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Assembly, function, and sensitivity of the skin microbiome of the Sierra Nevada yellow-legged frog (*Rana sierrae*) in the face of invasion by the fungal pathogen, *Batrachochytrium dendrobatidis*

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in Biochemistry and Molecular Biology

by

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December 2014

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I dedicate this work to the Sierra Nevada yellow-legged frog.

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PEER-REVIEWED PUBLICATIONS

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PRESENTATIONS

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ABSTRACT

Assembly, function, and sensitivity of the skin microbiome of the Sierra Nevada yellow-legged frog (*Rana sierrae*) in the face of invasion by the fungal pathogen, *Batrachochytrium dendrobatidis*

by

Andrea Jutta Jani

Symbiotic microbial communities (microbiomes) are ubiquitous inhabitants of multicellular organisms and are increasingly recognized to play roles in host health and development. The skin-associated microbiome of amphibians may affect resistance to the potentially lethal disease, chytridiomycosis, which is caused by the fungal pathogen *Batrachochytrium dendrobatidis* (Bd). The Sierra Nevada yellow-legged frog (*Rana sierrae*) is threatened with extinction, and Bd is a major driver of declines in these frogs. However, some *R. sierrae* populations are able to co-exist with the pathogen, persisting despite Bd infection. Understanding why some *R. sierrae* populations persist with Bd may provide clues for how to minimize the impact of Bd. However, while preliminary studies highlight the possibility that symbiotic bacteria affect disease resistance, very little is known about natural diversity, stability, or function of the amphibian skin microbiome.

I tested the hypothesis that differences in skin-associated bacterial communities (referred to simply as the microbiome for brevity) can account for differences in the outcome of *R. sierrae* infection, i.e. population extinction versus persistence. I surveyed microbiomes from multiple *R. sierrae* populations and showed that populations that persisted or declined to extinction due to Bd indeed harbored different skin bacterial communities, consistent with

a protective effect of bacteria in persistent populations. At the same time I found evidence that Bd infection itself may drive variation in the microbiome, both at the local scale (among frogs within a given population), and landscape scale (among populations).

Correlations between microbiome composition and Bd infection severity among wild frogs may be the result of either microbiome-mediated resistance to disease, in which bacterial assemblages control the degree of Bd infection, or, alternatively could result from Bd-induced disturbance causing changes in the microbiome. Using a laboratory experiment, I demonstrated that Bd infection alters the *R. sierrae* microbiome, while I found no direct evidence that the microbiome limits the severity of Bd infection, at least within the range of microbiome variability represented in this study. In addition, some of the bacterial taxa that were sensitive to Bd infection might have been predicted to inhibit the growth of Bd based on results in other study systems. These results highlight the importance of considering microbiome stability when assessing the potential for the microbiome to limit pathogen growth.

Understanding why microbial communities vary among individuals or populations is an important step toward understanding the diversity and function of the microbiome. I present an experiment that tested the effects of natural variation in the aquatic environment and genetic variation among *R. sierrae* individuals in shaping the skin microbiome.

By integrating controlled experiments with field surveys at multiple spatial scales, these studies reveal new insights into amphibian skin microbiome assembly, function, and sensitivity in the face of infection by an important fungal pathogen, and show the importance of these characteristics of the microbiome in wild amphibian populations.

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CHAPTER THREE:

INTRODUCTION

The microbial ecology of chytridiomycosis

Multi-cellular organisms are host to complex communities of symbiotic microbes, referred to as the microbiome. In recent years, microbiome research has sparked fundamental changes in our understanding of metabolism, development, and disease in metazoans (Dethlefsen et al., 2007; Grice and Segre, 2012; Heijtz et al., 2011; Honda and Littman, 2012; Turnbaugh et al., 2006). Variation in the composition of symbiotic microbial communities is now thought to play a role in human health (Huang et al., 2011; Kong et al., 2012; Ley et al., 2006; Naik et al., 2012), digestion in animals (Brulc et al., 2009; Liu et al., 2011; Pope et al., 2010), and bleaching of coral reefs (Bourne et al., 2008; Rosenberg et al., 2007; Thurber et al., 2009), but critical gaps in our understanding persist. The vast majority of microbiome research has focused on microbes inhabiting the gut, with much less known about the microflora of other organs or body regions, among them the skin. The skin is an important sensory organ and the first line of immune defense in most animals, and recent studies have begun to reveal surprising diversity in the microbes inhabiting human skin (Costello et al., 2009; Fierer et al., 2008; Grice and Segre, 2011), but we have barely begun to probe the skin microbiomes of other animals. Another knowledge gap exists in our understanding of how the microbiome interacts with infectious diseases. Studies working with laboratory models have shown that the microbiome can both affect resistance to infection and also itself be altered by pathogen infection (Lupp et al., 2007; Naik et al., 2012; Sekirov et al., 2008; Stecher et al., 2007). However, very few studies (Cariveau et al., 2014; Koch and Schmid-Hempel, 2012) have examined microbiome-pathogen interactions in nature. Microbial dynamics in nature may differ fundamentally from observations based on simplified laboratory models, requiring a concerted effort to draw connections between reductionist experiments in the laboratory and processes in the complex natural world.

Finally, we lack a solid understanding of what forces shape and regulate the microbiome and therefore ultimately determine its function. Addressing and integrating these question is important to a fundamental understanding of the assembly and function of symbiotic microbial communities. In the case of one group of animals, the amphibians, these answers may also be directly relevant to understanding and minimizing the loss of biodiversity due to an emerging infectious disease.

The world is losing its amphibians at an alarming rate. Forty-three percent of amphibian species are in decline (Wake and Vredenburg, 2008). Habitat destruction and climate change are likely major drivers of amphibian declines (Wake and Vredenburg, 2008) but in the last 15 years the emergence of a recently described infectious disease has become one of the most serious recognized threats to amphibian biodiversity (Kilpatrick et al., 2010; Wake and Vredenburg, 2008). The disease, chytridiomycosis, is caused by a chytrid fungal pathogen, *Batrachochytrium dendrobatidis* (Bd). Bd infects the skin of frogs, toads, and salamanders, where it disrupts osmoregulation and can cause lethal osmotic imbalance (Voyles et al., 2009). Bd is now known to have a broad global distribution and, in the short time since its discovery in 1999, has been implicated in the declines of over 200 amphibian species (Kilpatrick et al., 2010). Because of the threat Bd poses to amphibians worldwide, understanding the skin microbiome of amphibians and how it interacts with Bd infection is a key area of conservation research.

The Sierra Nevada yellow-legged frog (*Rana sierrae*) has been severely impacted by Bd. This frog species was once common in high elevation lakes throughout the Sierra Nevada, but has been extirpated from over 90% of historically inhabited sites (Vredenburg et al., 2007). The most serious known threats to *R. sierrae* are introduced trout and

chytridiomycosis. Non-native trout were stocked in Sierra Nevada lakes beginning in the 1850s for recreational fishing. Trout prey on both tadpole and adult stages of *R. sierrae* and can drive *R. sierrae* populations to local extinction (Bradford et al., 1998; Knapp, 2005; Knapp and Matthews, 2000). Although stocking of trout ended in National Parks of the Sierra Nevada in the 1990's, stocking continues in other region parts of the mountain range, and self-sustaining trout populations have become established in many localities. Removal of trout is labor intensive and expensive, but has been shown to lead to recovery of *R. sierrae* populations (Knapp and Matthews, 1998; Knapp et al., 2007). In contrast, there are no methods proven to prevent the impact of Bd on wild *R. sierrae* populations. The lack of tools to mitigate the impacts of Bd makes the pathogen arguably the gravest threat to the Sierra Nevada yellow-legged frog and its sister species, the mountain yellow-legged frog (*Rana muscosa*).

As Bd has spread across the Sierra Nevada, many *R. sierrae* and *R. muscosa* populations have been extirpated by the disease (Rachowicz et al., 2006; Vredenburg et al., 2010), and the few populations that currently remain unaffected by Bd are predicted to undergo catastrophic declines when the pathogen spreads to those localities. However, a key trait of *R. sierrae* is variability in the outcome of infection with Bd. While *R. sierrae* populations typically decline to extinction due to Bd, some populations have reached a state of co-existence with the pathogen (Briggs et al., 2010; Knapp et al., 2011). These co-existing populations are referred to as "persistent" populations because they persist and maintain stable population sizes despite the presence of the pathogen. That Bd infection does not necessarily lead to *R. sierrae* population extinction is encouraging from a conservation viewpoint. If we can understand what factors enable the persistence of some *R. sierrae*

populations in the presence of Bd, it may be possible to develop management strategies to help shift infection outcomes toward population persistence.

Recently, the hypothesis that symbiotic bacteria might affect resistance to chytridiomycosis has gained attention. Certain bacteria isolated from amphibians can inhibit the growth of Bd in laboratory culture (Becker et al., 2009), and treatment of *R. muscosa* individuals with a strain of the bacterial species *Janthinobacterium lividum* conferred resistance to Bd infection (Harris et al., 2009). Another study counted a greater number of Bd-inhibitory bacterial isolates in a persisting *R. sierrae* population than in a declining population, consistent with a protective role for bacteria in persisting frog populations (Woodhams et al., 2007). These results highlight the potential for symbiotic bacteria to affect resistance to Bd infection or disease, but we are a long way from understanding whether symbiotic bacteria explain natural variation in disease outcomes, or whether probiotic (inoculation with desirable bacteria) or prebiotic (manipulation of the environment to encourage the growth of certain bacteria) treatments can be used to enhance the survival of *R. sierrae* faced with Bd in the wild.

Although the potential for the microbiome to protect against chytridiomycosis has been proposed (Harris et al., 2009; Woodhams et al., 2007), most research has not considered the possibility that Bd infection itself alters the microbiome. Studies finding differences in the symbiotic bateria of persisting and declining populations are often interpreted to indicate that differences in bacterial symbionts lead to the different outcomes of infection: persistence or decline. However, it is also important to consider that infectious pathogens may disrupt the normal microbiome during the course of infection. If Bd alters the amphibian skin microbiome, then associations between Bd infection severity and differences in microbiome

composition in observational studies may be a result, rather than a cause, of differential resistance to pathogen infection. Additionally, the sensitivity of specific bacterial taxa to invasion by Bd may impact their effectiveness in slowing Bd growth: if a bacterial strain or species cannot persist on frogs when infected with Bd, it cannot contribute to slowing the growth of Bd once the pathogen is established. In addition to affecting susceptibility to pathogen infection, sensitivity of the microbiome to pathogen infection may also exacerbate disease symptoms. Dysbiosis, the emergence of disease symptoms due to "imbalances" in the microbiome, have been documented in other systems (Chow et al., 2011). Likewise, disturbance of the microbiome due to Bd infection could lead to secondary disease symptoms during chytridiomycosis, although this has not been tested. Knowledge of the sensitivity of the amphibian microbiome to Bd infection should lead to a clearer understanding of both susceptibility to Bd and disease symptoms caused by Bd infection. In Chapter 1, I test whether Bd infection alters the *R. sierrae* skin microbiome in a controlled experiment as well as during naturally occurring disease episodes in wild frog populations. I also examine characteristics of bacterial taxa that appear sensitive to Bd infection, and discuss implications for the development of bacterially-based strategies for the management of chytridiomycosis in wild amphibians.

Comparing the microbiomes of *R. sierrae* populations that either persist or are driven extinct by Bd may provide insights into the links between the microbiome and disease resistance. A previous study compared one persisting and one declining population, and found that the two populations differed in the number of bacterial isolates that inhibited the growth of Bd in vitro, consistent with the hypothesis that differences in symbiotic bacteria affect the outcomes of Bd infection (Woodhams et al., 2007). However, any two populations

are likely to differ in their microbiomes (as shown in Chapter 2), and these differences are not necessarily linked to differences in disease resistance. Other differences between populations, such as environmental differences, host genetic differences, or even the timing of sample collection, may affect the microbiome in ways not necessarily linked to the outcome of infection (persistence or extinction). Therefore, for a more general test of the hypothesis that microbiome differences explain differences in infection outcomes, it is critical to examine more than one population of each type, and to sample all populations in as short a time frame as possible. In Chapter 2, I present a synoptic survey of multiple *R. sierrae* populations, including persisting, declining, and Bd-free populations, to examine landscape-level relationships between Bd infection and microbiome composition. I test whether differences in the microbiome can consistently explain why some populations persist with Bd while others decline to extinction. I also examine the potential for Bd infection to drive variation in the microbiome among *R. sierrae* populations at the landscape level. I present analyses aimed at distinguishing effects of Bd infection on the microbiome from effects of microbiome variation on Bd infection severity using landscape level field data.

Observational field studies can provide some evidence for or against the role of bacterial communities in affecting the outcome of Bd infection, but field observations cannot distinguish cause from correlation. Controlled experiments are needed to demonstrate cause, and clear knowledge of causal relationships forms a fundamental foundation for our understanding of the microbiome's function with respect to disease resistance. A separate but tightly linked question is that of what factors shape the microbiome to begin with. If the microbiome does affect disease resistance, then understanding what factors control the microbiome takes on importance in the realm of applied conservation biology as well as

basic science. The dire outlook for *R. sierrae* and other amphibians threatened with Bd, together with the results of early studies indicating that bacteria can inhibit Bd growth, have led to preliminary trials of probiotic treatments to curb the effects of Bd in wild populations. At the same time, very little is known about the factors that shape the amphibian skin microbiome, making it difficult to predict how the microbiome will respond to probiotic treatments. So far, studies have shown that amphibian species maintain distinct skin microbiomes in the wild (Kueneman et al., 2014; McKenzie et al., 2012; Walke et al., 2014), indicating that innate differences between amphibian species contribute to microbiome composition. It has also been shown that populations of a given amphibian species exhibit differences in their skin-associated bacterial communities (Chapter 2; Kueneman et al., 2014; Walke et al., 2014). However, it is not possible from these results to clearly determine whether microbiome differences were driven by among-population genetic differences or environmental differences associated with different habitats. In Chapter 3, I present a fully crossed factorial experiment to disentangle the effects of within-species host genetic variation and the aquatic environment in shaping the amphibian skin microbiome. I then test how variation in the microbiome affects resistance to Bd infection and chytridiomycosis, directly addressing the question of cause and effect underlying associations between microbiome composition and Bd infection intensity.

The research conducted as part of this dissertation aimed to advance understanding of natural patterns in the amphibian skin bacterial microbiome and causal relationships between the microbiome and severity of infection by Bd. The results show that both genetic variation within host species and differences in aquatic environments contribute to shaping the phylogenetic composition of the amphibian skin microbiome. I found that persisting and

declining *R. sierrae* populations consistently harbor different skin-associated bacterial communities, and that these differences may have the potential to explain differences in the population-level outcomes of disease, consistent with a protective function of the microbiome. At the same time, I demonstrate that the *R. sierrae* microbiome is itself sensitive to Bd infection. Bd infection altered the *R. sierrae* microbiome, and this may in part drive landscape-level patterns of microbiome variation among wild frogs. Furthermore, examination of the specific bacterial taxa affected by Bd infection suggests that sensitivity of bacterial taxa to Bd infection may affect their effectiveness in preventing or limiting the growth of Bd *in vivo*. By coordinating laboratory and field studies, and also considering multiple spatial scales ranging from individual frogs to multiple populations at the landscape level, this work advances an integrated understanding of the amphibian microbiome and its interactions with the pathogen Bd. Taken together, the work presented here advances our understanding of the assembly, function, and sensitivity of the amphibian microbiome when faced with infection by Bd.

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CHAPTER ONE

The pathogen *Batrachochytrium dendrobatidis* disturbs the frog skin microbiome during natural epidemics and experimental infection.

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ABSTRACT

Symbiotic microbial communities may interact with infectious pathogens sharing a common host. The microbiome may limit pathogen infection or, conversely, an invading pathogen can disturb the microbiome. Documentation of such relationships during naturally occurring disease outbreaks is rare, and identifying causal links from field observations is difficult. This study documented the effects of an amphibian skin pathogen of global conservation concern (the chytrid fungus *Batrachochytrium dendrobatidis;* Bd*)* on the skin-associated bacterial microbiome of the endangered frog, *Rana sierrae*, using a combination of population surveys and laboratory experiments*.* We examined covariation of pathogen infection and bacterial microbiome composition in wild frogs, demonstrating a strong and consistent correlation between Bd infection load and bacterial community composition in multiple *R. sierrae* populations. Despite the correlation between Bd infection load and bacterial community composition, we observed 100% mortality of postmetamorphic frogs during a Bd epizootic, suggesting that the relationship between Bd and bacterial communities was not linked to variation in resistance to mortal disease and that Bd infection altered bacterial communities. In a controlled experiment, Bd infection significantly altered the *R. sierrae* microbiome, demonstrating a causal relationship. The response of microbial communities to Bd infection was remarkably consistent: several bacterial taxa showed the same response to Bd infection across multiple field populations and the laboratory experiment, indicating a somewhat predictable interaction between Bd and the microbiome. The laboratory experiment demonstrates that Bd infection causes changes to amphibian skin bacterial communities, while the laboratory and field results together strongly support Bd-disturbance as a driver of bacterial community change during natural disease dynamics.

INTRODUCTION

Symbiotic interactions between microbes and multicellular organisms are ubiquitous. In recent years, research to understand the complex microbial communities living in or on multicellular organisms (termed the microbiome) has sparked fundamental changes in our understanding of the biology of metazoans (Blaser et al., 2013; Dethlefsen et al., 2007; Engel and Moran, 2013; Grice and Segre, 2012; Philippot et al., 2013). The microbiome can affect host health directly by influencing metabolism (Turnbaugh et al., 2006), development (Heijtz et al., 2011), inflammation (Honda and Littman, 2012), or behavior (Theis et al., 2013), but may also influence host health indirectly through interactions with infectious pathogens. The microbiome may interact with pathogens through competition for resources, release of anti-microbial compounds, contact-dependent antagonism, or modulation of the host immune response (Round and Mazmanian, 2009), and an "imbalanced" microbiome may leave the host more susceptible to pathogen infection (Croswell et al., 2009; Khosravi and Mazmanian, 2013). At the same time, an invading pathogen may disrupt the microbiome (Barman et al., 2008; Round and Mazmanian, 2009; Stecher et al., 2007; Winter et al., 2010). Thus, the microbiome may play a role in disease resistance, or may itself be disturbed or altered by invading pathogens. While a wealth of recent research has described associations between microbiome composition and a variety of syndromes in both humans and animals (Cardenas et al., 2012; Closek et al., 2014; Huang et al., 2011; Kelly et al., 2012; Kong et al., 2012; Li et al., 2012; Mazmanian et al., 2008; Sato et al., 2013; Sunagawa et al., 2009; Thurber et al., 2009), documentation of microbiome responses to natural epidemics of known infectious pathogens is rare.

Chytridiomycosis is an emerging infectious disease of amphibians caused by the chytrid fungus *Batrachochytrium dendrobatidis* (hereafter Bd). Bd is an aquatic fungus that infects the skin of

amphibians and disrupts osmoregulation, a critical function of amphibian skin (Voyles et al., 2009). Chytridiomycosis can be fatal, and the severity of disease symptoms has been linked to Bd load, which is a measure of the density of Bd cells infecting the host (Briggs et al., 2010; Vredenburg et al., 2010). Bd has a broad host range spanning hundreds of amphibian species, and has been implicated in population extinctions and species declines worldwide (Berger et al., 1998; Crawford et al., 2010; Kilpatrick et al., 2010; Lips et al., 2006; Morgan et al., 2007; Pounds et al., 2006). Efforts to understand and mitigate the effects of Bd have led to research examining the potential for symbiotic bacteria to increase resistance to infection by the pathogen (Küng et al., 2014; Woodhams et al., 2014). Bacterial species isolated from the skin of amphibians have been shown to inhibit the growth of Bd and other fungal pathogens in culture (Lauer et al., 2007, 2008; Woodhams et al., 2007), possibly by producing anti-fungal metabolites (Becker et al., 2009; Brucker et al., 2008). In a controlled laboratory experiment, inundation of *Rana muscosa* with the bacterium *Janthinobacterium lividium* protected frogs from subsequent Bd infection (Harris et al., 2009). These and other studies highlight the possible role of bacteria in resistance to chytridiomycosis, but critical questions remain. First, most research has focused on the ability of cultured bacteria to prevent Bd infection, while very little is known about whether Bd infection alters the diverse skin microbiome. Examining this latter concept is critical both to a basic understanding of how the microbiome interacts with pathogens and to conservation efforts because Bd-induced perturbations of the microbiome could undermine attempts to mitigate effects of Bd infection through augmentation with particular bacteria. A second knowledge gap is the paucity of comprehensive culture-independent assessments of the amphibian microbiome, which are important because the vast majority of environmental and symbiotic microbes are not readily cultured, and culture-based methods can lead to severe underestimates of diversity and biased assessment of community composition (Rappé and Giovannoni, 2003). Few studies have applied Next-Generation sequencing methods to

characterize the microbial communities on amphibian skin (Kueneman et al., 2014; Loudon et al., 2013; McKenzie et al., 2012; Walke et al., 2014), and to our knowledge none have done so in the context of Bd infection. A final challenge to understanding interactions between Bd and bacteria stems from the difficulties of drawing direct connections between laboratory and field studies. Laboratory studies are essential for definitive identification of cause and effect. However, complex natural microbiomes can be impossible to recreate in the laboratory, and field studies are needed to show whether processes identified in lab are relevant in nature.

We present paired laboratory and field studies using high throughput 16S amplicon pyrosequencing both to document associations between Bd infection and amphibian skin bacterial microbiome in nature, and to deduce causal relationships in an experiment. Our work centers on the Sierra Nevada yellow-legged frog, *Rana sierrae*, which is severely threatened by, and has already suffered drastic declines due to, Bd (Rachowicz et al., 2006; Vredenburg et al., 2010). We surveyed frogs from four distinct *R. sierrae* populations to test if differences in skin bacterial communities are associated with the intensity of pathogen infection. We then conducted a laboratory experiment to establish causal relationships underlying Bd-bacterial community associations. The data establish a strong effect of Bd infection on the composition of the amphibian skin bacterial microbiome that is consistent between the laboratory experiment and naturally occurring Bd dynamics in wild frog populations.

