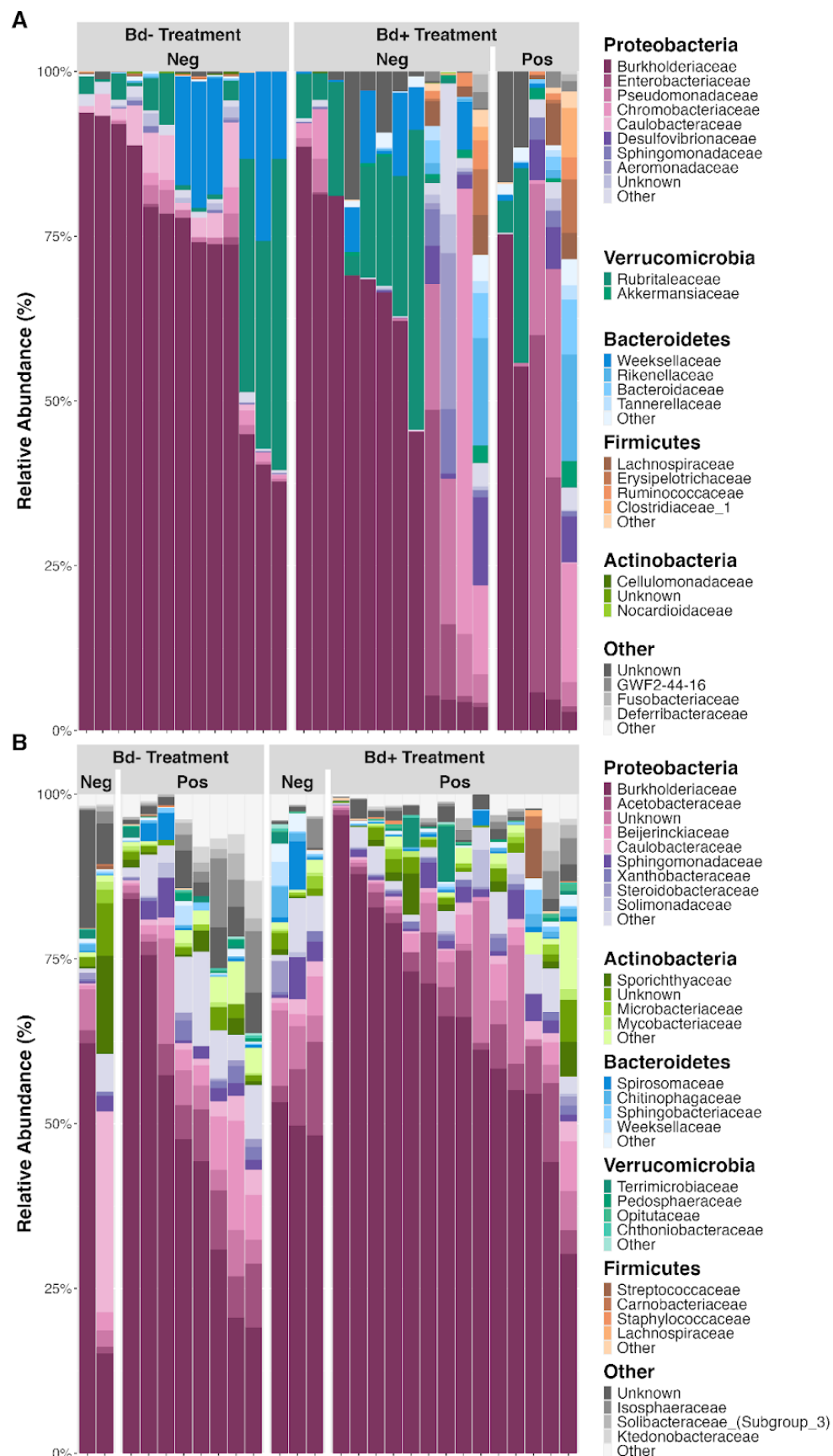
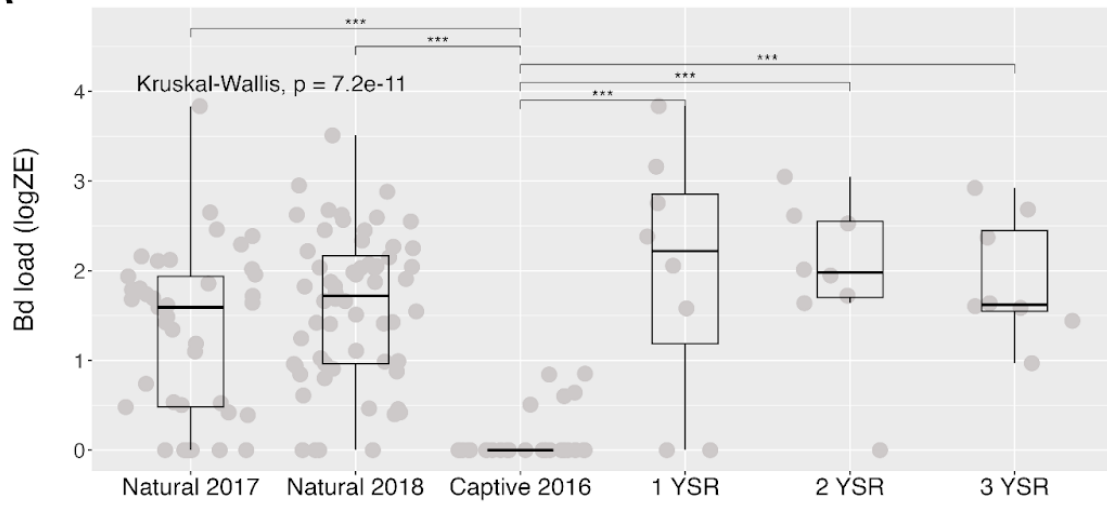
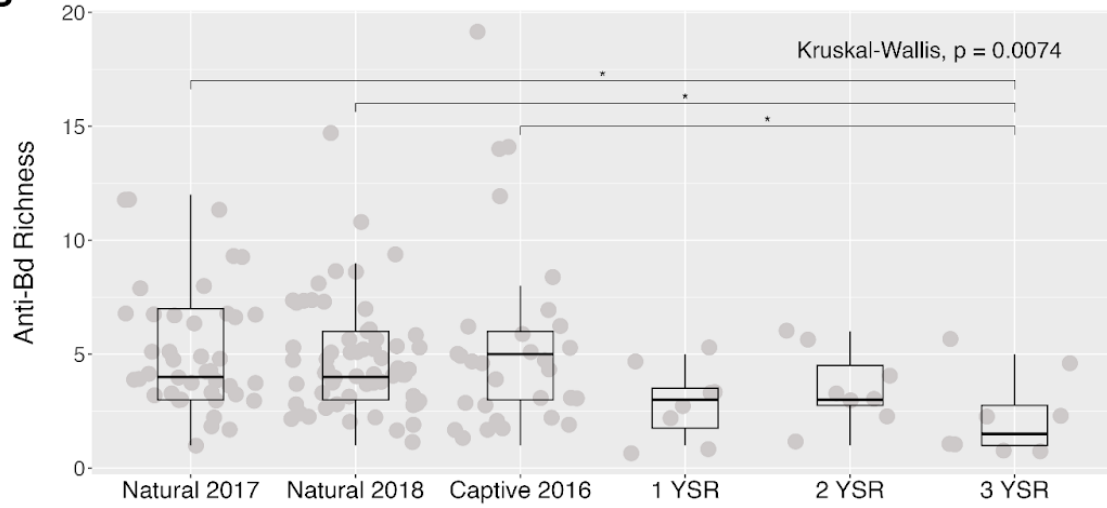
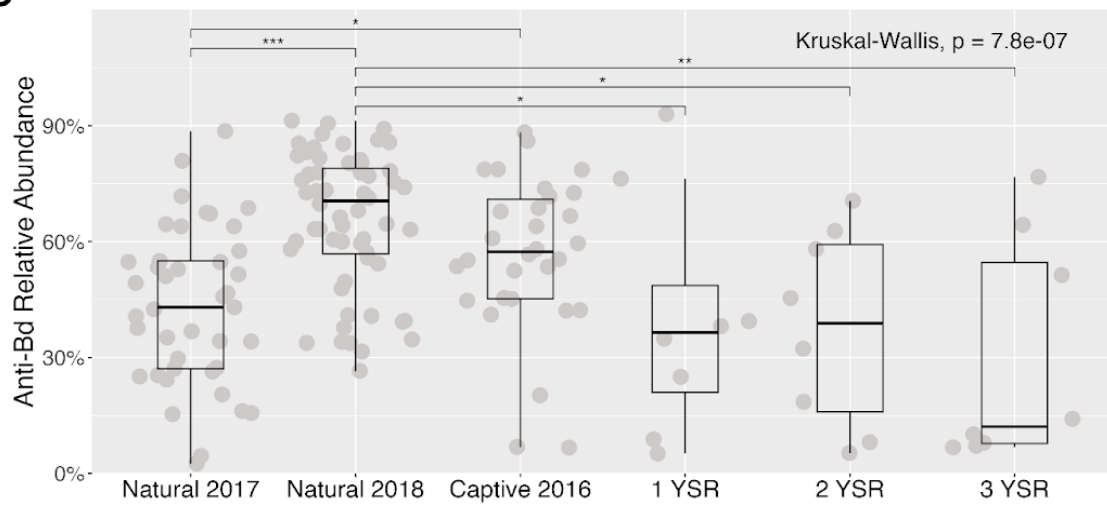


Figure S5-16. Sample values of microbial metrics for natural frogs, captive frogs, and reintroduced frogs across time intervals. (A) *Bd* load, (B) putative anti-*Bd* richness, and (C) putative anti-*Bd* relative abundance are shown for naturally occurring frog samples from the Rivendell natural site in 2017 and 2018 collection years, for captive frog samples from the Zoo in 2016, and for reintroduced frog samples collected across 1, 2, and 3 years since release (YSR) to two restored sites (*i.e.*, Tamarack and Lucille); **(A–C)** Results of Kruskal-Wallis and *post hoc* Dunn tests are shown (“*” = $p < 0.05$; “**” = $p < 0.01$; “***” = $p < 0.001$).

A**B****C**

“May the microbes be ever in your favor...”

-Jonathan Eisen