METHODS

Field surveys. This study was conducted in 4 *R. sierrae* populations in the Sierra Nevada of California, USA (specifically Yosemite National Park and Sierra National Forest). Our group has collected census and Bd infection data on these populations as well as others since 2004 or earlier (Briggs et al., 2010; Knapp et al., 2011). For the current study, each population was surveyed three to four times between July and September 2010, and at least once in 2011

(Table S1). Bd infection was analyzed for all swabs collected to establish temporal Bd trajectories, while bacterial community analysis was performed for a subset of swabs to target specific questions (detailed below). In each survey visit to each population, a target of 30 post-metamorphic (i.e. subadult and adult) frogs were captured by dip net and sampled for skin-associated microbes (symbiotic bacteria as well as Bd) with a sterile synthetic swab (Medical Wire and Equipment) using standard protocols (Briggs et al., 2010). Nets were rinsed thoroughly in lake water between each capture and new nitrile gloves were worn for each animal handled. During each visit to each population, the microbial communities present in lake water were sampled by filtration (mean volume filtered: 1198 ml, range: 560-2000 ml) through 0.22 μm pore polyethersulfone filters (Sterivex-GP, Millipore), which were immediately purged of remaining water and amended with 1.5 ml sucrose lysis buffer (40 mmol l^{-1} EDTA, 50 mmol l^{-1} Tris-HCl, 750 mmol l^{-1} sucrose, pH adjusted to 8.0), which has been shown to preserve bacterial DNA samples under field conditions for several days (Nelson, 2009). All samples were frozen immediately upon return to the laboratory.

Selection of samples for bacterial community analysis. We characterized bacterial communities from a subset of swabs to address two central questions. First, to examine associations between Bd infection and skin-associated bacteria independent of geographic or temporal variation, we characterized bacterial communities from 18-20 *R. sierrae* individuals from one sampling visit to each of the four populations. Second, to examine how Bd infection might affect temporal succession of bacterial communities, bacterial communities were analyzed from an additional sampling visit to each of three populations (n=8 to 10 per population, Table S1).

DNA extraction. Swab DNA was prepared for PCR using Prepman Ultra (Life Technologies) as described previously (Boyle et al., 2004; Briggs et al., 2010). Briefly, each swab was incubated with 40 μL Prepman Ultra at 95°C for 10 minutes, centrifuged (3 min, 16,000 G), and the

supernatant collected for use in PCR. Genomic DNA was extracted from water filter cartridges following Nelson (2009) (Nelson, 2009).

Quantification of Bd *loads*. Bd load (also referred to as infection intensity) is the number of Bd zoospores detected on a swab DNA sample of an animal following a standardized protocol (Briggs et al., 2010), and was measured by quantitative polymerase chain reaction (qPCR) following the methods of Boyle *et al.* (Boyle et al., 2004). Standards provided by the laboratory of Alex Hyatt (Commonwealth Scientific and Industrial Research Organization, Geelong, Australia) were prepared from known numbers of Bd cells, enabling estimates of cell counts (Bd load). Swab DNA extracts were diluted 1/10 in molecular biology grade water, and 5 μL of the diluted sample was used in each 25 μL qPCR reaction. Bd load for each sample was calculated in Bd Zoospore Equivalents after accounting for the proportion of the swab extract used in a qPCR reaction.

16S Sequencing and bioinformatic processing. Bacterial communities present on frog skin and in lake water were characterized by 16S ribosomal RNA gene amplicon pyrosequencing as detailed in the Supplementary Methods. Briefly, the V1-V2 regions of the 16S gene were amplified from each sample, and pooled, multiplexed PCR products were sequenced on a Roche/454 GS FLX using Titanium Chemistry. The program mothur v1.30 (Schloss et al., 2009) was used to quality-filter (de-noise and screen for short, potentially low-quality, or chimeric reads) sequences, align them to a curated 16S alignment database (Nelson et al., 2014), and cluster them into operational taxonomic units. Sequences were classified using the Bayesian classifier of Wang *et al.* (Wang et al., 2007) and each OTU was assigned a consensus taxonomy from SILVA v111. Pairwise phylogenetic community distances (weighted Unifrac, (Lozupone and Knight, 2005)) among all samples were calculated based on OTU relative abundances. Measures of bacterial richness and diversity (observed number of OTUs, Chao's estimated

richness (Chao, 1984), and Shannon diversity and evenness) were calculated after randomly subsampling to 500 sequences per sample. Pyrosequencing runs have been deposited in the NCBI Sequence Read Archive (http://www.ncbi.nlm.nih.gov/sra) under accession numbers SRR1598941, SRR1598942, SRR1598944. Sample barcodes for demultiplexing of sequence data are provided in Jani and Briggs (2014).

Statistical analyses. Multivariate analyses, including NMDS, ANOSIM, PERMANOVA, DISTLM, and Mantel tests, were conducted in Primer-E v6 (Clarke and Gorley, 2006). Data were transformed as needed to approximate a Gaussian distribution for parametric statistical analyses: $\log_{10}(X+1)$ for Bd load data, arcsine of square-root for bacterial relative abundance data. In Figures, OTU relative abundance data are shown untransformed to provide intuitively meaningful display of original data. Analyses of relative abundances of individual OTUs were performed using only common OTUs, defined as OTUs present in at least 25% of samples in the population, survey visit, or experiment being analyzed (yielding 51, 50, 38, 68, 33, and 60 common OTUs in the Marmot, Mono, Unicorn, Conness, Experiment, and Temporal analyses, respectively). The effect on Type I statistical error of individually testing multiple OTUs (i.e., multiple comparisons) was accounted for by calculating the false discovery rate, Q, using the program Qvalue (Storey, 2002), and applying a maximum threshold of Q=.05 (Storey and Tibshirani, 2003). All other statistical analyses were performed using JMP v. 10 (SAS Institute Inc., Cary, NC, USA, 1989–2012).

Bacterial community data analysis: We use the term "community composition" in reference to analyses based on the phylogenetic dissimilarity of bacterial communities (e.g. using weighted Unifrac distances), in contrast to community diversity analyses, which assess the number or distribution of taxa without regard to their identities. To visualize multivariate community composition, pairwise community weighted Unifrac distances were rendered in 2-dimensional

space using non-metric multidimensional scaling (NMDS). To test for relationships between bacterial community composition and factors of interest (Bd load, frog population, or time), we used multivariate permutation tests, specifically Mantel tests and DISTLM for continuous explanatory variables and ANOSIM and PERMANOVA (permutation-based MANOVA) for categorical variables. To provide visual representation of multivariate relationships, where relevant, we fit orthogonal regression lines to scatterplots of NMDS axes against Bd load.

Within-population microbiome analyses. To examine the within-population relationship between Bd load and bacterial community composition among individual frogs in the field, we separately analyzed the data from a single survey (visit) for each of the four frog populations (n=18-20 frogs per population, Table S1). We conducted DISTLM and Mantel tests to test for associations between Bd load and overall community composition. For bacterial diversity, we tested for correlations between Bd load and the specified diversity metrics. To identify individual bacterial taxa associated with Bd load, we calculated correlations between Bd load and relative abundance of common OTUs, adjusting significance for multiple comparisons as described above.

Temporal microbiome analysis. We compared temporal change in the microbiomes in three frog populations: one population experiencing increasing Bd loads during an active Bd outbreak, and two "reference" populations, i.e., enzootic populations that had stable Bd loads through time. (The third enzootic population was not included due to logistical limitations in sampling all populations within the same temporal window as well as limited number of samples that could be included in the pyrosequencing run.) This analysis of temporal change in the microbiome should be considered as exploratory : Ideally, multiple sampling dates from multiple reference and outbreak populations would be analyzed, but because Bd outbreaks are unpredictable and Bd loads often rapidly increase to homogenously high loads throughout a

population once an epizootic begins, samples collected during the period of increasing Bd loads within a given population are very rare and almost never synchronized between multiple outbreaks. For this analysis, we compared microbiomes from samples collected 15 days apart (referred to as the "early" and "late" survey visits) and spanning the same time period (August 29 to September 16) in each of the three frog populations (Table S1). Because frogs were not individually marked, it is possible that some frogs were sampled on both dates, which could affect independence of data between the two time points and robustness of statistical analyses. We used PERMANOVA, with time (early or late), host population, and the time*population interaction as explanatory factors, and multivariate bacterial community similarity (Unifrac distance) as the response, to test if bacterial community composition differs by time point or host population, and whether the time effect differs among populations. We used ANOVA (with explanatory factors the same as for PERMANOVA) to examine changes in bacterial diversity. We focus primarily on metrics of whole-community change (e.g. Unifrac distance) as our response variable in analyses of temporal variation because we expect Unifrac distance to be relatively robust to specific compositional differences between populations: We can reasonably compare the magnitude of change in the overall bacterial communities of different frog populations, even if the species composition of communities is different. In contrast, analysis of relative abundances of individual bacterial OTUs may be more sensitive to differences between frog populations in the specific composition of bacterial communities. Therefore, while we present analyses of individual OTUs that changed in time, we emphasize that these are exploratory. We examined temporal change in relative abundances of individual bacterial OTUs to test if these are consistent with Bd-driven change through time in the epizootic population, as detailed in the Supplementary Methods. Briefly, we calculated the magnitude of temporal change for OTUs in the outbreak population and adjusted that effect to remove baseline seasonal variation as estimated by temporal change in the reference populations. We then used ANOVA to test if,

across all OTUs that changed with time in the epizootic population, the magnitude and direction of change in OTU relative abundance was consistent with Bd driving temporal change. We predicted that OTUs that were positively correlated with Bd load in time-independent analysis (previous section) would increase in time, while OTUs that were negatively correlated with Bd would decrease in time.

Experiment to test effects of Bd *on microbiome*: We used experimental manipulations to test for a direct relationship between Bd infection and microbiome composition. We analyzed samples collected from two time points (before and after Bd infection) of a longer-term experiment examining Bd-microbiome relationships under controlled conditions. *R. sierrae* individuals were collected as eggs or tadpoles during the 2010 field season from two wild populations in accordance with National Park Service and US Forest Service permits and reared to the subadult stage in the laboratory. All experimental procedures were approved by the UCSB Institutional Animal Care and Use Committee (IACUC) prior to beginning the study. For 2 weeks prior to beginning the experiment, all frogs were housed in four large custom-built common tanks to standardize the pre-experiment environment: each tank held frogs from both populations, such that frogs from the two populations shared common tank water. Frogs from different source populations sharing a tank were separated by plexiglass dividers with holes to allow water (but not frogs) to pass through. Once weekly, water was also mixed between the tanks to maintain similar environments among the common-garden tanks. At the beginning of the experiment, in September 2011, animals were transferred to individual tanks and housed individually (1 frog per tank) for the duration of the experiment. Frogs were randomly assigned to artificial habitat treatments consisting of either sterilized bottled drinking water (autoclaved, cooled, and checked for sterility by plating aliquots on R2A and LB agar,) or non-sterile water collected from lakes within the habitat range of *R. sierrae* in the Sierra Nevada in an attempt to create more realistic laboratory conditions. Frogs were fed crickets weekly, and tank water was

changed after feeding to minimize introduction of bacteria to the tanks from food. Prior to being added to tanks, lake water was filtered through a 1.2 μm filter to remove larger particles including any Bd cells that might naturally occur in lake water. To characterize the bacterial community present in the experimental habitat, prior to adding lake water to tanks, bacteria present in lake water were sampled by filtration of 250 ml through a .22 μm pore polyethersulfone filter. Forty two frogs from each of the source populations were randomly assigned to the artificial habitats (total of 84 frogs). After a three week acclimation period, half of the frogs were challenged with Bd (3 doses of 200,000 zoospores, consisting of an equal mixture of four Bd strains isolated from wild *R. sierrae*: TST75, CJB4, CJB5, CJB7) released into the tank water of each frog for 3 consecutive days. Before inoculation, Bd cells were rinsed by gently pelleting and resuspending cells in 35 ml sterile water three times, and live cell counts were conducted after rinsing. Frogs in the uninfected (control) treatment received a sham inoculum prepared from Bd culture medium (without Bd) that was harvested and rinsed following the same procedure that was used for the live Bd inoculum. Bd loads and skin-associated bacterial communities were sampled before Bd challenge and weekly thereafter for 60 days post-infection. A new pair of nitrile gloves was used to handle each frog, and frogs were rinsed twice with 60 ml sterile water and swabbed as described above to collect Bd and bacterial cells. All samples were placed in sterile microcentrifuge tubes on ice immediately and frozen within 1 hour of collection. Bd loads were quantified from swab extracts by quantitative PCR, as described above. One frog in the Bd-free treatment group became contaminated with Bd and was excluded from analyses. Skin-associated bacteria were characterized by 16S amplicon pyrosequencing (as detailed above) from swabs collected before and after Bd infection (immediately prior to Bd inoculation and 3 weeks post-infection (PI); the 3-week-PI time point was chosen because loads at that time point were comparable to Bd loads observed in the field). Quantitative PCR, pyrosequencing, and bioinformatic processing, were conducted

as described above. PERMANOVA/ANOVA was used to test for differences in microbiome composition/diversity between Bd infected and uninfected frogs at 3 weeks post infection, with a mixed-effect linear model testing Bd treatment (infected or control, fixed factor), and including water source and frog source as random factors. These analyses were also performed on the pre-infection dataset to confirm that no difference between Bd treatment groups existed before the Bd treatment was applied. Specific OTUs differing between Bd treatment groups were identified using separate mixed-effect linear model tests for each of 33 common OTUs (present on at least 25% of frogs), with frog source and water source included as random factors and significance determined after accounting for multiple comparisons as described above.

Preventing researcher-mediated spread of Bd: To prevent researcher-mediated spread of Bd between *R. sierrae* populations, before conducting surveys or sampling at any field site, all field research equipment (including nets and shoes) that contact lake water or frogs was disinfected with 0.1% quaternary ammonium compound 128 solution and incubated for at least 5 minutes (Johnson et al., 2003), or in the case of small or sensitive equipment, disinfected with 70% ethanol.

RESULTS

Microbiome community composition is correlated with Bd *load in wild* R. sierrae *populations.* To investigate links between Bd infection and the *R. sierrae* skin bacterial microbiome (hereafter simply "microbiome" for brevity) in the wild, we examined both Bd infection loads and bacterial communities from multiple frogs in four *R. sierrae* populations (Table S1) and tested for correlations between Bd load and bacterial community composition or diversity. Bacterial communities and Bd loads were assessed by 16S ribosomal RNA gene amplicon pyrosequencing and quantitative PCR, respectively. An overview of sequence quality and quantity, diversity of

bacterial taxa observed, and baseline analyses of bacterial communities, including tests for differences between *R. sierrae* life stages and comparison of bacterial communities from the lab, field, frogs, and water, are available in the Supplementary Results and Figure S1. Microbial communities can change with time and between geographic locations, and we wished to exclude confounding effects due to temporal or geographic variation when asking specifically how Bd load is correlated with bacterial communities. Therefore, in addressing this question each population was analyzed separately using only samples from a single sampling date (N=18-20 per population, Table S1). Bd load was strongly and consistently correlated with multivariate microbiome composition in all four populations: distance-based linear model (DISTLM) analysis showed a significant linear relationship between Bd load and multivariate bacterial community composition (P values: 0.0005, 0.0075, 0.0002, 0.0012, and proportion of variance explained: 0.37, 0.23, 0.30, 0.26 for the Marmot, Mono, Unicorn, and Conness populations, respectively), and permutation-based Mantel tests showed significant correlation between the distance matrices of Bd loads and bacterial communities (P values: 0.0006, 0.0008, 0.0006, 0.0023, Spearman's rank correlation coefficients: 0.49, 0.42, 0.37, 0.31 for Marmot, Mono, Unicorn, and Conness, respectively). These results demonstrate that multivariate bacterial community composition is correlated with the intensity of Bd infection, which is visualized by fitting an orthogonal linear regression to NMDS ordination axis 1 plotted against Bd load (Figure 1 a,b). In contrast to bacterial community composition, bacterial diversity appeared unrelated to Bd load ($P>0.05$ for all richness/diversity measures tested).

To identify specific bacterial taxa that change with Bd load, we tested for correlations between Bd load and the relative abundance of each common bacterial operational taxonomic unit (OTU). We define common OTUs as those found in at least 25% of samples in each population (see Methods). Bd load was significantly correlated with the relative abundance of 27 OTUs in at least one frog population after corrections for multiple comparisons (Figure 2). There was

remarkable consistency in bacteria-Bd correlations among the four frog populations: 7 of the 27 OTUs were significantly associated with Bd in more than one frog population and the direction of the Bd-OTU correlations was the same across frog populations in all 7 cases (Figure 2b,c). In addition, examining both significant and non-significant associations, the direction of the trend of relationships between Bd load and OTU relative abundance was consistent among all populations tested for 18 of the 19 OTUs for which data was available from multiple populations (Figure 2b). There was also taxonomic consistency in the relationships between Bd and bacteria (Figure 2a): OTUs that were negatively associated with Bd belonged primarily to the Betaproteobacteria, Gammaproteobacteria or Actinobacteria, with a few representatives from the Acidobacteria and Alphaproteobacteria, while the few bacterial OTUs that were positively correlated with Bd load belonged primarily to the Betaproteobacteria, with a few representatives from the Flavobacteria and Verrucomicrobia. OTUs in the same bacterial class or family tended to show consistent relationships (either all negative or all positive) with Bd. This pattern held consistent at the genus level: Among OTUs that were significantly correlated with Bd load, OTUs from the same genus always exhibited the same type of relationship with Bd across frog populations. We found no correlation between Bd load and any OTUs of the genus *Janthinobacterium*, a group from which isolates have been shown to prevent Bd infection in lab studies (Harris et al., 2009), and in general sequences classified to this genus were rare in the field study, representing only 0.026% of all sequences across the four populations.

Bd-*microbiome associations are similar for enzootic and epizootic* Bd *episodes*. *R. sierrae* populations infected with Bd can exhibit enzootic or epizootic disease dynamics. (Enzootic and epizootic refer to disease dynamics in animals, analagous to endemic and epidemic dynamics of human diseases). Three of the populations in this study (Mono, Unicorn, Conness) show enzootic Bd dynamics, characterized by moderate Bd loads and long-term frog population persistence despite Bd infection (Briggs et al., 2010; Knapp et al., 2011). Census and infection

data show these populations have been infected with Bd since 2004 or earlier, with no sign of population decline (Briggs et al., 2010; Knapp et al., 2011). The fourth population (Marmot) first showed signs of Bd infection in late 2010, which rapidly developed into an epizootic (with maximum Bd loads rising from 0 to over 150,000 zoospores in under 2 months) that resulted in catastrophic population decline, with no post-metamorphic frogs found during censuses the following summer (Table S1). Despite these differences in disease dynamics, the relationship between Bd load and bacterial community composition was documented across all four populations (Figures 1 and 2).

Bd *causes changes in the microbiome during experimental infection*. To clarify causal relationships underlying correlations between Bd infection and bacterial community composition, we conducted a laboratory study in which 42 frogs were experimentally infected with Bd and their skin bacterial communities compared to 42 uninfected control animals both pre-infection and 3 weeks post-infection. All animals in the Bd+ treatment became infected, with Bd loads at 3 weeks post-infection comparable to those observed in field surveys (mean $\log_{10}$(Bd load): 3.35, standard deviation: 0.67). Infected and uninfected frogs harbored significantly different bacterial communities (permutation-based multivariate ANOVA, PERMANOVA, P=0.0001). In addition, observed bacterial OTU richness was significantly lower on Bd-infected frogs compared with uninfected frogs (P=0.0129; uninfected: mean = 38.3 OTUs, standard error = 2.09; infected: mean = 33.5 OTUs, standard error = 2.08, calculated after subsampling to 500 sequences per sample), but other diversity metrics (Shannon diversity and evenness and Chao's estimated richness) did not differ between Bd-infected and uninfected frogs. No differences in bacterial community composition or diversity existed between Bd treatment groups before Bd infection (all tests: $p>0.05$), indicating that the observed differences between microbiomes of infected and uninfected frogs were a result of Bd infection treatments.

To identify specific bacterial taxa affected by Bd infection, we tested if the mean relative abundances of common OTUs differed between experimentally infected and uninfected frogs. Bd infection significantly altered the relative abundance of 19 OTUs representing 18 genera, including an OTU in the genus *Janthinobacterium*, which was on average nearly twice as abundant on infected relative to uninfected frogs (Table S2). There was strong concordance in the taxa affected by Bd infection in the experiment and taxa correlated with Bd load in the field surveys. Consistent with the field survey results, taxa negatively affected by Bd in the experiment belonged primarily to the Actinobacteria, Betaproteobacteria, and Gammaproteobacteria, and taxa that were positively affected by Bd were overwhelmingly from the Betaproteobacteria (Table S2). OTUs in 8 genera showed significant effects in both the lab and the field and for all 8 genera the direction of the relationship between OTU abundance and Bd was consistent across experimental and field data: genera that were positively correlated with Bd load in the field were more abundant on Bd-infected frogs in the experiment, while genera that were negatively correlated with Bd load in the field were more abundant on uninfected frogs in the experiment (Figures 3 and S2).

Bd *outbreak is linked to increased temporal change in bacterial communities*. The analyses presented up to this point focus on individual populations, each analyzed within a single time point, or from a controlled experiment, specifically to avoid temporal or spatial confounding in the data. Because those analyses indicated that Bd infection induces changes in the skin microbiome and that Bd load in natural populations covaries with microbiome community structure, we hypothesized that Bd epizootics, where Bd loads increase through time, would be accompanied by temporal shifts in the frog skin microbiome. To analyze temporal shifts in frog skin bacterial communities we analyzed bacterial communities from a second sampling date in three of the field populations to obtain two sampling dates for each population spanning the same two-week period (Table S1). We assessed the magnitude of temporal change in the

microbiomes of frogs in the three populations over the same two-week period, representing two cases of enzootic Bd dynamics and one epizootic Bd outbreak. In the epizootic population (Marmot) Bd loads increased significantly between the two sampling dates (P=0.0017, Figure 4a) while the two enzootic frog populations (Mono and Unicorn) showed no significant change in Bd loads over the same time period (P=0.7210, P=0.7841, respectively). Bacterial community composition showed significant change over time in all three frog populations, but the magnitude of change was greater in the population undergoing the Bd outbreak (Figure 4b,c, permutation-based analysis of similarity, ANOSIM: Marmot P=0.0001, Global R=0.55; Mono P=0.033, Global R=0.18; Unicorn P=0.047, Global R=0.19). PERMANOVA using the combined dataset of all three populations to test the effects of frog population and time on microbiome variation showed that in addition to both main effects ($P_{population}$<.0001, P_{time}<.0001) the interaction between time and frog population was significant ($P_{population\ x\ time}$=.0007). Examination of Global R values (from ANOSIM tests of temporal change within populations) from the outbreak and enzootic populations reveals that the significant Time*Population interaction is due to an increased rate of temporal change of bacterial communities in the epizootic frog population that experienced significant increases in Bd loads. In contrast, ANOVA examining the response of bacterial diversity to time in the three populations showed that temporal dynamics in bacterial diversity did not differ between the outbreak and enzootic populations (Time*Population effect, P>0.05 for all richness and diversity metrics), indicating that the Bd outbreak did not lead to changes in bacterial diversity. These results are consistent with analyses of Bd-bacterial community correlations in the previous field analyses (first paragraph of Results), which showed that Bd load was correlated with bacterial community composition but not diversity. Exploratory analyses of temporal changes in relative abundances of individual OTUs showed that, in the epizootic population, the magnitude and direction of temporal change was consistent with Bd-driven change. Specifically, OTUs that were negatively

correlated with Bd (in analyses restricted to single sampling visits, Figure 2) tended to decrease with time in the analysis of temporal change across two sampling visits. Similarly, OTUs that were positively correlated with Bd (within sampling visits) tended to increase in time across two sampling visits (ANOVA with Tukey HSD post-hoc tests: $P<0.05$; Figure S3 and Table S3).

DISCUSSION

Background and Key Findings: Symbiotic microbial communities are likely to interact with invading pathogens. The microbiome has the potential to mediate disease resistance, but can also be altered by pathogen infection. Both types of interactions have been demonstrated in experiments (Koch and Schmid-Hempel, 2012), usually under laboratory conditions (Barman et al., 2008; Harris et al., 2009; Naik et al., 2012; Sekirov et al., 2008; Stecher et al., 2007), but rarely documented during natural outbreaks of known infectious pathogens in the wild. A few studies have demonstrated associations between pathogen infection and bacterial diversity (Cariveau et al., 2014) or the abundance of certain cultured isolates in the field (Woodhams et al., 2007), but it is often challenging to determine whether observational correlations stem from microbiome variation affecting resistance to pathogen infection, or instead from pathogen infection altering the microbiome. Our data suggest a causal relationship between infection by a fungal pathogen and changes to the skin microbiome of wild frogs during a natural disease outbreak. The intensity of Bd infection was strongly correlated with microbiome community composition in multiple *R. sierrae* populations. One of the populations appeared to suffer 100% mortality due to Bd (Table S1), indicating that the observed link between bacterial community composition and Bd loads did not represent a protective effect of bacteria, at least with respect to fatal disease; the data are more consistent with Bd disturbing bacterial communities (discussed in detail below). This hypothesis is further supported by our laboratory experiment showing that Bd infection induced changes in the skin microbiome, and the similarities between

those experimental results and the Bd-microbiome associations we observed in the field. These findings are directly relevant to amphibian conservation efforts: Bd is one of the leading causes of amphibian declines, and there is a dire need for methods to manage the disease in wild amphibians. Manipulation of amphibian skin-associated bacterial communities to manage the disease have shown some promise; our results indicate that further work to understand the stability of the microbiome in the face of Bd infection may advance microbially-based conservation efforts.

Bd *infection is consistently correlated with bacterial community change during both enzootic and epizootic disease dynamics in nature.* We found that increasing Bd loads were consistently associated with changes in bacterial communities on frogs within multiple *R. sierrae* populations. In addition, the taxonomy of many of the bacteria that varied with Bd load was consistent among frog populations, suggesting a somewhat predictable, non-random relationship between Bd and the *R. sierrae* microbiome. Because the analysis for each population is based on frogs that share a common lake habitat and were sampled on the same day, environmental and temporal variation is minimized and it is likely that the correlation between Bd and microbiome represents a relationship between these two factors rather than a coincidence due to spatial or temporal autocorrelation. Still, two fundamentally different processes could drive the association between Bd and the microbiome. Variation in bacterial communities that exists before Bd invades could determine pathogen resistance, affecting downstream infection intensity. Conversely, Bd infection may perturb bacterial communities and alter their composition. Our microbiome data from the epizootic *R. sierrae* population provide suggestive insights regarding cause and effect: within five weeks of the first signs of Bd infection in this population, Bd infection prevalence reached 100% and the mean Bd load was well above 10,000 Bd cells, the threshold at which *R. sierrae* are thought to succumb to chytridiomycosis (Vredenburg et al., 2010), and by the following season a complete population

crash had ensued, with a census finding no post-metamorphic frogs (Table S1). Thus, the correlation between bacterial communities and Bd loads was not accompanied by measured variation in disease resistance (no frogs were observed to resist infection or to survive the Bd epidemic), indicating that bacterial communities were ultimately not protective in the epizootic population. Instead, the correlation between microbiome and Bd in the epizootic event likely indicates that Bd infection induced changes in bacterial communities. In addition, exploratory analyses of temporal patterns in bacterial communities (Figure 4) are consistent with the Bd-disturbance hypothesis: the rate of bacterial community change through time was greater in a population experiencing increasing Bd loads than in populations where average Bd loads were constant through time. While these results do not rule out the possibility that bacterial communities influence Bd dynamics (discussed further below), taken together they are strongly suggestive of Bd disturbing bacterial communities, a hypothesis that is further supported by our experimental evidence.

Coordinated laboratory and field studies show Bd *infection drives bacterial community change.* A key challenge in microbiome research involves drawing clear connections between mechanistic laboratory experiments and field observations. Due to their diversity and complex assembly, natural symbiotic communities can be nearly impossible to accurately reconstruct in laboratory experiments, making field observations essential for characterizing microbiomes. At the same time, field patterns generally cannot clearly conclude cause or effect, and much of what is known about the workings of microbiomes relies on insights from simplified microbiota of model organisms. By coordinating field studies with experimental studies of the same wildlife host species, we aimed to bridge the gap between describing natural patterns and understanding the underlying processes. We found that experimental Bd infection induced clear changes in the *R. sierrae* skin microbiome. Moreover, in many cases the bacterial taxa that responded to Bd infection in the experiment were also correlated with Bd load in the field

observations, with particular bacterial taxa showing consistent relationships (increasing or decreasing with Bd infection) in both the field and laboratory (Figure 3). This consistency is remarkable given that overall microbiome composition differed between frogs in the laboratory and field (Supplementary Results and Figure S1), and indicates that despite those differences the processes documented in our laboratory experiment are relevant in the field setting. The consistency in bacterial taxa affected also suggests that the effect of Bd infection on the microbiome may be a somewhat predictable process among hosts of the same species. This consistency within a host species is striking given the phenotypic variability in bacterial symbionts among different host species and studies (discussed below). Together, these data show that Bd infection causes changes in the skin microbiome under controlled conditions, and further strongly support Bd-induced disturbance as a process underlying field correlations between Bd and the microbiome. This finding raises the possibility that Bd-induced disturbance of the microbiome contributes to disease symptoms during chytridiomycosis (i.e., Bd infection may cause dysbiosis in addition to direct effects of the pathogen on its host,) a hypothesis that is intriguing but will be challenging to test.

Bacterial OTUs classified to eight known genera responded to Bd infection in both the field survey and laboratory experiment (Figure 3). Bacteria in the genera *Rubrivivax* and *Undibacterium* responded positively to Bd infection in the lab and field. *Rubrivivax* are purple non-sulfur Betaproteobacteria in the widespread freshwater family Comamonadaceae that have been isolated from several environmental sources (Nagashima et al., 2012). While a number of studies have examined metabolic properties of cultured isolates belonging to this genus, little is known about its ecology. *Undibacterium* species are Betaproteobacteria in the family Oxalobacteraceae that have been isolated from soil and freshwater (Kim et al., 2014) and documented in shrimp intestines (Rungrassamee et al., 2014). *Janthinobacterium*, a relative of *Undibacterium* also in the family Oxalobacteraceae, did not show consistent effects between the

laboratory and field studies, but still merits discussion given that it is possibly the best-studied bacterial genus in the context of Bd infection. An OTU in this genus was positively associated with Bd infection in the experiment, but in the field *Janthinobacterium* was rare and not significantly correlated with Bd load. The increases in relative abundances of *Janthinobacterium* and its relative *Undibacterium* in response to Bd infection are interesting given the previously documented protective effects of *Janthinobacterium* (Harris et al., 2009; Muletz et al., 2012) because they raise the possibility that these taxa may act as opportunistic colonists of compromised *R. sierrae* skin in some cases. In general these results highlight the phenotypic and ecological diversity of even closely related bacteria in different host systems and the importance of ecological context for understanding microbial interactions.

Bacteria that were negatively affected by Bd infection in both the laboratory experiment and field survey belonged to six known genera: *Pseudomonas, Stenotrophomonas*, *Methylotenera*, and three Actinomycetes (*Rhodococcus*, *Microbacterium*, and *Sanguibacter*). *Pseudomonas* is a large, ecologically diverse group and members of this genus include known pathogens, common environmental bacteria, commensals, and have been found on the skin of amphibians in previous studies (Woodhams et al., 2007), sometimes at very high relative abundances comparable to those observed in the current study (Roth et al., 2013). *Pseudomonas* species are commonly used in biological control in agriculture (Philippot et al., 2013) and have been shown to have anti-Bd activity in laboratory tests (Flechas et al., 2012), although isolates from *R. cascadae*, a close relative of *R. sierrae*, showed no anti-Bd activity (Roth et al., 2013). Our finding that the abundance of *Pseudomonas* species declined sharply with Bd infection suggests that in the *R. sierrae* system, the sensitivity of *Pseudomonas* species to disturbance by Bd may render these bacteria ineffective at providing protection against Bd infection, regardless of whether they produce anti-Bd metabolites. *Stenotrophomonas* species are broadly present in the environment, including freshwater habitats, sewage, plankton samples, and soil (Piccini et

al., 2006), are also known to cause nosocomial infections in human patients, and have been found to be resistant to broad-spectrum antibiotics (Denton and Kerr, 1998). *Stenotrophomonas* species have been isolated from amphibian skin (Flechas et al., 2012; Woodhams et al., 2007) and an isolate from harlequin toads (*Atelopus elegans*) inhibited Bd growth in laboratory tests (Flechas et al., 2012). *Sanguibacter*, *Rhodococcus*, and *Microbacterium* belong to the Actinomycetales, a group with important applications in human medicine and long appreciated for broad anti-microbial activity (Waksman and Lechevalier, 1962), often through the production of secondary metabolites including polyketides, alkaloids, peptides, and terpenes (Abdelmohsen et al., 2014). *Rhodococcus* and *Microbacterium* species isolated from marine sponges showed activity against diverse pathogens, including bacteria and Trypanosomes (*Microbacterium*), and viruses and fungi (*Rhodococcus*) (Abdelmohsen et al., 2014).

The available data characterizing the taxa that we found to be affected by Bd infection highlight an apparent paradox: many of the taxa that declined in abundance due to Bd infection have been found to have anti-microbial, in some cases specifically anti-Bd, properties, yet these taxa failed to prevent Bd infection in the current study despite often being numerically dominant members of the microbiome. This may indicate that either the sensitivity of these taxa to Bd renders them ineffective at mediating Bd infection, or that they do not produce sufficient quantities of anti-Bd metabolites under the field or laboratory conditions of this study, or that the species or strains observed here do not have the capacity to produce anti-Bd metabolites even though closely related species do. In general these results highlight the difficulty in predicting protective effects of bacterial taxa based on data from even closely related bacteria in another study system or ecological context. Small genetic differences between closely related bacteria can lead to considerable phenotypic diversity. For example, the species *Salmonella enterica* comprises six subspecies, only one of which is pathogenic to mammals (Porwollik et al., 2004). Furthermore, expression of phenotypic traits of a given species vary with ecological

contexts, such as single-strain versus whole community context, culture media versus amphibian host, or laboratory versus field. This diversity and context-dependence may help explain why augmentation with *Janthinobacterium* isolates have been found to increase amphibian resistance to Bd infection in some cases (Harris et al., 2009; Muletz et al., 2012) but failed to protect amphibians in another study (Becker et al., 2011).

Several of the bacterial taxa affected by Bd infection in the current study, such as the Pseudomonadaceae, Opitutae, and Comamonadaceae, are common members of the amphibian microbiome, both in this and other studies (Kueneman et al., 2014; Loudon et al., 2013; McKenzie et al., 2012), and it would be interesting to find out whether these taxa respond similarly to Bd infection in different amphibian species. In general it will be important to determine if the disturbance effect of Bd on the *R. sierrae* microbiome that we observed is generally true for other amphibian species. Roth and colleagues (Roth et al., 2013) tested for differences in cultured bacterial isolates between infected and uninfected *Rana cascadae* and found no effect, although differences between the findings of Roth *et al*. and the current study may be affected by methods (culture-based versus pyrosequencing) or, potentially, differences in severity of infection. (Roth *et al.* report binary Bd infection rather than Bd load, but another study found relatively low loads in *R. cascadae* (Gervasi et al., 2013), compared with the Bd loads observed in *R. sierrae* in the current study.)

Implications for the role of the skin microbiome in disease resistance. Amphibians are experiencing alarmingly high extinction rates (Wake and Vredenburg, 2008), and Bd has caused large-scale declines of amphibian species, including *R. sierrae*. Understanding the potential for symbiotic microbes to mediate disease resistance may provide critical tools for amphibian conservation, and to this end several bacterial isolates have been shown to inhibit Bd growth under laboratory conditions. The current study contributes a natural context to this body of

research. *R. sierrae* populations exhibit natural variability in their response to Bd infection, with epizootic populations undergoing catastrophic declines due to Bd while enzootic populations remain stable despite infection (Briggs et al., 2010; Vredenburg et al., 2010). We found that many of the same bacterial taxa are correlated with Bd load during enzootic and epizootic Bd dynamics (Figure 2), consistent with a similar process (Bd-induced disturbance) occurring in both types of pathogen dynamics. Thus even frogs that are able to tolerate Bd infection may be sensitive to disturbance of the microbiome by the pathogen. However, this does not rule out the possibility that aspects of bacterial community composition could play a protective role. Bd-induced disturbance and bacteria-induced resistance are not mutually exclusive: Even while Bd disturbs the microbiome, the rate of increase in Bd load, the maximum load reached, or the clinical effects of a given pathogen load can still be affected by pre-existing properties of the bacterial community. We also note that the current study focuses primarily on microbiome dynamics within populations, rather than differences between host populations. It is possible that differences between populations in the bacterial communities existing before Bd invades affect rates of Bd load increase or the magnitude or rate of Bd-induced disturbance of the microbiome. In this study, multivariate microbiome composition appeared to be more tightly correlated with Bd load in the epizootic population (Mantel test, R=0.49) than in the enzootic populations (R=0.42, 0.37, 0.31), suggestive of a more stable microbiome in persistent populations, although the hypothesis cannot be tested formally here because only one epizootic event was observed. In addition, although many of the bacterial taxa associated with changes in Bd load were consistent among frog populations, this was not necessarily the case across all taxa, and differences between Bd-bacteria dynamics in enzootic and epizootic populations may still be relevant to variation in disease resistance. Much work in this and other study systems is still needed to achieve a fully integrated understanding of the variability and stability of the skin microbiome and its role in disease resistance.

SUPPLEMENTARY METHODS

Preparation of Libraries for Multiplexed 16S Amplicon Sequencing. Bacterial communities were analyzed by 16S amplicon pyrosequencing as follows. The V1-V2 regions of the 16S gene were amplified using primers 8f with 5' Roche FLX Amplicon Adapter B (underlined in primer sequence) and TC linker (denoted in lower case): GCCTTGCCAGCCCGCTCAG-**tc-**AGRGTTYGATYMTGGCTCAG, and 338r with 5'Roche FLX Amplicon Adapter A, 8-basepair barcode ((Hamady et al., 2008), denoted by "xxxxxxxx",), and CA linker: GCCTCCCTCGCGCCATCAG-xxxxxxxx-**ca-**TGCWGCCWCCCGTAGGWGT. For the 16S amplicon library of samples from the laboratory experiment, the 8-basepair barcode was incorporated into the forward primer. Each 25 µL PCR reaction consisted of 1.25 U DNA polymerase (5Prime) in proprietary buffer, 200 µmol L^{-1} each dNTP, 2 mmol L^{-1} MgCl2, 5% Acetamide, 200 nmol L^{-1} each primer, and 1 µL DNA template. PCR conditions were 94°C/120s followed by 35 cycles of 94°C/30s, 58°C/45s, 72°C/60s, and final extension (72°C/300s). A subset of samples and all negative controls were visualized by electrophoresis in a 1% agarose gel stained with ethidium bromide, and further quantified using PicoGreen fluorescence on a Qubit fluorometer (Invitrogen). All PCR products were pooled in equimolar quantities for sequencing on one half picotitre plate on a Roche/454 GS FLX using Titanium Chemistry (laboratory of Stefan Schuster, Pennsylvania State University). Samples generated in the experiment (168 frog swabs, 12 water samples) and field survey (99 frog swabs, 7 water samples) were analyzed on separate pyrosequencing runs.

Bioinformatic Processing: The program mothur v 1.30 (Schloss et al., 2009) was used to quality filter and align sequences, cluster into operational taxonomic units (OTUs), classify OTUs, and calculate per-sample diversity measures and among-sample distance measures as follows. Pyrosequence flowgrams were first quality-filtered using PyroNoise (Quince et al., 2009).

Sequence reads were then screened to remove reads with any ambiguous base call, any barcode mismatch, more than 1 primer mismatches, homopolymers > 8 bases, or read length < 200 bases. Sequences identified as chimeric PCR products were removed using Perseus (Quince et al., 2011). Sequences were aligned to a non-redundant representative subset of approximately 50,000 template sequences (Nelson et al., 2014) of the SILVA v111 SSU Ref 16S curated alignment database (Quast et al., 2013); sequences beginning after or ending before 95% of sequences, or with fewer than 200 bases, were considered potential sequencing artefacts and removed from the data set. To further minimize generation of spurious OTUs due to PCR or sequencing error, sequences differing by <1% were grouped using average neighbor single linkage preclustering (Huse et al., 2010). Sequences were then assigned to operational taxonomic units (OTUs) by average-neighbor hierarchical clustering at the 95% identity level (95% identity across the sequenced V1-V2 region of the 16S gene best approximates 97% identity across the entire 16S gene, a standard benchmark for assigning bacterial taxa; (Schloss, 2010)). OTU relative abundances were used to calculate pairwise among-sample phylogenetic distances (weighted Unifrac; (Lozupone and Knight, 2005)). Sequences were classified using the Bayesian classifier of Wang *et al.* (Wang et al., 2007) with minimum 70% confidence, and each OTU was assigned a consensus taxonomy from SILVA v111. Sample-based richness and diversity measures (observed OTU richness, Chao's richness estimate (Chao, 1984), Shannon diversity, Shannon evenness) were calculated after randomly subsampling to 500 sequences per sample. 11 field and 2 experiment samples had fewer than 500 sequences and were excluded from diversity analyses. Across the 106 field collected samples, 159,126 sequences passed the quality control pipeline, with median sequence length of 323 bp and a mean of 1,508 sequences per sample. From the 180 experimental samples analyzed here (see below for experiment details), 393,119 sequences passed quality control, with median sequence length 323 bp and a mean of 2,184 sequences per sample.

Analysis of temporal change in OTU relative abundances. We conducted analyses to examine whether patterns of change in bacterial OTU relative abundances over time are consistent with Bd driving temporal change in the outbreak population; these are intended to be exploratory rather than conclusive because analyses of OTU relative abundances may be sensitive to specifics of community composition, which may be particularly important when incorporating data from multiple host populations and survey dates into the analysis. Unlike the time-independent analyses (within-population-visit analyses and experimental study), in the temporal analysis it is critical to consider not only the direction of OTU change, but also the magnitude of change. This is because a given OTU may be affected by both Bd and baseline seasonal change, in which case it would change with time in both the epizootic population (where temporal change potentially includes effects of both Bd and seasonal variation) and reference populations (where only seasonal variation is relevant). Therefore it is not informative to simply compare lists of OTUs that change with time in each frog population. Instead, we accounted for baseline seasonal change in the outbreak population and then conducted statistical analyses to ask if, across all OTUs that changed with time in the outbreak population, the magnitude and/or direction of change in time is predicted by patterns of Bd-OTU correlations that were calculated in the absence of temporal or geographic confounding (e.g. Figure 2). To do this, we first used t-tests (with FDR correction for multiple tests as described in *Methods: Statistical Analyses*) to identify OTUs that had significant differences in relative abundance between the Early and Late sampling dates in the outbreak population. Only common OTUs were tested, with "common" defined as the 60 OTUs that were present in at least 25% of samples across the three populations, as described in *Methods:Statistical Analyses*. For each OTU that showed significant temporal change, we calculated an estimate of the magnitude and direction of temporal change in the outbreak population after accounting for baseline seasonal variation, which we refer to as the reference-adjusted effect of time, $D_{\text{reference-adjusted}}$:

$$D_{reference\text{-}adjusted} = D_{outbreak} - D_{reference}$$

where $D_{outbreak}$ is the time parameter estimate for the effect of Time on relative abundance of a given OTU in the one-way ANOVA (t-test), for the outbreak population, and $D_{reference}$ is the mean parameter estimate for time in the two reference (enzootic) populations. Only parameters from significant t-tests are considered since parameters from non-significant t-tests are not significantly different from zero. If Bd drives temporal change in OTU relative abundances, we expect those OTUs that were correlated with Bd in the within-date analyses (Figure 2) to exhibit greater change through time, after accounting for seasonal variation, than OTUs that were not correlated with Bd load. To statistically test this hypothesis, we categorized each OTU as positively correlated, negatively correlated, or uncorrelated with Bd based on within-date analyses (from Figure 2) and conducted ANOVA (with Tukey HSD post-hoc pairwise comparison of means) to test if the reference-adjusted time parameter ($D_{reference\text{-}adjusted}$) in the outbreak population differs among the three Bd-correlation groups. For this analysis we used the population-specific-Bd correlations in Figure 2 (i.e. Bd correlations from Marmot) since the objective is to predict change within the same population. However, we also conducted the same analysis using a less conservative definition to group OTUs into Bd-correlation categories: Here, OTUs were categorized based on within-visit correlation in *any* population (any of the OTUs listed in Figure 2). This did not change the qualitative outcome of the analyses.

SUPPLEMENTARY RESULTS

Overview of R. sierrae microbiome and aquatic bacterial communities. We identified 5,188 bacterial OTUs (mean 139 OTUs per sample) in the field-collected samples, representing 38 phyla and 998 phylotypes, 580 of which could be classified to the genus level. In the experiment there were 1,162 OTUs (mean 38 OTUs per sample), representing 26 phyla and 346 phylotypes, with 254 classified to genus. Bacterial communities differed between lab-reared and wild frogs: in particular bacterial communities from lab-reared frogs had lower richness and diversity (observed OTU richness $P<0.0001$, Chao richness estimate $P<0.0001$, Shannon diversity $p<0.0001$, Shannon evenness $P=0.0006$). However, lab and field-collected microbiomes clustered together and were distinct from bacterial communities from ambient water in an ordination based on phylotypes of the combined lab and field data (Figure S1), indicating that community composition of lab-reared frogs was a reasonable representation of the *R. sierrae* microbiome.

No effect of life stage on microbiome composition. Because our analysis included samples from both adult and subadult *R. sierrae*, we tested for effects of life stage on microbiome community composition. We used PERMANOVA to test for differences in the bacterial communities of adult versus subadult frogs, based on the multivariate phylogenetic Unifrac distance and using data from the four focal surveys (i.e., visits with >10 frogs sampled, see Table S1.) The analysis were conducted separately for each population visit to avoid confounding with temporal or among-population variation. In three of the populations, Bd loads were higher in subadults than adults (t-tests conducted separately within each population, $P<0.0001$ for Mono, Unicorn, and Conness), therefore we included $\log_{10}$(Bd load) as a covariate in the analysis. (In the Marmot population, Bd load did not differ significantly by life stage, probably because during epizootics adult loads increase to similar levels to those generally seen in subadults. To ensure robust

results, in Marmot we conducted PERMANOVA tests for the effect of life stage on community composition both with and without Bd load in the model, and obtained the same result: no significant effect of life stage.) Life stage had no significant effect on bacterial community composition in any of the four population visits (Marmot: $P_{lifestage}$=0.2840, P_{BdLoad}=0.0007; Mono: $P_{lifestage}$=0.6960, P_{BdLoad}=0.0271; Unicorn: $P_{lifestage}$=0.1034, P_{BdLoad}=0.0008; Conness: $P_{lifestage}$=0.8022, P_{BdLoad}=0.0452). This result is consistent with a previous study (Kueneman et al., 2014), which found that skin-associated bacterial communities did not differ between subadult and adult *Rana cascadae*.

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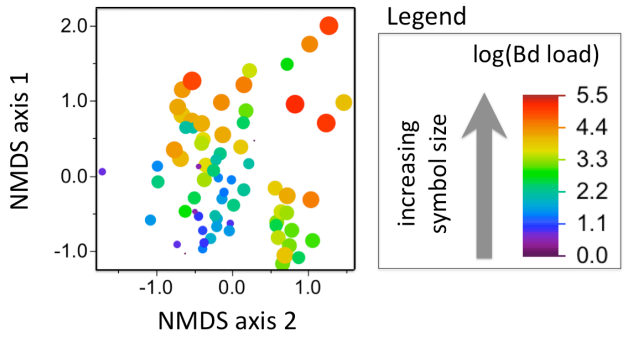
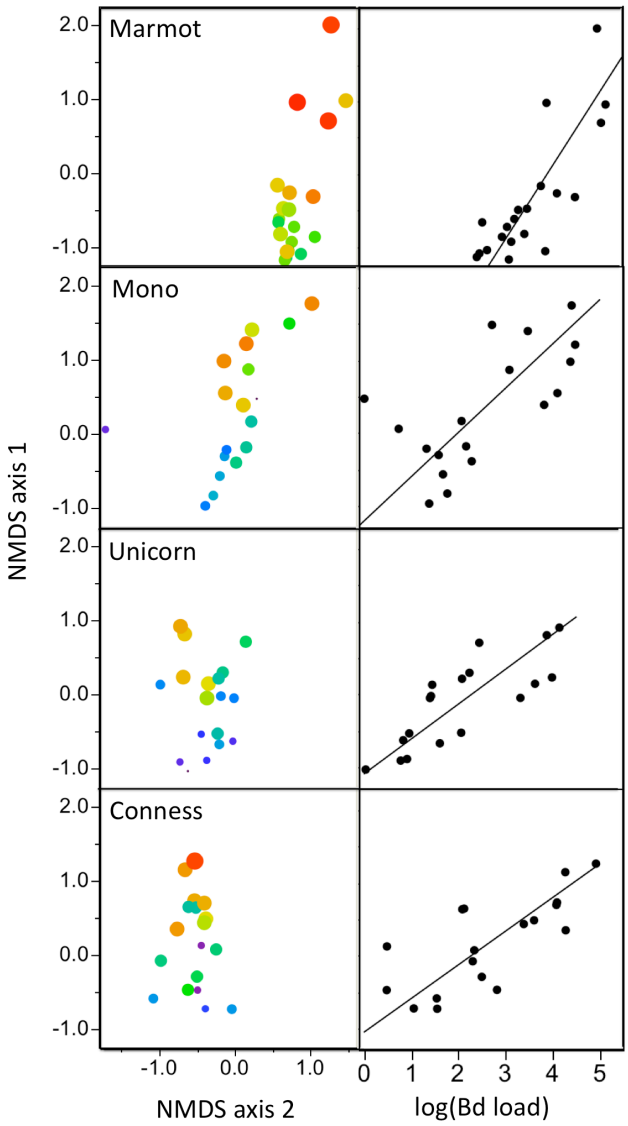
TABLES AND FIGURES

Figure 1. Skin bacterial community composition covaries with Bd load in wild frogs. (a) NMDS ordination of bacterial communities from the four frog population visits (stress = 0.17; marker color and size indicate Bd load.). (b) NMDS ordination from panel (a) separated by frog population visit (left, labeled by population) and correlations between Bd load and NMDS axis 1 (right). Analyses were performed within a single sampling visit to each population to avoid temporal or spatial confounding. Lines of fit represent significant orthogonal regressions ($P < 0.05$) and are intended for visualization purposes only: formal hypothesis tests (all significant, $P < 0.05$) for multivariate community data were performed using multivariate statistical methods (Mantel tests, DISTLM) as reported in Methods.

a


NMDS axis 1
2.0
1.0
0.0
-1.0
-1.0
0.0
1.0
NMDS axis 2
Legend
log(Bd load)
increasing symbol size
5.5
4.4
3.3
2.2
1.1
0.0

b


Marmot
Mono
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NMDS axis 1
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log(Bd load)
2.0
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1
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Figure 2. Bacterial taxa show consistent associations with Bd load across frog populations. (a) Bacterial OTUs with negative (top) versus positive (bottom) associations with Bd fall into distinct taxonomic groups (listed at left, with OTU identifiers in parentheses). (b) Bacterial associations with Bd are consistent across four frog populations. Values are coefficients of correlation between Bd load and bacterial OTU relative abundances within one sampling visit for each frog population. Text color indicates direction of correlation or trend (red: positive, blue: negative); asterisks indicate statistical significance (* marginally significant trend with $P<0.05$; ** significant relationship with $P<0.05$ and $Q<0.05$). Shown are the 27 OTUs that are significantly correlated with Bd load in at least 1 population. (c) Representative scatterplots of correlations between OTU relative abundance and Bd load for three common bacterial OTUs. For each OTU, the direction of the relationship is the same across frog populations. Scatterplots fit orthogonal regression lines to each population where a significant relationship or marginally significant trend was detected. Relative abundances are proportions of the total sampled community. Analyses were performed within a single sampling visit to each population to avoid temporal or spatial confounding.

a

b

	Bacterial OTU	Marmot epizootic	Mono	Conness enzootic	Unicorn
taxa negatively correlated with Bd load	Acidobacteria-Acidobacteriaceae-Bryocella (F-177)			-0.67**	
	Actinobacteria-Nocardiaceae-Rhodococcus (F-15)	-0.65**	-0.42	-0.25	-0.32
	Actinobacteria-Sporichthyaceae-hgcI_clade (F-13)	-0.60**	-0.62**	-0.42	
	Actinobacteria-Sporichthyaceae (F-29)	-0.17	-0.66**		
	Actinobacteria-Microbacteriaceae-Microbacterium (F-12)	-0.80**	-0.56**	-0.48*	-0.47
	Actinobacteria-Microbacteriaceae-Microbacterium (F-30)	-0.69**	-0.38		-0.27
	Actinobacteria-Microbacteriaceae-Microbacterium (F-33)			-0.58**	-0.27
	Actinobacteria-Sanguibacteraceae-Sanguibacter (F-11)	-0.76**	-0.20	-0.42	-0.24
	Alphaproteobacteria-Caulobacteraceae-Brevundimonas (F-52)			-0.56**	-0.17
	Gammaproteobacteria-Pseudomonadaceae-Pseudomonas (F-1)	-0.81**	-0.42	-0.60**	-0.61**
	Gammaproteobacteria-Pseudomonadaceae-Pseudomonas (F-5)	-0.81**	-0.35	-0.69**	-0.13
	Gammaproteobacteria-Pseudomonadaceae-Pseudomonas (F-18)	-0.76**			
	Gammaproteobacteria-Xanthomonadaceae-Stenotrophomonas (F-3)	-0.77**	-0.52*	-0.58**	-0.52*
	Betaproteobacteria-Methylophilaceae-Methylotenera (F-20)	-0.41	-0.58**	-0.11	-0.07
	Betaproteobacteria-Alcaligenaceae (F-323)		-0.62**		
	Betaproteobacteria-Alcaligenaceae-GKS98_freshwater_group (F-31)	-0.58**			
	Betaproteobacteria-Burkholderiaceae-Polynucleobacter (F-6)	-0.21	-0.75**	-0.66**	-0.28
	Betaproteobacteria-Comamonadaceae-Chlorochromatium (F-78)		-0.56**	-0.21	
	Betaproteobacteria-Comamonadaceae-Limnohabitans (F-10)		-0.64**		
	Betaproteobacteria-Comamonadaceae-Variovorax (F-36)	-0.71**			
taxa positively correlated with Bd load	Betaproteobacteria-Comamonadaceae-Rubrivivax (F-2)	0.66**	0.69**	0.63**	0.87**
	Betaproteobacteria-Comamonadaceae (F-56)		0.66**	0.04	0.06
	Betaproteobacteria-Oxalobacteraceae-Undibacterium (F-8)	0.45*	0.60**	0.53*	
	Betaproteobacteria-Oxalobacteraceae-Undibacterium (F-16)	0.23	0.51*	0.63**	0.24
	Flavobacteria-Flavobacteriaceae-Soonwooa (F-4)	0.66**	0.49*		-0.02
	Verrucomicrobia-Opitutae (F-55)		0.61**		
	Verrucomicrobia-Opitutae (F-146)		0.56**		

c

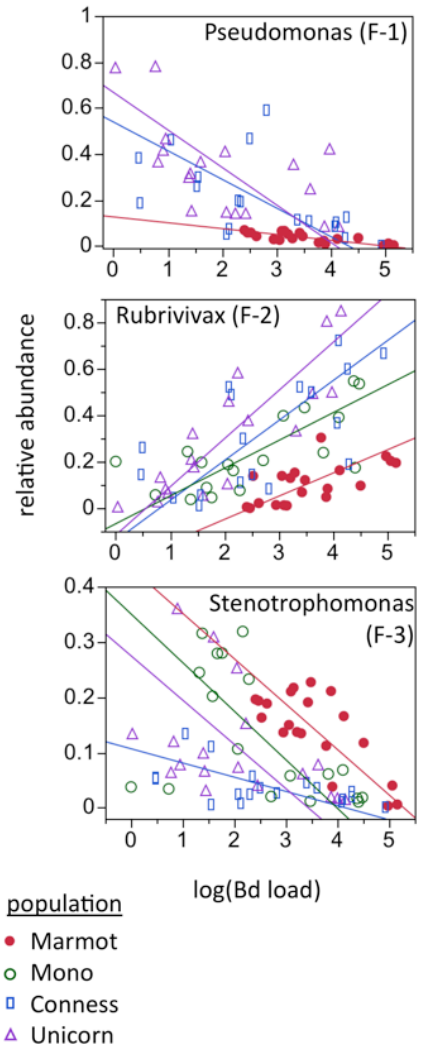


Figure 3. Bd-bacterial relationships are consistent across the laboratory experiment and field surveys. Left panel: comparison of bacterial OTU mean relative abundance between Bd infected and uninfected frogs in laboratory experiment. Right panel: correlations between OTU relative abundances and Bd load in field survey. In all cases the direction of the relationship between bacterial OTU relative abundance and Bd is consistent between laboratory and field data. Except where noted ("NS", not significant) all relationships are significant ($P<0.05$ and $Q<0.05$). For clarity, for each genus only one representative OTU from the lab and one field population visit (Marmot sampled on August 30) is shown; see Figure S2 for complete results. The eight genera shown are those from which OTUs were significantly associated with Bd infection in the lab experiment and at least one field population. Relative abundances are proportions of the total sampled community. Error bars are standard error.

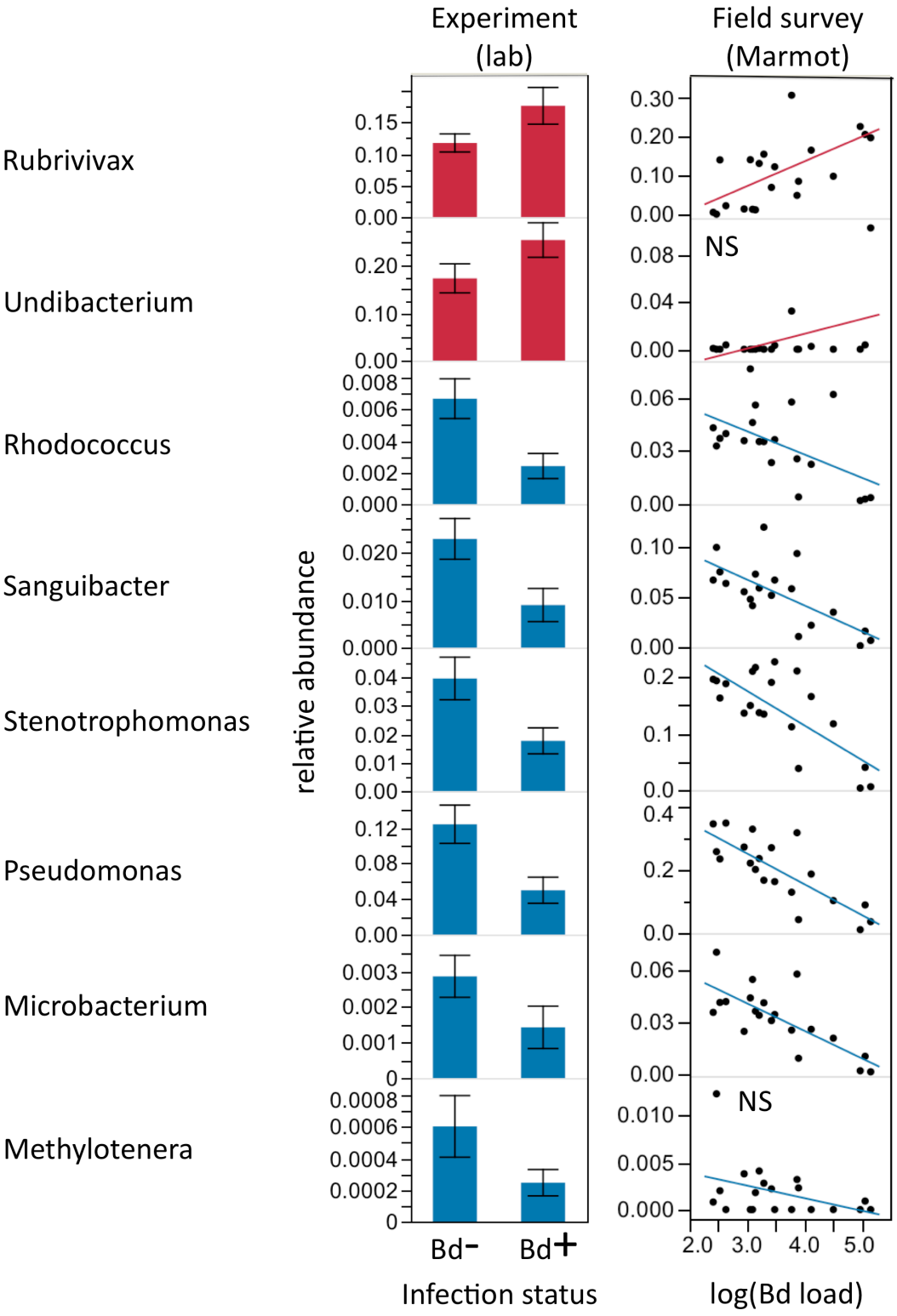
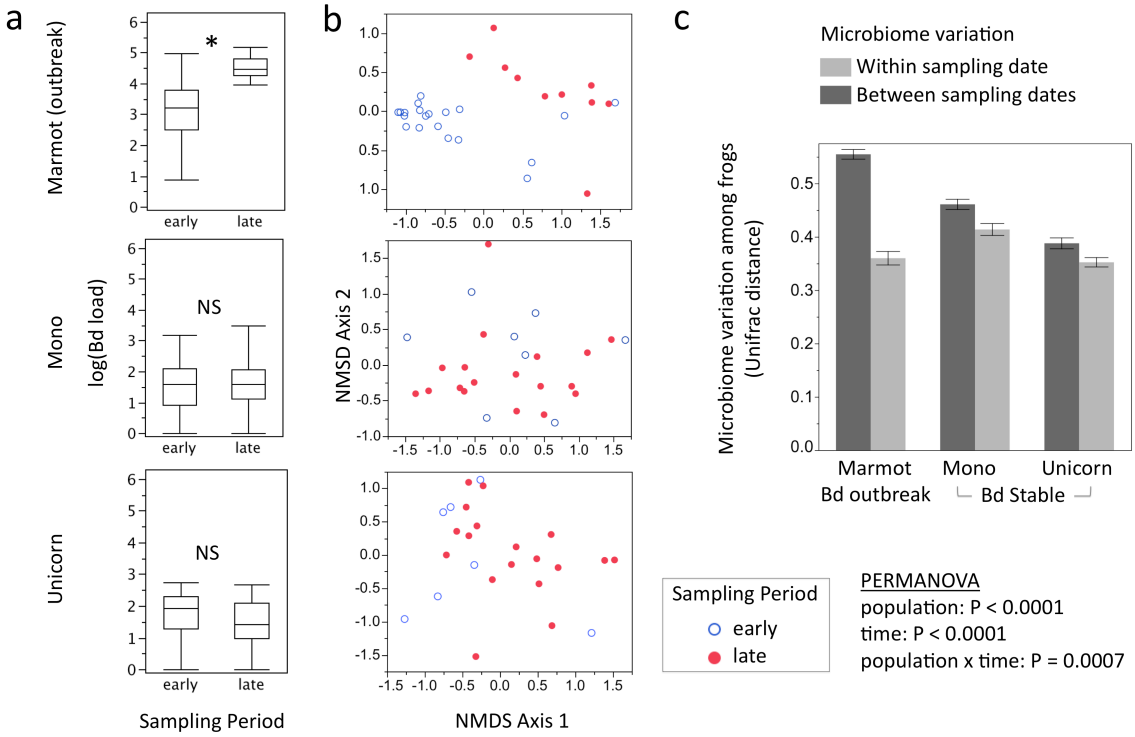

Experiment
(lab)
Field survey
(Marmot)
Rubrivivax
Undibacterium
Rhodococcus
Sanguibacter
Stenotrophomonas
Pseudomonas
Microbacterium
Methylotenera
relative abundance
NS
NS
Bd−
Bd+
Infection status
log(Bd load)

Figure 4. Increased temporal change in the skin microbiome during a Bd outbreak relative to enzootic infection episodes. (a) Bd loads increased through time in the epizootic population experiencing Bd outbreak (Marmot, P<0.0001) but were stable in the enzootic populations (Mono, Unicorn, P>0.05). (b) NMDS ordination of bacterial communities in each frog population shows greater temporal distinction in the epizootic population compared with the two enzootic populations. Ordination stress: Marmot 0.08, Mono 0.14, Unicorn 0.11. (c) Microbiome variation (mean pairwise weighted Unifrac distance among individuals) between the early and late samples (dark bars) was greater in the epizootic than in the enzootic populations. Variation in a given population within sampling dates (light bars) provides a baseline of variation not related to temporal change. Early and late time points are 15 days apart in each population, with all samples collected August 29 through September 16, 2010.



SUPPLEMENTARY TABLES AND FIGURES

Table S1. Field surveys conducted in the study populations, 2010-2011. Bd infection status was determined by quantitative PCR. Disease dynamics (enzootic, epizootic) were determined by historic data (Briggs *et al*. 2010, Knapp *et al.* 2013) together with data presented here on Bd load trajectories and *R. sierrae* population stability (see Methods). Population visits analyzed individually for within-population visit association between Bd and bacterial communities are shown in **bold text**.

* In the Marmot population, no post-metamorphic frogs were found in the 2011 census due to Bd-induced population crash, but presence of Bd at the site could be determined based on swab samples of *R. sierrae* tadpoles. (Tadpoles can persist after extinction of post-metamorphic *R. sierrae* because Bd does not cause lethal disease in tadpoles of this species.)

Population Nickname	Survey Date	Number of frog swabs analyzed for Bd load: total (adults,subadults)	Number of frog swabs analyzed for bacterial communities	Bd infection status	Bd load (log_{10}) for Adult frogs: mean (SD)	Bd disease dynamics
Marmot	July 16, 2010	30 (30, 0)		uninfected	0.00 (0.00)	epizootic
	August 8, 2010	30 (27, 3)		uninfected	0.06 (0.24)	
	August 30, 2010	**30 (22, 8)**	**20**	**infected**	**3.18 (0.88)**	
	September 14, 2010	14 (10, 4)	10	infected	4.58 (0.37)	
	September 15, 2010	29 (27, 2)		infected	4.38 (0.38)	
	September 6, 2011	Population crashed; no frogs encountered.		infected *	N/A	
Mono	July 7, 2010	6 (6, 0)		infected	0.63 (0.60)	enzootic
	August 11, 2010	20 (17, 3)		infected	1.5 (0.77)	
	September 1, 2010	33 (26, 7)	8	infected	1.49 (0.85)	
	September 16, 2010	**40 (31, 9)**	**18**	**infected**	**1.58 (0.80)**	
	July 21, 2011	28 (27, 1)		infected	1.58 (0.83)	
Unicorn	July 28, 2010	19 (19, 0)		infected	0.45 (0.65)	enzootic
	August 29, 2010	31 (22, 9)	7	infected	1.74 (0.80)	
	September 13, 2010	**37 (24, 13)**	**18**	**infected**	**1.47 (0.71)**	
	August 5, 2011	31 (28, 3)		infected	1.08 (0.98)	
Conness	July 9, 2010	27 (20, 7)		infected	1.31 (0.88)	enzootic
	August 18, 2010	**30 (18, 12)**	**18**	**infected**	**1.85 (0.75)**	
	September 10, 2010	30 (19, 11)		infected	2.08 (0.63)	
	July 17, 2011	14 (13, 1)		infected	1.58 (0.96)	

Table S2. Bacterial OTUs significantly affected by Bd infection in the laboratory experiment. Experimental group (Bd-infected or uninfected) in which relative abundance of each OTU was higher is indicated. Least squares means are based on arcsine-square-root transformed data. Criteria for statistical significance: $P<0.05$ and $Q<0.05$.

Bacterial OTU	Group with Higher Relative Abundance	Least Squares Mean	
		Bd-	Bd+
Actinobacteria-Nocardiaceae-Rhodococcus (E-24)	uninfected	0.0650	0.0297
Actinobacteria-Cellulomonadaceae-uncultured (E-47)	uninfected	0.0123	0.0052
Actinobacteria-Microbacteriaceae-Microbacterium (E-26)	uninfected	0.0400	0.0179
Actinobacteria-Sanguibacteraceae-Sanguibacter (E-9)	uninfected	0.1205	0.0576
Sphingobacteriia-Chitinophagaceae-Filimonas (E-55)	uninfected	0.0202	0.0075
Alphaproteobacteria-Brucellaceae-Ochrobactrum (E-41)	uninfected	0.0362	0.0178
Alphaproteobacteria-Rhizobiaceae-Rhizobium (E-49)	infected	0.0153	0.0345
Gammaproteobacteria-Enterobacteriaceae-Pantoea (E-20)	uninfected	0.0739	0.0335
Gammaproteobacteria-Pseudomonadaceae-Pseudomonas (E-2)	uninfected	0.3078	0.1595
Gammaproteobacteria-Xanthomonadaceae-Stenotrophomonas (E-7)	uninfected	0.1650	0.0954
Gammaproteobacteria-Xanthomonadaceae-Stenotrophomonas (E-105)	uninfected	0.0096	0.0035
Betaproteobacteria-Methylophilaceae-Methylotenera (E-57)	uninfected	0.0131	0.0071
Betaproteobacteria-Neisseriaceae-Aquitalea (E-6)	uninfected	0.2609	0.0801
Betaproteobacteria-Comamonadaceae-Acidovorax (E-11)	infected	0.0546	0.1980
Betaproteobacteria-Comamonadaceae-Curvibacter (E-5)	infected	0.1701	0.3246
Betaproteobacteria-Comamonadaceae-Rhodoferax (E-25)	infected	0.0159	0.0540
Betaproteobacteria-Comamonadaceae-Rubrivivax (E-4)	infected	0.3203	0.4033
Betaproteobacteria-Oxalobacteraceae-Janthinobacterium (E-34)	infected	0.0129	0.0247
Betaproteobacteria-Oxalobacteraceae-Undibacterium (E-1)	infected	0.3605	0.4719

Table S3. Bacterial OTUs that changed in relative abundance between the early and late sampling points in the outbreak population. Of 17 OTUs that changed with time, 11 were also correlated with Bd load in the same population in previous analyses (shown in Figure 2). OTUs here are grouped by Relationship with Bd (independent of time), corresponding to groups in ANOVA (Figure S3), and sorted taxonomically within groups. "Time parameter" is the parameter estimate for the effect of Time (comparing early and late time points in 1-way ANOVA, where the response variable is relative abundance of a given OTU, arcsine-square-root transformed) in the outbreak population after subtracting the corresponding Time effect in the reference populations (see Supplementary Methods for details). "Change with time" denotes the qualitative direction of the time parameter. "Relationship with Bd" refers to the OTU-Bd correlations, calculated on a single sampling date to avoid confounding with time, for each OTU in the outbreak population (as shown in Figure 2).

Bacterial OTU	time parameter	change with time	Relationship with Bd (independent of time)
Betaproteobacteria-Comamonadaceae-Rubrivivax (F-2)	0.1668	increase	positive
Flavobacteria-Flavobacteriaceae-Soonwooa (F-4)	0.2986	increase	positive
Actinobacteria-Microbacteriaceae-Microbacterium (F-12)	-0.0909	decline	negative
Actinobacteria-Microbacteriaceae-Microbacterium (F-30)	-0.0730	decline	negative
Actinobacteria-Nocardiaceae-Rhodococcus (F-15)	-0.0691	decline	negative
Actinobacteria-Sanguibacteraceae-Sanguibacter (F-11)	-0.1521	decline	negative
Actinobacteria-Sporichthyaceae-hgcI_clade (F-13)	-0.0285	decline	negative
Betaproteobacteria-Comamonadaceae-Variovorax (F-36)	-0.0376	decline	negative
Gammaproteobacteria-Pseudomonadaceae-Pseudomonas (F-1)	-0.1172	decline	negative
Gammaproteobacteria-Pseudomonadaceae-Pseudomonas (F-18)	-0.1188	decline	negative
Gammaproteobacteria-Pseudomonadaceae-Pseudomonas (F-5)	-0.1967	decline	negative
Actinobacteria-Cellulomonadaceae-uncultured (F-35)	-0.0495	decline	no correlation
Betaproteobacteria-Burkholderiaceae-Polynucleobacter (F-6)	-0.0312	decline	no correlation
Flavobacteria-Flavobacteriaceae-Flavobacterium (F-26)	0.0490	increase	no correlation
Gammaproteobacteria-Aeromonadaceae-Aeromonas (F-57)	0.0308	increase	no correlation
Gammaproteobacteria-Enterobacteriaceae-Enterobacter (F-98)	0.0022	increase	no correlation
SubsectionIII-Pseudanabaena-unclassified Pseudanabaena (F-79)	0.0389	increase	no correlation

Figure S1. NMDS ordination of microbial communities from frog skin and environmental water in the laboratory and field studies. Phylotype relative abundance data from laboratory and field were combined and Bray Curtis distances calculated based on relative abundances of all phylotypes. To avoid pseudoreplication, only one sampling date from the lab and field is shown although results are qualitatively the same when all data are included. Ordination stress=0.17

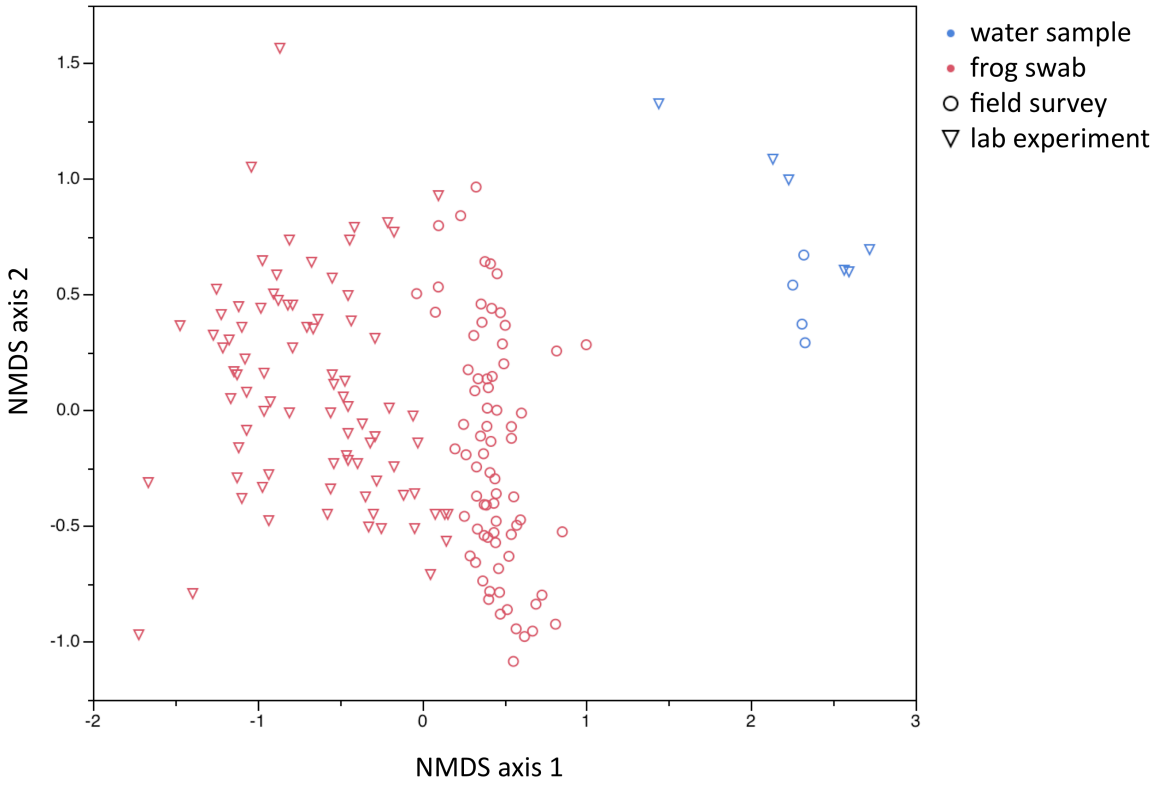


Figure S2. OTUs classified to eight genera were associated with Bd infection in both the laboratory and field studies. Scatterplots show OTU relative abundances plotted against $\log_{10}$(Bd load). Data for the laboratory experiment and representative data for the Marmot field population on August 30 are shown in Fig. 3. Shown here are additional OTUs (genus Pseudomonas) correlated with Bd load in Marmot on August 30, as well as data for the remaining three frog population visits: Conness on August 18, Mono on September 16, and Unicorn on September 13. Blue lines indicate significant negative correlations, red lines indicate significant positive correlations, and gray lines indicate relationships that are not statistically significant.

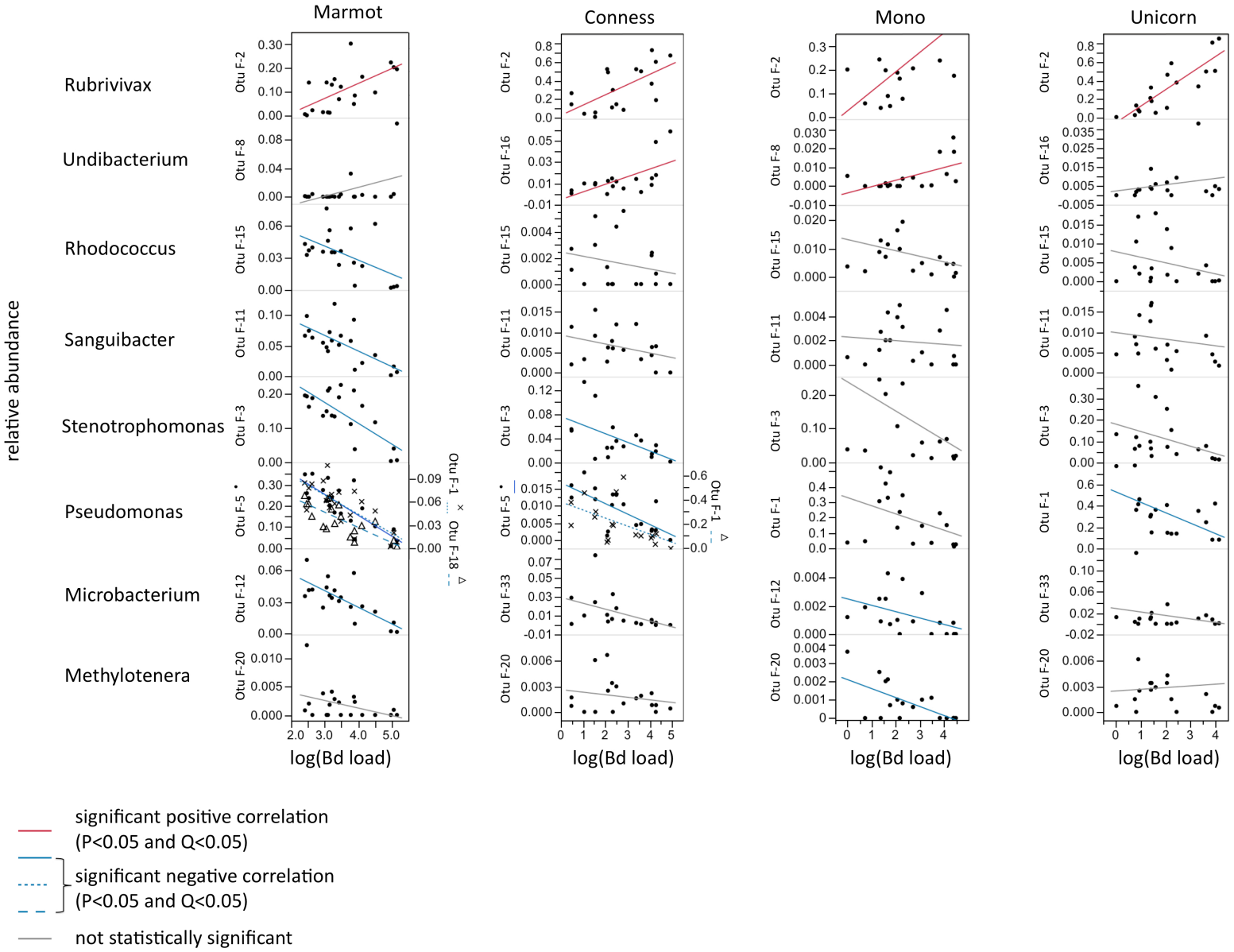
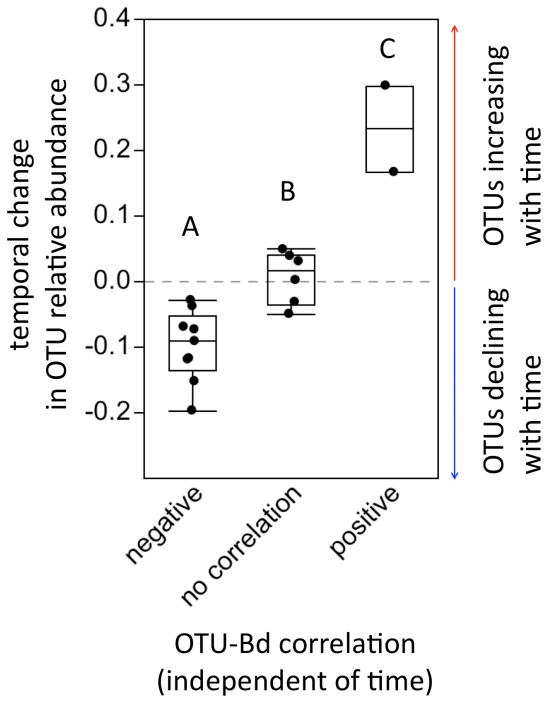

Marmot
Conness
Mono
Unicorn
relative abundance
Rubrivivax
Undibacterium
Rhodococcus
Sanguibacter
Stenotrophomonas
Pseudomonas
Microbacterium
Methylotenera
Otu F-2
Otu F-8
Otu F-15
Otu F-11
Otu F-3
Otu F-5
Otu F-18
Otu F-1
Otu F-12
Otu F-20
Otu F-16
Otu F-33
log(Bd load)
significant positive correlation
(P<0.05 and Q<0.05)
significant negative correlation
(P<0.05 and Q<0.05)
not statistically significant

Figure S3. Correlations between Bd and bacterial OTUs predict temporal change during a Bd outbreak. Shown are data from Marmot during a Bd epizootic that resulted in population collapse. OTUs that were positively correlated with Bd load increased with time, while OTUs that were negatively correlated with BD load declined. Vertical axis shows the effect of time (parameter estimate for Time) from 1-way ANOVA in the outbreak population after accounting for baseline seasonal change as estimated from the two reference (enzootic) populations (see Supplementary Methods for details). Only the 17 OTUs that changed significantly with time are included. OTUs are grouped based on their relationship to Bd (relative abundance positively correlated, negatively correlated, or uncorrelated with Bd load) based on analysis within a single survey date in the outbreak population, such that correlation results are not confounded with time. Groups annotated with different letters above the box plot are significantly different based on Tukey HSD post-hoc comparison of means.



CHAPTER TWO

Landscape-level covariation in amphibian skin bacterial communities and pathogen infection is consistent with both bacterially-mediated disease resistance and pathogen-induced disturbance of the microbiome.

ABSTRACT

The fungal pathogen *Batrachochytrium dendrobatidis* (Bd) is a leading cause of amphibian declines, yet there is variation in amphibian host response to infection, and understanding why some amphibians are able to resist lethal disease may be critical to improving management of threatened species. The Sierra Nevada Yellow-Legged frog, *Rana sierrae*, exhibits variability in response to Bd infection: many *R. sierrae* populations have declined due to Bd, but some are able to persist despite Bd infection. Previous studies support a possible role for skin-associated bacteria in limiting Bd infection. However, a recent study found that Bd infection alters the amphibian microbiome, and this is important to consider when assessing the potential for skin-associated bacteria to confer protection against disease because correlations between Bd infection and microbiome composition may be the result of Bd-induced alterations of the microbiome, as opposed to bacterially-mediated resistance to the pathogen. The current study tested for associations between the amphibian skin microbiome and apparent disease resistance while taking into account the potential for the pathogen to alter the microbiome. Bd infection intensity and skin bacterial microbiome composition were sampled across ten *R. sierrae* frog populations that varied in Bd infection status and in the population-level outcome of infection (persisting or declining). Bd infection was associated with changes in bacterial communities among host populations, indicating that Bd may affect landscape-level patterns in skin microbiome variation among host populations. However, the skin microbiome also differed between host populations that declined due to Bd and those that persisted with the pathogen, and this difference appears to be partially independent of the effects of Bd on the microbiome. Specifically, multivariate microbiome composition differed between declining and persisting *R. sierrae* populations

even after accounting for the effect of Bd. In addition, some of the bacterial taxa that differed between declining and persisting host populations showed no association with Bd infection intensity, indicating that differences in their abundance between persisting and declining populations is probably not due to differences in the degree of Bd-induced alteration of the microbiome. The potential effects of Bd infection and host population type (persisting or declining) on microbiome composition were compared with potential environmental drivers of community assembly, including the environmental bacterial species pool and large scale spatial variables. Skin microbiome composition was most tightly correlated with Bd load, but was also associated with environmental species pool and latitude, which coincides with host population response to infection. Together, these data suggest that both bacterially-mediated disease resistance and pathogen-induced microbiome disturbance likely occur simultaneously, but somewhat independently.

INTRODUCTION

Emerging infectious diseases pose a threat to global biodiversity (Daszak et al., 2000), and the unprecedented impact of the amphibian chytrid pathogen, *Batrachochytrium dendrobatidis*, is a well-documented example of disease-driven biodiversity loss (Kilpatrick et al., 2010; Wake and Vredenburg, 2008). First described in 1999 (Longcore et al., 1999), *Batrachochytrium dendrobatidis* (Bd) is a fungus that infects the skin of amphibians and causes chytridiomycosis, a disease characterized by skin-sloughing, weight loss, disorientation, and disruption of osmotic balance, which can be lethal (Voyles et al., 2009). In the 15 years since its discovery, Bd has been implicated in the decline of over 200 amphibian species (Berger et al., 1998; Crawford et al., 2010; Kilpatrick et al., 2010; Lips et al., 2006; Rachowicz et al., 2006; Vredenburg et al., 2010). However, the effects of infection vary among host species, and even among individuals of the same species, with outcomes ranging from asymptomatic infection to lethal disease (Briggs et al., 2010; Kilpatrick et al., 2010; Vredenburg et al., 2010). The factors underlying this variation in the outcomes of infection could inform management strategies for threatened amphibians, but are currently poorly understood.

The Sierra Nevada yellow-legged Frog (*Rana sierrae*) has been severely impacted by Bd, but is also one of the few amphibian species that show natural within-species variation in response to Bd infection. *R. sierrae* and its sister species *Rana muscosa* were once common in alpine lakes of the Sierra Nevada, but have been reduced to less than 5% of their historic population numbers (Vredenburg et al., 2007). Introduced trout and Bd pose the greatest threats to these endangered frogs (Knapp, 2005; Knapp et al., 2007; Rachowicz et al., 2006; Vredenburg et al., 2010). Trout eradication is expensive, labor-intensive, and often

controversial due to conflicting interests between conservation and recreational fishing interests. Bd poses a still greater management problem because, regardless of cost or political will, no proven methods exist to prevent Bd-induced extirpations of amphibian populations in the wild. As Bd has spread across the Sierra Nevada, entire metapopulations of *R. sierrae* have collapsed due to chytridiomycosis. However, previous studies have identified two distinct types of population-level outcomes of Bd infection among *R. sierrae* populations: enzootic (with population persistence) and epizootic (resulting in population decline) (Briggs et al., 2005, 2010; Knapp et al., 2011; Rachowicz et al., 2006; Vredenburg et al., 2010). In populations experiencing epizootic disease dynamics, frogs develop high Bd loads (mean Bd loads reaching 10,000 to 100,000 Bd cells/frog) and population decline or extinction ensues within months to years after initial arrival of the pathogen (Vredenburg et al., 2010). In contrast, in populations experiencing enzootic disease dynamics, frogs maintain moderate Bd loads (generally << 10,000 Bd cells/adult frog), infected frogs survive for years with no obvious signs of disease, and population numbers remain stable over multiple years despite Bd infection (Briggs et al., 2010; Knapp et al., 2011). The factors that lead to either enzootic (population persistence) or epizootic disease dynamics (population decline) are not known. Furthermore, for most persisting populations the initial Bd invasion was not observed and probably occurred before the discovery of Bd in 1999 (Fellers et al., 2001; Longcore et al., 1999; Ouellet et al., 2005). It is possible that many currently persisting populations experienced initial declines due to Bd, and that the important difference in response to infection lies in whether a given population can transition to the enzootic/persistent state after the initial decline rather than declining completely to extinction (Briggs et al., 2010). Disease models indicate that demographic traits such as frog population density can influence the rate

of increase in Bd loads and likelihood of catastrophic outbreaks leading to host population extinction (Briggs et al., 2010), although empirical support for this hypothesis is currently lacking. Similarly, pathogen population genetics do not clearly delineate between strains isolated from persistent and die-off frog populations (Morgan et al., 2007). The outcomes of infection do show geographic patterns, with enzootic populations generally found in Yosemite National Park and northward, while the vast majority of populations in Kings Canyon National Park and southward undergo epizootic dynamics and population declines when Bd invades. This geographic divide coincides with the timing of Bd invasion: populations in Yosemite have likely been infected for decades (Fellers et al., 2001; Ouellet et al., 2005), while invasion by Bd of populations in the southern range has been more recent (Rachowicz et al., 2006; Vredenburg et al., 2010). The geographic divide also coincides in part with genetic differences within *R. sierrae*: northern populations (Yosemite and northward) belong to *R. sierrae* clades 1 and 2, while southern populations belong to clade 3 (Vredenburg et al., 2007). Thus, there is some association between geographic or genetic groups and population response to infection, although no causal link has been demonstrated. In addition, if host genetics were to affect disease response, the mechanisms underlying variable resistance would need to be examined, and could include acquired immunity, innate immunity, tolerance of infection, host-controlled selection of defensive microbial symbionts, or behavioral traits. A better understanding of the underlying causes of variation in infection outcomes is critical to a general understanding of disease dynamics and conservation management of *R. sierrae* as well as for other threatened amphibians.

Recently, the possibility that symbiotic skin bacteria affect frog resistance to disease has gained attention, and augmentation of amphibian skin with high concentrations of certain

bacterial isolates (most notably isolates identified as *Janthinobacterium lividum*) conferred disease resistance under laboratory conditions in some cases (Harris et al., 2009; Muletz et al., 2012). However, in other studies bacterial augmentation failed to protect frogs from disease (Becker et al., 2011; Woodhams et al., 2012). In addition, skin-associated bacterial communities differ between wild and laboratory-held frogs (Jani and Briggs, in press), and it is not clear whether bacteria contribute to natural disease resistance in wild amphibians. Woodhams *et al.* (2014) found that the complete mucus of amphibians, which includes microbes, host-produced antimicrobial compounds, and possibly other factors, was correlated with the severity of Bd infection among frog species across Europe. Because *R. sierrae* exhibits variation in disease response among conspecific populations, it may provide an opportunity to understand the factors contributing to disease resistance or tolerance while minimizing confounding variables. Two previous studies have asked if bacterial symbionts differ between enzootic and epizootic, or infected and uninfected, *R. sierrae* or *R. muscosa* populations. Woodhams *et al.* (2007) used bacterial culture methods to test if the number of bacterial isolates with in vitro anti-Bd activity (determined by agarose plate co-culture tests) differed between an enzootic *R. sierrae* population and an epizootic *R. muscosa* population. The authors found more anti-Bd bacterial isolates in the enzootic population, consistent with bacteria playing a role in disease resistance. In a similar study, Lam *et al.* (2010) compared an uninfected *R. muscosa* population that was expected to undergo epizootic disease dynamics with an enzootic *R. sierrae* population, and found no difference among populations in the number of anti-Bd bacterial isolates. Based on these results and possible signs of population persistence in the *R. muscosa* population, the authors concluded that anti-Bd bacteria may contribute to population persistence.

In the current study, we further examine the potential for skin-associated bacteria to contribute to disease resistance with four advances: First, we employ culture-independent, Next-Generation marker sequencing methods, which provide a much more complete characterization of bacterial communities than bacterial culture approaches (Rappé and Giovannoni, 2003). Second, we reduce confounding variables by restricting our analysis to only one species, comparing populations of the same host species that differ in disease response. Third, and more importantly, we use multiple *R. sierrae* populations to obtain a more generalizable assessment of differences between enzootic and epizootic populations. This is important because it enables us to distinguish variation associated with population type (enzootic versus epizootic) from variation that exists between a given pair of populations that may not be specifically relevant to differences in response to Bd infection. Fourth, we account for possible effects of Bd on the skin microbiome. We previously showed that Bd infection alters the *R. sierrae* skin microbiome (Jani and Briggs, in press), and this is directly relevant to understanding differences between enzootic and epizootic *R. sierrae* populations because epizootic populations have higher Bd loads than enzootic populations (Briggs et al., 2010; Vredenburg et al., 2010). It is therefore important to consider the possibility that differences in the skin microflora of enzootic and epizootic *R. sierrae* populations could be caused by differences in Bd infection intensity, as opposed to differences in bacterial communities leading to differences in Bd infection intensity and concomitant host response to infection. We present coordinated analyses of skin-associated symbiotic bacterial communities (hereafter simply "microbiome" for brevity), Bd infection loads, and variation in host response to infection across ten *R. sierrae* populations to better understand the potential contribution of symbiotic bacteria to disease resistance.

Because previous work indicates that Bd affects the *R. sierrae* microbiome among frogs within a given population, we begin the current study by asking if this effect scales up to the landscape level. In other words, can differences in Bd load potentially explain microbiome differences between frog populations? Second, we test if the skin microbiome differs consistently between enzootic and epizootic *R. sierrae* populations, and examine the extent to which those differences can be explained by Bd-induced disturbance. Third, we compare the potential effects of Bd infection and environmental factors in shaping the *R. sierrae* skin microbiome. We discuss the implications of our results for testing and refining the hypothesis that the skin microbiome contributes to the natural ability of some amphibian populations to persist in the face of Bd infection.

METHODS

Population surveys and determination of disease dynamic type. This study was conducted in ten *R. sierrae* populations spanning a large portion of the species' range, including populations in Yosemite, Kings Canyon National Park, and Sierra National Forest, California (Figure 1). *R. sierrae* has an aquatic life history, being completely restricted to the aquatic environment during the 2-3 year tadpole stage, and requiring regular contact with water for survival of the subadult and adult stages. This enables us to reasonably designate each lake inhabited by *R. sierrae* as a separate population, provided lakes are not closely connected by streams. To estimate population sizes, visual encounter surveys were conducted: trained research staff walked the lake perimeter and, where accessible, 100m of the inlet and outlet streams, while noting the number of adult, subadult, and tadpole *R. sierrae* observed, as described in Knapp *et al.* (2011). *R. sierrae* is a diurnal species and individuals spend most of their time within one to two meters of the shoreline (Bradford,

1984), making visual encounter surveys a reasonably accurate method for estimating population size, particularly when multiple surveys of each population are conducted. Previous work showed that visual encounter surveys yield reproducible results (Knapp and Matthews, 2000).

During the 2010 field season, each *R. sierrae* population was classified with respect to Bd-infection at each sampling time point (referred to as a survey visit) as operationally uninfected (which includes populations that are completely free of Bd as well as those with very low levels of infection, with maximum Bd load < 10 Bd cells detected per frog) or infected, based on qPCR data from skin swabs (Figure 1, Table 1). We chose to pool populations that were completely uninfected with those that showed low levels of infection because our goal is not to identify the exact transition point between 100% uninfected and early infection, but to distinguish uninfected/lightly-infected populations from those where enzootic or epizootic dynamics are clearly underway. We note that infection status (uninfected, infected) refers to a given population survey, taken at a given point in time: a population may be uninfected in one survey and infected at a later date.

We used long-term population census and Bd infection data to categorize populations as enzootic or epizootic with respect to disease dynamics. All populations in this study have been censused regularly since 2002 or earlier. Beginning in 2005, when Bd-specific qPCR protocols became widely available (Boyle et al., 2004), surveys included collection of standardized skin swabs for quantification of Bd loads, as described previously (Briggs et al., 2010). Populations that maintained stable Bd loads on adults and stable populations through time were categorized as enzootic. Populations that exhibited adult Bd load trajectories that increased through time, followed by population collapse (no post-metamorphic frogs

observed in surveys the following year) were categorized as epizootic (Table 1, Figure 1). In addition, because this and previous studies have shown a consistent geographic pattern in the response of mountain yellow legged frogs (*Rana sierrae* and *Rana muscosa*) to Bd infection, we were able to make reasonable predictions (based on geographic region) about the disease-resistance of populations that were still uninfected in 2010. Populations in the southern habitat range have consistently responded to Bd infection with dramatic population declines and often extinction (R. A. Knapp personal communication, and Vredenburg 2010 PNAS), and those populations that are currently uninfected are predicted to undergo enzootic dynamics and population declines as well. Thus, all northern populations in this study are documented enzootic populations, and all southern populations in this study are either documented or predicted to undergo epizootic dynamics and declines. Notably, surveying both uninfected and infected populations in the southern region enables analysis of changes in the microbiome that are likely to be due to change in infection status, and not associated with differences in disease resistance.

Collection of skin-associated bacterial community samples. During the 2010 field season, we conducted surveys to simultaneously sample *R. sierrae* skin-associated microbial communities and Bd loads from individual frogs in the 10 populations. We also sampled planktonic microbial communities present in lake water to allow assessment of the bacterial species pool present in these frogs' immediate environment. Detailed sample collection, preservation, and storage methods for bacterial community samples are provided in Jani and Briggs (in press). Briefly, in each survey visit to each population, a target of 30 post-metamorphic (i.e. subadult and adult) *R. sierrae* were captured by dip net and swabbed for skin-associated microbes (bacteria and Bd) using sterile synthetic swabs and wearing new

nitrile gloves for each frog handled. Nets were rinsed thoroughly in lake water between each frog captured. Bacterial communities present in lake water were sampled at each survey visit to each lake by filtration of water through 0.22 micron filters. We quantified Bd loads from all swabs collected. However, because bacterial communities were characterized using in-depth pyrosequencing, it was not feasible to characterize bacterial communities from all swabs collected. Therefore, bacterial communities were characterized for a subset of samples selected to provide microbiome data from as many *R. sierrae* populations as possible, synoptically sampled (all within a period of less than 30 days) to enable among-population comparisons while minimizing effects due to seasonal variation. For each of the visits, one sample of the lake water bacterial community was also sequenced to provide information on the ambient bacterial communities experienced by these frogs. Sampling dates and sample sizes for 2010 field surveys are provided in Table 1.

To prevent researcher-mediated spread of Bd, any equipment that contacted frogs or lake water (including shoes) was disinfected by immersion in 0.1% quaternary ammonium compound 128 for at least 5 minutes (Johnson et al., 2003), or, for small or sensitive equipment, disinfected with 70% ethanol.

16S sequencing and bioinformatic processing. Bacterial communities present on frog skin and in lake water were characterized by 16S amplicon pyrosequencing as described previously (Jani and Briggs in press). Briefly, the V1-V2 regions of the 16S gene were amplified using primers 8f and 338r with sample-specific barcodes and Roche FLX amplicon adapters. PCR products were quantitated and pooled in equimolar quantities for sequencing on a Roche/454 GS FLX using Titanium Chemistry. Sequences were bioinformatically processed using the program Mothur (v1.30) as described Jani and Briggs (in Press). Briefly,

sequences were quality-filtered (de-noised and screened for short, potentially low-quality, or chimeric sequences), aligned to a non-redundant representative subset of the SILVA v111 SSU Ref 16S curated alignment database (Nelson et al., 2014), and clustered into operational taxonomic units (95% identity OTUs) and phylotypes. Sequences were classified using the Bayesian classifier of Wang *et al.* (2007) and each OTU was assigned a consensus taxonomy from SILVA v111. Pairwise phylogenetic community distances (weighted Unifrac, (Lozupone and Knight, 2005)) among all samples were calculated based on relative abundance of OTU relative abundances. Richness and diversity of each sample was estimated after randomly subsampling 500 sequences per sample to equalize detection effort among samples, using four metrics: observed OTU richness (S_{OBS}), Chao's richness estimate (Chao, 1984), Shannon diversity, and Shannon evenness.

Statistical details: All statistical analyses were performed using JMP v. 10 (SAS Institute Inc., Cary, NC, USA, 1989-1212), with the following exceptions: Multivariate bacterial community data were analyzed using nonparametric, permutation-based methods (NMDS, ANOSIM, PERMANOVA, DistLM, and Mantel tests) in the software package Primer-E v6 (Clarke and Gorley, 2006). Patterns in the relative abundances of individual OTUs were tested for only common OTUs, which we define as any OTU comprising at least 0.1% of the total sequence reads. Relative abundance data were arcsine square root transformed for parametric analyses. To prevent inflation of Type I statistical error due to testing multiple OTUs (i.e., multiple comparisons), statistical significance of tests was adjusted by calculating the false discovery rate, q, using the program Qvalue (Storey and Tibshirani, 2003). The Q-value is an estimate of the proportion of tests that appear to reject the null hypothesis ($P< \alpha$, in this case $P<0.05$) when in fact the null hypothesis is true ("false positives"). Bd load data

were $\log_{10}$ transformed as: $\log_{10}$(Bd load +1). (1 is added to each value before transformation because the base 10 logarithm is undefined for values less than 1. This adjustment should have negligible effects on our analyses, given that Bd loads ranged from 0 to more than 10^5.)

Structure of statistical models for testing landscape-level hypotheses. Our data are sampled at the individual frog (skin swab) level, however many of our questions are focused on differences among frog populations, not individuals. We therefore include population survey ("visit") as a random factor in all analyses of population-level variation. Where relevant, population survey is nested in population type (enzootic/epizootic or infected/uninfected). This approach helps maintain low Type I statistical error by accounting for the fact that frogs from the same populations survey (sharing the same lake environment and sampling date) are less independent of each other (due to shared environment and probably closer genetic relatedness) than frogs from different populations. We chose visit (rather than population) as the random variable because we found significant differences in bacterial communities sampled on different dates from the same population (Table S1). This statistical model structure comes at the cost of statistical power, potentially overlooking some patterns that are biologically relevant but not statistically significant, but enables identification of consistent differences between enzootic and epizootic populations or landscape level associations between Bd load and bacterial communities.

Testing effects of Bd infection on bacterial communities. We previously showed that Bd infection was associated with changes in the *R. sierrae* skin microbiome during experimental infection and within individual populations (Jani and Briggs, 2014). In the current study, we expand to the landscape scale and test if Bd infection or infection severity is associated with changes in the *R. sierrae* skin microbiome across multiple *R. sierrae* populations in the wild.

We first asked if bacterial communities differ between Bd-infected and uninfected host populations. We used PERMANOVA (permutation-based MANOVA), with population visit as a random factor (nested in population infection status) to test if multivariate bacterial community composition differs between Bd-infected and uninfected populations. To test for effects of infection status on diversity, we used mixed models (ANOVA, with visit as a random nested factor) to detect differences between infected and uninfected populations with respect to four metrics of bacterial diversity: observed number of bacterial OTUs (S_{OBS}), Chao's richness estimate, Shannon diversity index, and Shannon evenness. We also tested for a continuous relationship between Bd load and bacterial communities. We used mixed models, with Bd load as a continuous fixed factor and visit as a random factor, to test for linear relationships between Bd load and the four diversity metrics. We used a distance-based linear model (DistLM, [ref]) to test for a linear relationship between Bd load bacterial community composition. When testing for effects of Bd load on bacterial community composition, we pooled data across all frogs within a population visit and used population visit as the unit of replication. Bacterial community data were pooled across all frog swabs within a given population visit and used to calculate among visit Unifrac distances. Mean Bd load was calculated for each population visit, and used to test for a linear relationship among populations between Bd load and bacterial community composition. Our reasoning for pooling data within each population was that Bd load, being a continuous variable, varies both within and among populations and time points, such that a significant relationship between Bd load and bacterial communities at the individual frog level can be due to within-visit effects, among-visit effects, or both. Analysis with visit as the unit of replication allows

us to focus on whether Bd load is associated with differences among populations at the landscape scale.

Testing for differences in bacterial communities of enzootic and epizootic populations. We used PERMANOVA, with visit as a random factor nested in population type (enzootic or epizootic), to test for differences in the overall composition of bacterial communities between enzootic and epizootic host populations. Because Bd loads are generally higher during epizootic than enzootic disease episodes, and increasing Bd loads may lead to changes in the skin microbiome, it is important to test whether differences in the bacterial microbiomes of enzootic and epizootic frog populations could be due to differences in Bd loads. To test this, we added Bd load ($\log_{10}$) to the model as a covariate. We used Type Three Sums of Squares (T3SS), such that effects of each variable are calculated after accounting for other factors in the model.

Identifying bacterial taxa associated with disease severity or resistance. We tested all common OTUs to identify bacterial taxa for which relative abundance is linked to Bd load or differs between populations based on their response to Bd infection (enzootic or epizootic disease dynamics). To identify bacterial taxa that are correlated with Bd load, we used a mixed model to test for a relationship between bacterial relative abundance (response variable) and Bd load, with population visit included as a random factor. To identify bacterial taxa that differ between enzootic and epizootic populations, we used a mixed model to test for differences in OTU relative abundance between enzootic and epizootic populations, with visit included as a random factor, nested in population type (enzootic, epizootic). We then re-ran these analyses with Bd load included as a covariate (ANCOVA, with T3SS). We used the False Discovery Rate ($Q<0.05$) to control Type I error due to multiple tests, as described in

Statistical details. We used two approaches to try to distinguish OTUs that differed between epizootic and enzootic populations due to differences in Bd from those that differed due to other causes (and therefore may still have the potential to contribute to variation in Bd resistance). First, we compared the identities of OTUs that differed between enzootic and epizootic populations (without Bd as a covariate), with OTUs that were correlated with Bd load (without population disease response included in the model). For OTUs that differ between enzootic and epizootic populations but are also correlated with Bd load, the effect of Bd load is confounded with the effect of population type. However, when OTUs are not correlated with Bd load but differ between enzootic and epizootic populations, we infer that the difference between enzootic and epizootic populations is probably not due to differences in Bd load. As a complementary approach, we used a mixed model with Bd load included as a covariate to identify OTUs that differed between enzootic and epizootic populations after accounting for the effect of Bd load. For this analysis, only OTUs for which the interaction between Bd load and population type (enzootic, epizootic) was non-significant were considered. While it is not possible to definitively conclude cause from observational data, the statistical and comparative approaches described here provide a first assessment of bacterial taxa that might be indicators of different disease responses (enzootic, epizootic).

Community assembly: comparison of environmental and amphibian-associated bacterial communities. We assessed the potential for planktonic aquatic bacterial communities to shape the *R. sierrae* microbiome. We first tested if bacterial communities on *R. sierrae* skin are different from bacterial communities present in the surrounding lake water, using ANOSIM. Next, we tested for covariation across *R. sierrae* populations in the bacterial communities present in water and on frogs. Because both aquatic and frog-associated bacterial community

composition are multivariate data, we used Mantel tests to test for correlations between them. This analysis requires a one-to-one correspondence between amphibian and lake water data, therefore we pooled data across *R. sierrae* swabs within each population survey. We conducted a Mantel test to assess the correlation between among-population distance matrices based on frog-associated bacterial communities compared with aquatic bacterial communities. To quantify the correlation between Bd load and the *R. sierrae* microbiome using a metric that can be compared with the effect of aquatic bacteria on the *R. sierrae* microbiome, we conducted a similar analysis for the relationship between Bd load and bacterial communities. For the latter analysis, we first converted population mean Bd load data to among-population Bd load distances (the difference in Bd load between two population surveys), and then compared the Bd-load distance matrix with the microbiome (Unifrac) distance matrix using a Mantel test.

Community assembly: testing the relative contribution of Bd and environment on microbiome composition. To begin to understand the factors shaping the amphibian skin microbiome, we examined the relative importance of landscape-level environmental factors, the bacterial species pool present in lake water, and Bd load in shaping the *R. sierrae* microbiome. Environmental variables included elevation (m), latitude (North UTM), and lake size measured as $\log_{10}$(surface area). Elevation was chosen as a variable because it is correlated with temperature (Knapp et al., 2011). Latitude was included because *R. sierrae* genetic clades largely follow a latitudinal gradient. Lake size was included because it may indicate the relative importance of terrestrial inputs to the aquatic environment. We tested for linear relationships between each of these predictor variables and the multivariate bacterial community distance among samples (weighted Unifrac distance) using DistLM, (a

permutation-based multivariate linear regression analog) in the software package PRIMER v.6. For types of data collected at the individual frog level (Bd load, microbiome data), we pooled all swabs within a given population visit such that each pooled data point corresponds to exactly one value for each environmental variable. First, the statistical significance and proportion variance explained was calculated for each predictor variable separately. Next, we identified the combination of predictor variables that minimized the AICc criterion, maximizing the variance explained while penalizing for the number of variables added. Finally, starting with only the variables identified in the "best" model, we constructed a model by adding the variables one by one, ordered based on the proportion of variance explained in the absence of other factors, to obtain a final model.

RESULTS

Different Bd dynamics among Bd-uninfected, enzootic, and epizootic populations. We observed clearly distinguishable Bd load and census count trajectories between enzootic and epizootic populations (Figure 2a,c; long-term data in Figure 2c courtesy of Roland Knapp). Bd loads on adult frogs differed significantly among enzootic and epizootic populations (Figure 2b, $P<0.0001$). Among surveys conducted in 2010, six surveys across four populations were categorized as enzootic based on stable Bd loads and population numbers through time. Three surveys across two populations were categorized as epizootic based on (1) recent transition from uninfected to infected state, (2) rapid increases in Bd loads through time, and (3) no post-metamorphic frogs found during census surveys in 2011. In one population (Dusy-2), the Bd epizootic had already reached the state when no adult frogs remain by 2010, but Bd trajectories in subadults show a clear increase through time (Figure 2a, inset). Five population surveys across four populations were categorized as operationally

uninfected with Bd in 2010. This includes surveys showing very low levels of infection (maximum Bd load < 10). Two of the uninfected populations can be seen to begin the transition to the infected state late in the 2010 field season (Figure 2a). Based on previous studies (Briggs et al., 2010; Knapp et al., 2011; Vredenburg et al., 2010), we had predicted that populations in the southern study region would experience epizootic disease dynamics. Consistent with this prediction, the two populations that began the transition from Bd-free to infected late in 2010 suffered population declines by the following year (Table 1).

Bd load is correlated with landscape-scale skin-microbiome composition. *R. sierrae* skin microbiome composition did not differ by infection status (i.e., between infected and uninfected populations) when all populations were considered (PERMANOVA, P=0.1162). When only Southern populations were included in the analysis, infection status did have an effect on microbiome composition (P=0.0296, Figure 3), possibly due to reduced noise in the data since only one geographic region and host clade is included. The continuous variable Bd load had a more robust effect on bacterial communities than binary infection status, with Bd load significantly predicting community composition regardless of whether analyses included all enzootic and epizootic populations (DistLM P=0.0003), infected and uninfected populations (P=0.0001), only Southern populations (P=0.0030), or only Northern populations (P=0.0120). The relationship between Bd load and multivariate bacterial community composition is visualized using NMDS ordination, which shows Bd loads covarying with bacterial community change along axis 1 (Figure 4a-c).

Enzootic and epizootic R. sierrae harbor different bacterial communities. Bacterial communities differed significantly between enzootic and epizootic frog populations (PERMANOVA P=0.0330), as visualized using NMDS ordination (Figure 4d-f). When Bd

load was added to the model as a covariate, the difference between enzootic and epizootic populations remained significant (P=0.0031), and Bd load was also highly significant (P=0.0001).

Bacterial richness (S_{OBS} and Chao's richness), but not diversity (Shannon diversity and evenness) was higher in enzootic populations compared with epizootic populations (P_{SOBS}=0.0232, P_{Chao}=0.0105, Figure 5). All richness and diversity metrics were negatively associated with Bd load when Bd load was tested alone (P_{SOBS}<0.0002 P_{Chao}=0.0003, $P_{Shannon}$<0.0001, $P_{evenness}$=0.0001). When both population type (enzootic vs. epizootic) and Bd load were included in the model, Bd load was still negatively associated with all richness and diversity metrics (P_{SOBS}=0.0003, P_{Chao}=0.0050, $P_{Shannon}$<0.0001, $P_{evenness}$<0.0001), but the effect of population type (enzootic, epizootic) was no longer significant (P<0.05 for all metrics).

Bacterial taxa that differ between Bd-susceptible and –resistant populations. The relative abundance of seven bacterial OTUs differed between enzootic and epizootic *R. sierrae* populations (Table 2). Three of these OTUs are assigned to the family Comamonadaceae, while the remaining four OTUs are from diverse families and orders. For two OTUs (classified as Flavobacterium and Ferruginibacter), relative abundances were not correlated with Bd load, indicating that the difference in abundance between enzootic and epizootic populations is not caused by differences in Bd load. An additional two OTUs (classified to the genus Rubrivivax and an unclassified Comamonad) differed between enzootic and epizootic populations in a statistical model that also accounted for the effect of Bd load (i.e., ANCOVA mixed model). Both of these OTUs were positively correlated with Bd load, such that an absolute relative abundance that is higher in enzootic populations relative to epizootic

populations is notable because one would expect a lower relative abundance in enzootic populations based on the effects of Bd load alone. The remaining three OTUs that differed in relative abundance between enzootic and epizootic populations when Bd load was not taken into account were also correlated with Bd load. For these three OTUs, differences among enzootic and epizootic populations was confounded with effects of Bd. In other words relative abundances of these OTUs were higher in epizootic populations, but relative abundance was also positively associated with Bd load, such that the high relative abundances in epizootic populations could be due to disturbance of the microbiome by Bd.

Bacterial taxa that vary with Bd load. Twenty-four bacterial OTUs classified to 19 phylotypes were correlated with Bd load (Table 2). In most cases the correlation between Bd and bacterial communities was not confounded with differences between enzootic and epizootic populations: Only three of the OTUs exhibited covariation between the effects of Bd load and population type (discussed above). The vast majority of OTUs that were correlated with Bd load belong to the Actinobacteria (7 OTUs), Betaproteobacteria (8 OTUs), and Gammaproteobacteria (5 OTUs). Multiple OTUs within the Undibacterium (2 OTUs), Microbacterium (3 OTUs), and Pseudomonas (3 OTUs) were correlated with Bd load, and in all cases the direction of the correlation was consistent across OTUs within a genus. A previous study identified bacterial OTUs that were causally affected by Bd during experimental infection, as well as OTUs that were correlated with Bd load within individual *R. sierrae* populations, in the absence of confounding spatial and temporal variables (Jani and Briggs, 2014). We compared the OTUs that were correlated with landscape-level variation in Bd load in the current study with the OTUs identified in the previous study, and found that the results were remarkably consistent. Of the 19 phylotypes correlated with Bd in

the current study, 13 were also correlated with Bd in the previous within-population analyses, and eight phylotypes were also affected by experimental Bd infection. In each of these cases, the direction of the relationship between bacterial relative abundance and Bd load was the same across studies (Table 2).

Community assembly: comparing the contribution of aquatic bacteria, landscape level spatial variables, and Bd in shaping the microbiome. Bacterial communities present on *R. sierrae* skin were clearly distinct from bacterial communities present in the water column (ANOSIM, P=0.0001, Figures 6 and 7). However, despite the clear differences between them, amphibian-associated and aquatic bacterial communities covaried among population surveys, based on a Mantel test revealing a significant correlation between the Unifrac distance matrices based on frog-associated compared with aquatic bacterial communities (P=0.0229, ρ=0.342). A Mantel test comparing Bd-load distances with frog skin bacterial community distances (Unifrac distances) revealed that *R. sierrae* skin bacterial communities are more tightly correlated with Bd load (P=0.0002, ρ=0.532) than with aquatic bacterial communities. We used DistLM to quantify the amount of microbiome variation explained by spatial variables (elevation, lake area, latitude) or Bd load. When explanatory variables were tested individually, only Bd load explained a significant proportion of variation in microbiome composition (DistLM P=0.0003). One landscape variable (latitude) had a significant effect (P=0.0154) when added to the model sequentially after Bd load, indicating that latitude explains a portion of the variation not already accounted for by Bd load. The best model explained a total of 45.7% of the variation in bacterial communities and included Bd load (P=0.0008, explaining 35.2% of variation) and latitude (P=0.0154, explaining 15.2% of variation).

DISCUSSION

Bd appears to affect landscape-level patterns in R. sierrae skin microbiome. We previously showed that Bd infection alters the *R. sierrae* microbiome during experimental infection, and that Bd load was correlated with bacterial community composition within *R. sierrae* populations (Jani and Briggs, 2014). In the current study, we scale up to the landscape level by examining relationships between Bd infection intensity and *R. sierrae* skin microbiome composition among host populations. We analyzed skin-associated bacterial communities and Bd loads from 14 surveys of 10 *R. sierrae* populations that varied in Bd infection status and intensity. When Bd infection was treated as a binary variable (infected/uninfected), *R. sierrae* skin microflora differed between infected and uninfected populations but only when analyses focused on a single geographic region: When all populations were considered, the difference between infected and uninfected populations was not significant, but when the analysis was constrained to only populations within the Southern region, microbiome composition differed significantly based on Bd infection status. (A similar analysis focused on only Northern populations was not possible because all known R. sierrae populations in the Northern region are infected with Bd.) We speculate that the larger geographic range introduces variation in skin bacterial communities that reduces the power to detect differences among population types. In addition, the relationship between Bd and the microbiome may be affected not simply by infection status but also by the intensity of infection. Indeed, when we considered Bd load rather than binary infection status, we found a significant linear relationship between Bd load and multivariate bacterial community composition, and this result was significant regardless of whether all populations or only Southern populations were included in the analysis.

Nineteen bacterial phylotypes were significantly correlated with Bd load at the landscape scale. Due to the observational nature and broad geographic range covered in the current study, it is not possible to determine whether associations between bacterial communities and Bd are causal (representing effects of Bd on bacteria or of bacteria on Bd), or are a result of other factors (e.g. environmental or host genetic factors that co-vary with Bd infection, particularly due to spatial or temporal auto-correlation). However, a previous study (Jani and Briggs in press) examined Bd–microbiome relationships within populations, in the absence of large-scale spatial and temporal covariation, to identify variation in microbial communities that is likely to be directly linked to Bd load, i.e., not due to spatial or temporal autocorrelation. In addition, the previous study used a controlled laboratory experiment to show a causal relationship in which Bd disturbed the *R. sierrae* skin microbiome. Notably, the OTUs that were found to correlate with Bd load at the landscape level in the current study largely match the results of the previous study: many of the same bacterial taxa were correlated with Bd load or infection status in the current (landscape-scale) study and previous (within-population and within-experiment) study. Furthermore, for bacterial taxa that were associated with Bd load in both studies, the direction of the Bd-bacteria association (negative or positive) was always the same across the landscape survey, within-population survey, and laboratory experiment. This consistency between the current landscape-level study and previous within-population survey and controlled infection experiment supports the hypothesis that the correlation between Bd and bacterial communities in the current landscape-level study is due, at least in part, to Bd disturbing bacterial communities.

Skin bacterial communities differ between R. sierrae populations experiencing enzootic versus epizootic disease dynamics. Understanding natural variation in how amphibians

respond to infection by Bd may provide clues to the prevention of Bd-induced amphibian declines. *R. sierrae* populations vary in their response to Bd infection, with most populations suffering epizootic disease dynamics and eventual population extinction, while a few populations exhibit enzootic infection dynamics and population persistence. We tested the hypothesis that skin-associated bacterial communities differ between enzootic and epizootic populations. We found that overall bacterial community composition (based on Unifrac distance), as well as the relative abundance of particular bacterial taxa, differed between *R. sierrae* populations undergoing enzootic versus epizootic disease dynamics. This result is consistent with the hypothesis that bacterial communities contribute to the ability of frogs to survive Bd infection and populations to persist with Bd. However, Bd infection can alter the amphibian skin microbiome (Jani and Briggs in press), and Bd loads are higher during epizootic events than enzootic events. Thus, a plausible alternative hypothesis explaining microbiome differences between the two population types is that higher Bd loads lead to greater Bd-induced disturbance of the microbiome in epizootic populations, and this leads to differences in bacterial communities between epizootic and enzootic host populations. We therefore included Bd load as a covariate in the multivariate mixed PERMANOVA model, and found that microbiome differences between enzootic and epizootic populations are still significant after accounting for the potential effect of Bd load. The independence between the effects of Bd on bacteria and the differences between microbiomes in enzootic versus epizootic frog populations can be observed in the ordination of bacterial communities, which shows that correlation between microbiomes and Bd load is primarily along ordination axis 1, while enzootic and epizootic populations separate along both axes (Figure 4). In addition, the bacterial taxa that differed between enzootic and epizootic host populations were largely

different from the taxa affected by Bd load (Table 2). Of the seven bacterial OTUs that differed in relative abundance between enzootic and epizootic host populations, two showed no association with Bd load. Another two OTUs were positively correlated with Bd load, but were more abundant in enzootic populations (which have lower Bd loads than epizootic populations). Thus, Bd disturbance alone does not explain all of the variation between the skin microbiomes of enzootic compared with epizootic populations, and the hypothesis that microbiome differences lead to variation in disease dynamics (enzootic vs. epizootic) is still viable. However, it is not possible based on survey data alone to conclude a causal relationship wherein bacteria confer infection resistance or tolerance. To clearly support this hypothesis, it will be necessary to distinguish between effects of microbial symbionts, environmental variation, and host genetic variation. Ideally, one would obtain data from experiments showing that frogs from enzootic and epizootic populations have different responses to Bd infection under the same environmental or laboratory conditions, *and* that those differences in infection response are due to symbiotic bacteria. To obtain this type of experimental data, a number of technical challenges, most notably, the need to alter or clear the skin-associated microbiome without introducing experimental artifacts, and the need to maintain ecologically relevant bacterial communities when frogs are held in the laboratory, must be overcome.

Understanding microbiome assembly: Effects of Bd load and environmental variables on skin microbiome composition. Skin-associated bacteria have shown promise in mitigating the effects of Bd infection under laboratory conditions, and efforts are underway to develop bacterial augmentation methods to control Bd outbreaks in the wild. A better understanding of how the microbiome is assembled and stabilized will be critical to microbiome-

augmentation approaches to disease control. We examined the potential for environmental and spatial factors to shape the microbiome. We first compared the composition of the *R. sierrae* skin microbiome with the bacterial communities found in the lake habitat. Because *R. sierrae* require regular contact with water and spend most of their time either in or immediately adjacent to lakes (Bradford, 1984), we expected the bacterial species pool of lake water to provide an important source of bacterial colonists to amphibian skin. However, we found that the bacterial communities present on *R. sierrae* skin and in lake water are highly distinct (Figure 6). Consistent with a recent study (Walke et al., 2014), our results suggest that bacterial communities on amphibian skin are actively selected rather than randomly assembled from the aquatic environment. However, we further examined the relationship between aquatic and amphibian-associated bacterial communities by using Mantel tests and found that the similarity in the skin microbiomes among host populations is correlated with similarity between the bacterial communities found in the lake water inhabited by those populations. Thus, although frog-associated and aquatic bacterial communities are distinct, they have somewhat parallel patterns in landscape scale variation. We speculate that environmental factors affect both the aquatic and amphibian-associated bacterial communities, but further research is needed to test this hypothesis and identify driving factors. Notably, the *R. sierrae* skin microbiome was more tightly correlated with Bd load (ρ=0.532) than with aquatic bacterial communities (ρ=0.342). To examine effects of large scale spatial variables on the *R. sierrae* skin microbiome, we conducted a model-building exercise to test the effects of spatial factors (latitude, elevation, lake size) as well as Bd load in explaining microbiome variation. Bd load explained 35.2% of microbiome variation, which could be due to Bd disturbance of the microbiome, microbiome control of

Bd load, or both. Of the spatial factors tested, only latitude had a significant effect on bacterial communities, explaining 15.2% of variation. Notably, latitude covaries with transitions between genetic clades in *R. sierrae*, and the effect of latitude may indicate environmental effects, host genetic effects, or both. Together, these analyses of bacterial community assembly indicate that the *R. sierrae* skin microbiome is distinct from, but covaries with, the aquatic bacterial community, and is also affected by environmental factors linked to latitude. However, Bd load had a stronger correlation with the microbiome than any spatial or environmental factor measured.

Identifying generalizable differences between enzootic and epizootic populations. In the current study, we examined bacterial communities from 14 surveys across 10 *R. sierrae* populations, with at least 7 individuals sampled per survey. In exploratory analyses, we tested for differences between every pair of surveys and found that 75 out of the 94 possible pairwise comparisons indicated differences in the bacterial communities of two population surveys, even when those populations exhibit the same disease dynamics, or when the two surveys compared are from the same population taken at different time points (Table S1). This result emphasizes the importance of considering multiple populations when trying to understand differences between enzootic and epizootic populations: Comparison of any two populations will likely indicate significant differences in skin-associated bacterial communities, but whether those differences are linked to differences in disease dynamics (enzootic, epizootic) cannot be determined from a single population pair. To examine differences in the microbiome between enzootic and epizootic populations, we analyzed 9 population surveys (6 enzootic, 3 epizootic) sampled within one month, taking care to intersperse trips to the two population types to avoid temporal aggregation of surveys to

either enzootic or epizootic populations. In statistical analyses, we included population survey as a random factor, nested in population type (enzootic or epizootic), to avoid pseudoreplication. With these constraints to study design and statistical analyses, we still found differences between the skin-associated bacterial communities of enzootic and epizootic populations, indicating that indeed there are general differences in the microflora of frogs undergoing the two distinct types of disease dynamics. However, the current study is still limited to only a few surveys of epizootic populations, largely because few epizootic events co-occurred in the same time period. In particular, the window of time during an epizootic when post-metamorphic *R. sierrae* are present can be short (sometimes only a few months), making synoptic sampling of multiple epizootic events challenging. Ideally, many populations of each type (enzootic, epizootic) would be included, and this is an important goal for future studies.

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Table 1. Populations surveys of R. sierrae skin bacterial communities and Bd infection during the 2010 field season.

Population Name	Region	Date Collected	N (Bd load) [total (adults,subadults)][a]	N (bacterial community)[b]	Bd infection status at time of sampling	2011 population status[c]	response to Bd infection	Mean Bd load (adults, log_{10})	Maximum Bd load (adults, log_{10})	Mean Bd load (adults & subadults, log_{10})
Conness	North	7/9/10	27 (20,7)		infected	extant	persist (enzootic)	1.31	3.04	3.04
		8/18/10	30 (18,12)	18	infected		persist (enzootic)	1.85	2.95	5.02
		9/10/10	30 (19,11)		infected		persist (enzootic)	2.08	3.36	4.22
Kuna	North	8/6/10	27 (16,10)		infected	extant	persist (enzootic)	0.69	2.46	2.46
		8/27/10	31 (13,17)	8	infected		persist (enzootic)	0.94	2.12	3.99
Mono	North	7/7/10	6 (6,0)		infected	extant	persist (enzootic)	0.63	1.74	1.74
		8/11/10	20 (17,3)		infected		persist (enzootic)	1.50	2.53	4.30
		9/1/10	33 (26,7)	8	infected		persist (enzootic)	1.49	3.19	5.37
		9/16/10	39 (31,9)	18	infected		persist (enzootic)	1.58	3.47	4.91
Unicorn	North	7/28/10	19 (19,0)		infected	extant	persist (enzootic)	0.45	1.69	1.69
		8/29/10	31 (22,9)	7	infected		persist (enzootic)	1.74	2.76	3.72
		9/13/10	37 (24,13)	18	infected		persist (enzootic)	1.47	2.66	4.15
Dusy-1	South	7/24/10	11 (1,10)		uninfected	extinct		0.00	0.00	0.40
		8/22/10	22 (3,19)	8	uninfected			0.00	0.00	0.00
		9/9/10	20 (2,18)	10	uninfected			0.14	0.29	1.86
Dusy-2	South	8/23/10	18 (0,18)	8	infected	extinct	decline (epizootic)			5.17
LeConte	South	8/14/10	42 (33,10)		uninfected	extant		0.01	0.47	0.47
		9/4/10	29 (20,9)	8	uninfected			0.05	0.71	0.71
Marmot	South	7/16/10	30 (30,0)		uninfected	extinct		0.00	0.00	0.00
		8/8/10	30 (27,3)	9	uninfected*			0.06	1.13	1.13
		8/30/10	30 (22,8)	20	infected		decline (epizootic)	3.18	4.98	5.16
		9/14/10	14 (9,5)	10	infected		decline (epizootic)	4.58	5.19	5.19
		9/15/10	29 (27,2)		infected		decline (epizootic)	4.38	5.08	5.08
Rambaud	South	7/15/10	15 (15,0)		uninfected	extant		0.00	0.00	0.00
		9/6/10	30 (18,12)	8	uninfected			0.00	0.00	0.23
Snowpole	South	8/9/10	1 (1,0)		uninfected	extinct		0.00	0.00	0.00
		8/31/10	24 (13,11)	8	uninfected			0.04	0.48	0.48
		9/15/10	15 (6,8)		uninfected			0.48	0.97	0.97

Table 1 footnotes:

a. number of frog swabs that were analyzed for Bd load.

b. number of frog swabs that were analyzed for bacterial communities.

c. Populations were designated “extant” if any adult or subadult *R. sierrae* were observed during a 2011 census. Populations were designated “extinct” if no adult or subadult *R. sierrae* were observed.

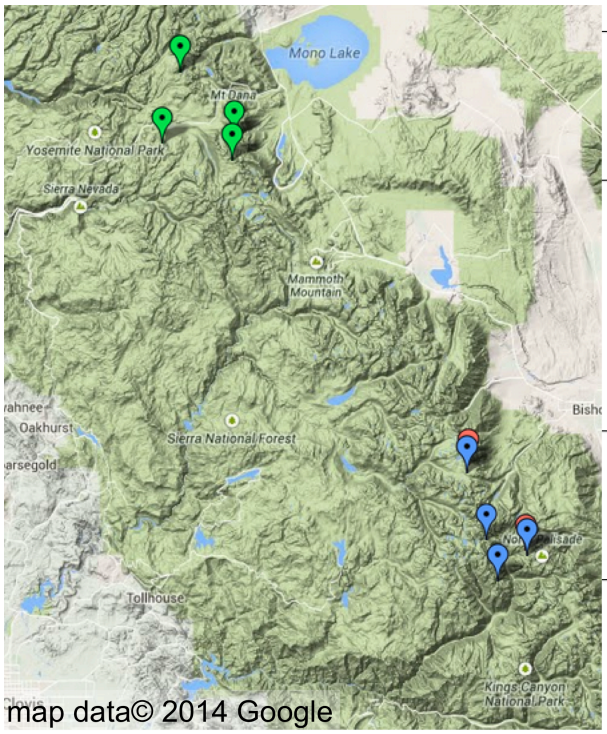
* The Marmot population survey dated 8/8/2010 was designated operationally uninfected, although the maximum Bd load was slightly above 10, because prevalence was still very low (2 Bd-positive swabs out of 30 total swabs) and the previous survey only 3 weeks prior had found no infected frogs.

Table 2. Bacterial taxa that differed between enzootic and epizootic populations, or were correlated with Bd load. Right three columns compare Bd-bacterial relationship in the current landscape-scale study and previous within-population survey and experimental study. "No test" indicates that a phylotype was not tested in a given study because it was absent or rare.

Bacterial Taxon	Type of population in which taxon was more abundant	Landscape-level correlation with Bd load	Within-population correlation with Bd load[a]	Effect of experimental Bd infection[a]
Actinobacteria-Cellulomonadaceae	ns	negative	ns	negative
Actinobacteria-Microbacteriaceae-Microbacterium	ns	negative	negative	negative
Actinobacteria-Nocardiaceae-Rhodococcus	ns	negative	negative	negative
Actinobacteria-Sanguibacteraceae-Sanguibacter	ns	negative	negative	negative
Actinobacteria-Sporichthyaceae-hgcI_clade	ns	negative	negative	no test
Alphaproteobacteria-Sphingomonadaceae-Sandarakinorhabdus	enzootic	negative	ns	no test
Betaproteobacteria-Burkholderiaceae-Polynucleobacter	ns	negative	negative	no test
Betaproteobacteria-Comamonadaceae	enzootic*	positive	positive	ns
Betaproteobacteria-Comamonadaceae-Rhizobacter	enzootic	negative	ns	no test
Betaproteobacteria-Comamonadaceae-Rubrivivax	enzootic*	positive	positive	positive
Betaproteobacteria-Methylophilaceae-Methylotenera	ns	negative	negative	no test
Betaproteobacteria-Neisseriaceae-Iodobacter	ns	positive	ns	no test
Betaproteobacteria-Oxalobacteraceae-Undibacterium	ns	positive	positive	positive
Flavobacteria-Flavobacteriaceae-Flavobacterium	epizootic	ns	ns	no test
Flavobacteria-Flavobacteriaceae-Soonwooa	ns	positive	positive	no test
Gammaproteobacteria-Enterobacteriaceae	ns	negative	ns	no test
Gammaproteobacteria-Pseudomonadaceae-Pseudomonas	ns	negative	negative	negative
Gammaproteobacteria-Xanthomonadaceae-Stenotrophomonas	ns	negative	negative	negative
Sphingobacteriia-Chitinophagaceae-Ferruginibacter	epizootic	ns	ns	no test
unclassified Cyanobacteria	enzootic	negative	ns	no test
unclassified Opitutae	ns	positive	positive	no test

* OTUs classified as Rubrivivax and unclassified Comamonadaceae differed between enzootic and epizootic populations after accounting for the effect of Bd load on bacterial relative abundance. a. reference: Jani and Briggs 2014

Figure 1. Map showing division between "northern" and "southern" *R. sierrae* populations in the current study. Northern populations are infected and exhibit enzootic dynamics (green markers). Southern locations include populations that were uninfected (blue markers) as of the 2010 surveys as well as populations that underwent epizootic disease dynamics and population declines (red markers). Note: red markers are partly obscured by blue markers because epizootic populations are very close to some uninfected populations.



"northern" populations
- Yosemite National Park
- *R. sierrae* clade 2
- enzootic populations (green markers)

"southern" populations
- Kings Canyon National Park, & Sierra National Forest
- *R. sierrae* clade 3
- uninfected (blue) and epizootic (red)

Figure 2. Distinct Bd load trajectories distinguish populations undergoing enzootic and epizootic disease dynamics. (a) Bd load trajectories during the 2010 field season. Inset shows subadult Bd loads over several years prior to the current study in population Dusy-2. (b) Mean Bd loads are higher during epizootic than epizootic disease dynamics ($P<0.0001$). (c) Long-term Bd load and population census data in three populations. Top: An uninfected population showing stable population numbers until invasion by Bd that resulted in epizootic dynamics and population decline. Middle: An enzootic population exhibiting stable Bd loads and population numbers through time. Bottom: A population that remained free of Bd infection and maintained stable population numbers through time. Long-term Bd load data courtesy of Roland Knapp.

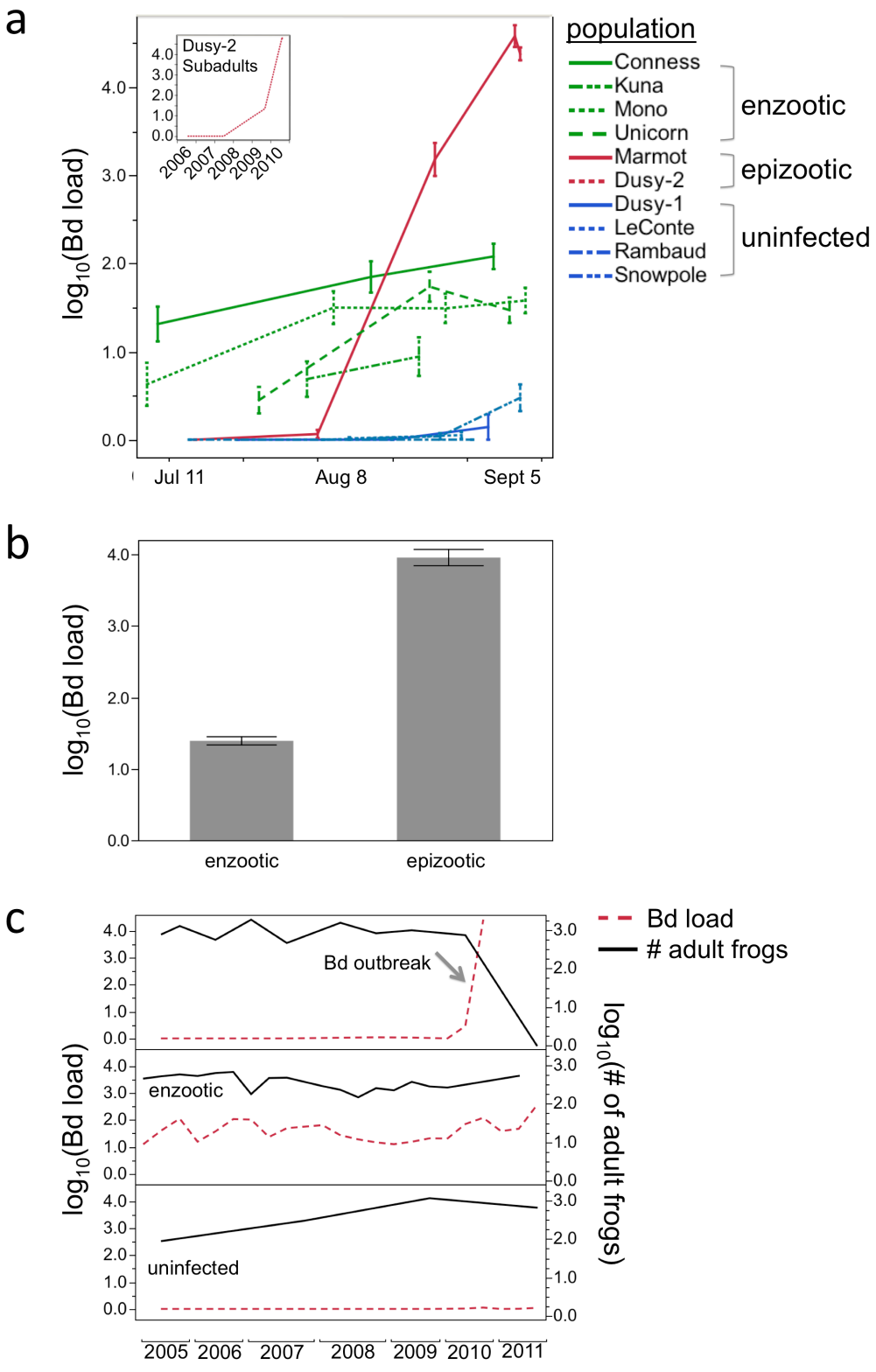

a
log10(Bd load)
4.0
3.0
2.0
1.0
0.0
Jul 11
Aug 8
Sept 5
Dusy-2
Subadults
2006
2007
2008
2009
2010
population
Conness
Kuna
Mono
Unicorn
Marmot
Dusy-2
Dusy-1
LeConte
Rambaud
Snowpole
enzootic
epizootic
uninfected
b
log10(Bd load)
enzootic
epizootic
c
Bd load
adult frogs
Bd outbreak
enzootic
uninfected
log10(Bd load)
log10(# of adult frogs)
2005
2006
2007
2008
2009
2010
2011

Figure 3. Among southern populations, skin bacterial communities differ based on Bd-infection status.

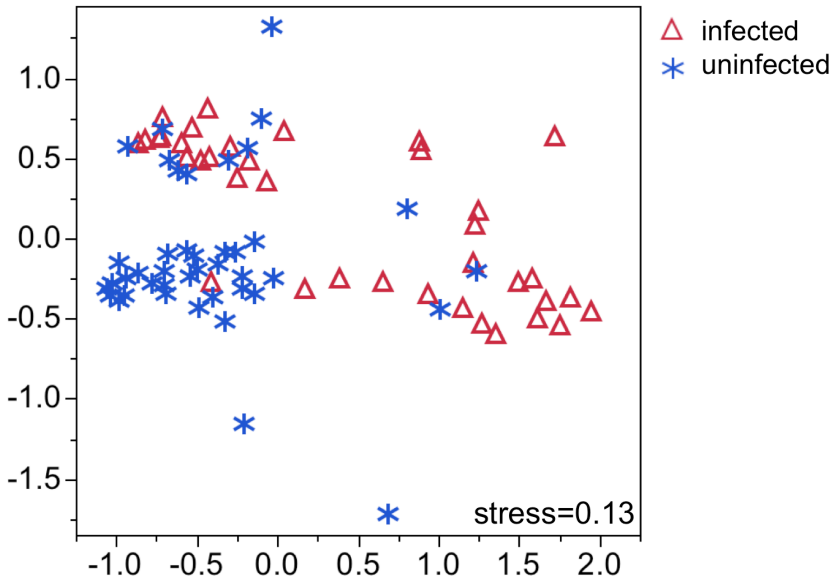


Figure 4. Difference between microbiomes of enzootic and epizootic frog populations appears partially independent of correlation with Bd load. (a) NMDS ordination of bacterial communities from frogs in enzootic and epizootic populations. Marker size and color indicate Bd load. Bd loads primarily increase from left to right along axis 1 and shown in orthogonal regression between $\log_{10}$(Bd load) and NMDS axes (b, c). (d) Same ordination as in left panel, now coded by frog population disease dynamics. Differences between enzootic and epizootic populations are apparent along both axis 1 and axis 2 (e, f).

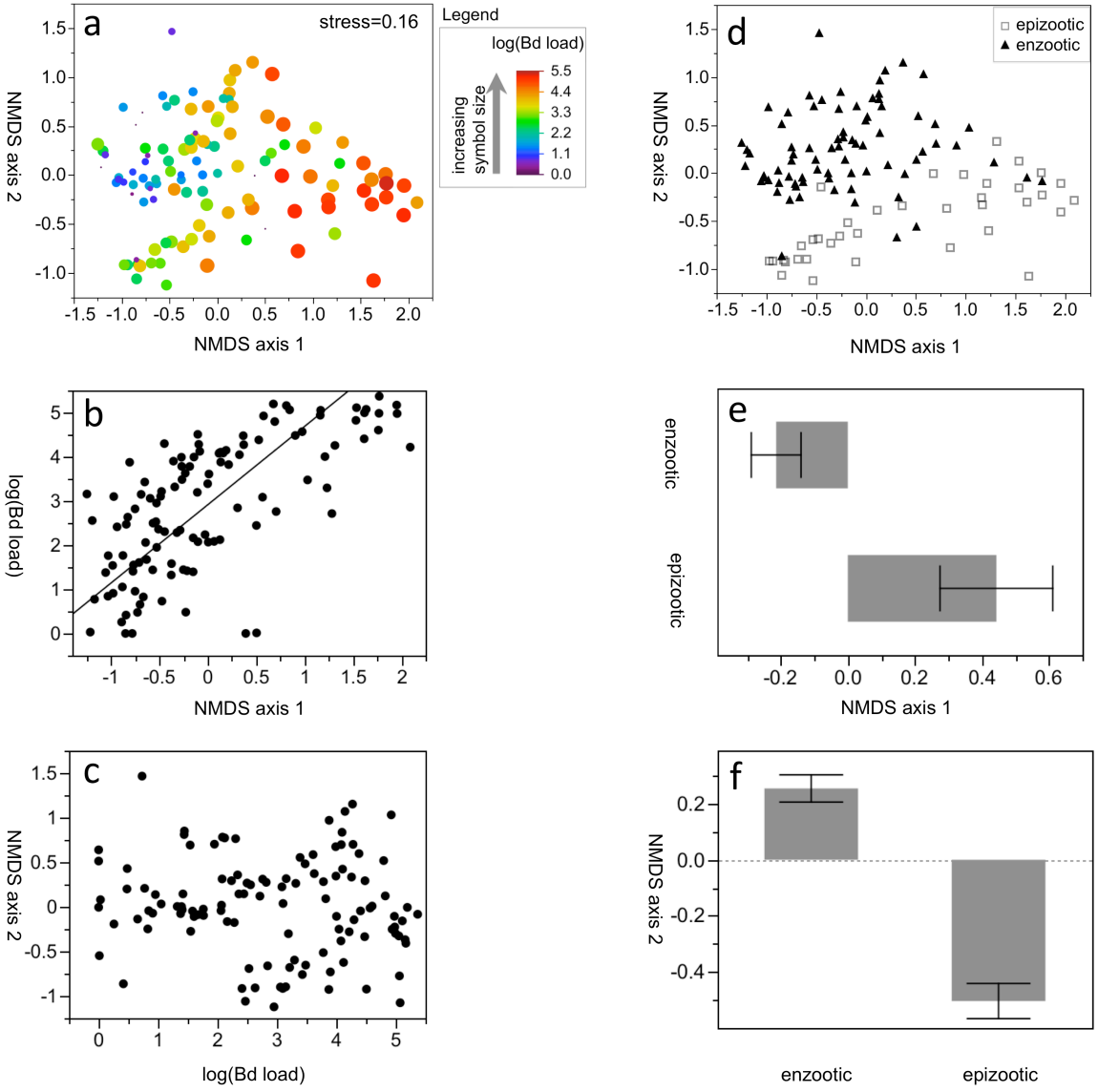

a
stress=0.16
Legend
log(Bd load)
increasing symbol size
5.5
4.4
3.3
2.2
1.1
0.0
NMDS axis 2
NMDS axis 1
d
epizootic
enzootic
b
log(Bd load)
e
enzootic
epizootic
c
f
enzootic
epizootic

Figure 5. Bacterial richness covaries with both Bd load and host response to infection. (a) Bacterial richness (number of OTUs observed) is correlated with Bd load. (b) Bacterial richness is higher in enzootic than epizootic *R. sierrae* populations. Note that the difference between enzootic and epizootic populations becomes non-significant when Bd load is added to the model.

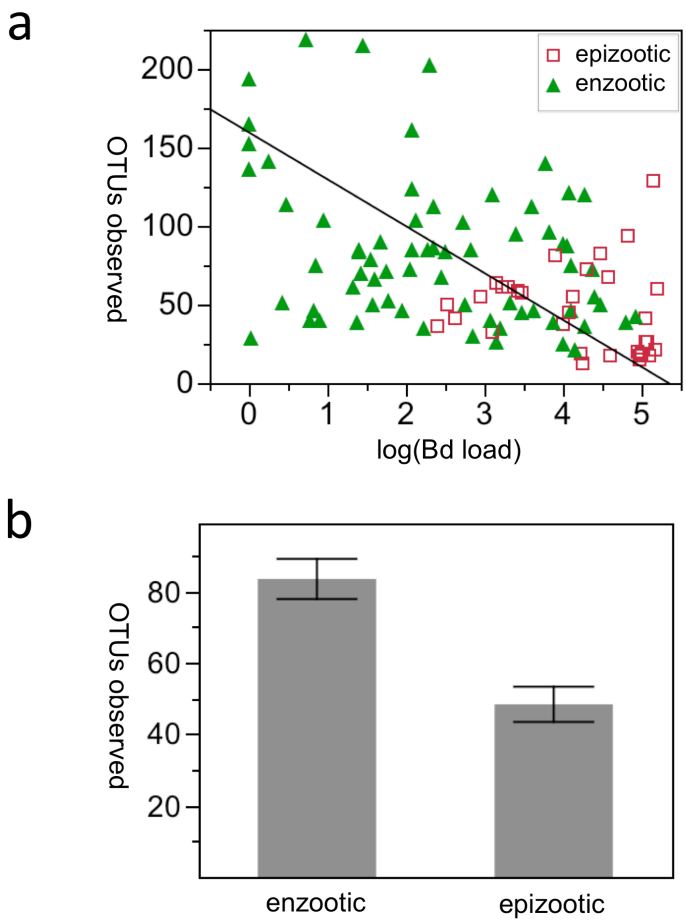


Figure 6. Bacterial communities on frog skin are distinct from surrounding aquatic bacterial communities. NMDS ordination shows separation between bacterial communities of frog skin compared and bacterial communities sampled from lake water. ANOSIM: P=0.0001; Global R=0.98. Each data point represents one population survey. Frog skin microbiome data are pooled within frog populations, such that each frog data point is a pooled sample for all frogs swabbed in a given lake on a given date.

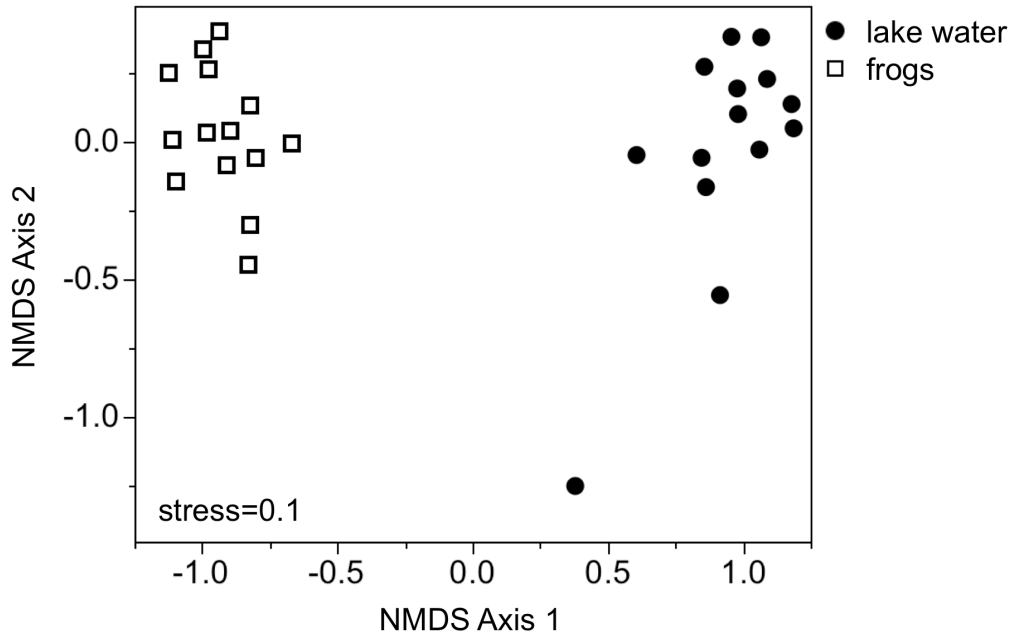
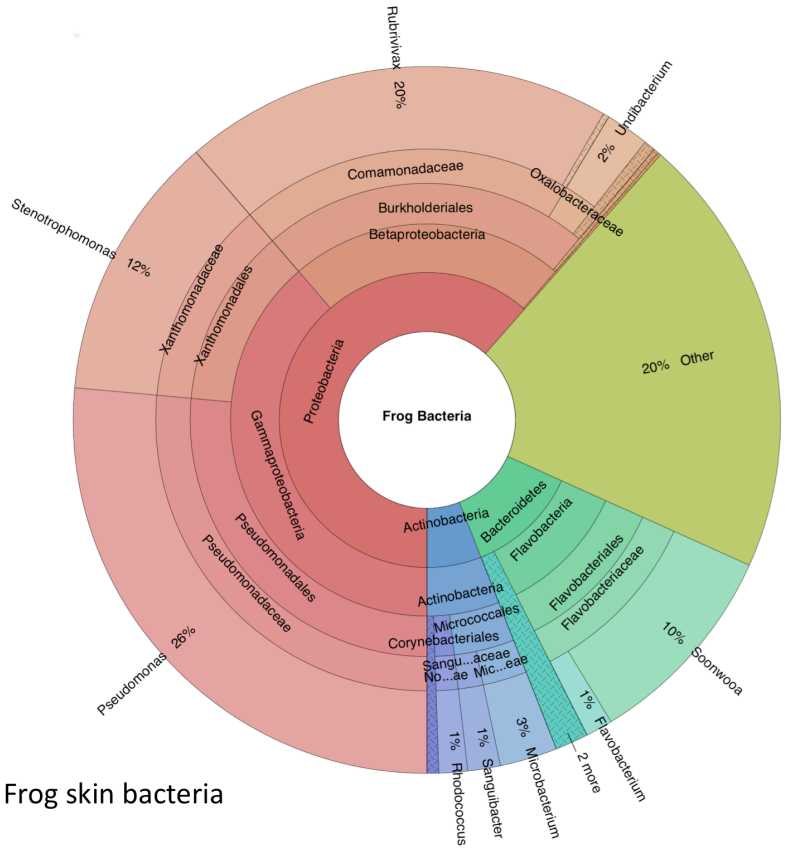


Figure 7. Phylogenetic composition of bacterial communities from (a) frog skin and (b) lake water. Shown are taxa that make-up at least 1% of mean per-sample relative abundance. Taxa with less than 1% relative abundance, and unclassified taxa, are pooled and shown a "other". Plots constructed using the program Krona (Ondov et al., 2011).



a. Frog skin bacteria

b. Lake water bacteria

SUPPLEMENTARY TABLE

Table S1. Pairwise comparisons of skin bacterial communities among all population surveys.

survey 1		survey 2		compares surveys from the same population	Global R	p	q
population	survey date	population	survey date				
LeConte	September 4	Conness	August 18		0.615	0.0001	7.92E-06
Rambaud	September 6	Conness	August 18		0.47	0.0001	7.92E-06
Dusy-1	September 9	Dusy-2	August 23		1	0.0001	7.92E-06
Dusy-1	September 9	Marmot	September 14		0.566	0.0001	7.92E-06
Dusy-2	August 23	Marmot	August 30		0.723	0.0001	7.92E-06
Marmot	September 14	Conness	August 18		0.687	0.0001	7.92E-06
Mono	September 1	Dusy-1	September 9		0.63	0.0001	7.92E-06
Mono	September 1	Unicorn	September 13		0.572	0.0001	7.92E-06
Mono	September 1	Conness	August 18		0.538	0.0001	7.92E-06
Mono	September 16	Dusy-2	August 23		0.518	0.0001	7.92E-06
Mono	September 16	Marmot	August 30		0.465	0.0001	7.92E-06
Unicorn	September 13	Dusy-2	August 23		0.961	0.0001	7.92E-06
Unicorn	September 13	Marmot	August 30		0.601	0.0001	7.92E-06
Unicorn	September 13	Marmot	September 14		0.588	0.0001	7.92E-06
Conness	August 18	Dusy-2	August 23		0.964	0.0001	7.92E-06
Conness	August 18	Marmot	August 30		0.63	0.0001	7.92E-06
LeConte	September 4	Dusy-2	August 23		0.865	0.0002	9.74E-06
LeConte	September 4	Unicorn	September 13		0.471	0.0002	9.74E-06
Rambaud	September 6	Dusy-2	August 23		0.878	0.0002	9.74E-06
Dusy-1	August 22	Dusy-2	August 23		0.987	0.0002	9.74E-06
Dusy-1	September 9	Dusy-1	August 22	X	0.324	0.0002	9.74E-06
Dusy-2	August 23	Unicorn	August 29		0.902	0.0002	9.74E-06
Dusy-2	August 23	Kuna	August 27		1	0.0002	9.74E-06
Marmot	September 14	Marmot	August 30	X	0.55	0.0002	9.74E-06
Mono	September 1	Kuna	August 27		0.516	0.0002	9.74E-06
Mono	September 16	Conness	August 18		0.277	0.0002	9.74E-06
LeConte	September 4	Dusy-1	September 9		0.468	0.0003	1.23E-05
Dusy-1	September 9	Marmot	August 30		0.505	0.0003	1.23E-05
Mono	September 1	Dusy-1	August 22		0.454	0.0003	1.23E-05
Kuna	August 27	Marmot	August 30		0.528	0.0003	1.23E-05
Conness	August 18	Snowpole	August 31		0.411	0.0003	1.23E-05
Marmot	September 14	Dusy-1	August 22		0.572	0.0004	1.58E-05
Rambaud	September 6	Dusy-1	August 22		0.285	0.0005	1.86E-05
Dusy-2	August 23	Snowpole	August 31		0.686	0.0005	1.86E-05
LeConte	September 4	Kuna	August 27		0.391	0.0006	2.00E-05
Dusy-1	August 22	Marmot	August 30		0.473	0.0006	2.00E-05
Marmot	September 14	Kuna	August 27		0.532	0.0006	2.00E-05
Mono	September 1	Rambaud	September 6		0.425	0.0006	2.00E-05
Mono	September 1	Dusy-2	August 23		0.545	0.0008	2.60E-05
LeConte	September 4	Dusy-1	August 22		0.325	0.0010	3.02E-05
Rambaud	September 6	Marmot	September 14		0.507	0.0010	3.02E-05
Dusy-1	September 9	Conness	August 18		0.309	0.0010	3.02E-05
LeConte	September 4	Marmot	September 14		0.533	0.0020	5.76E-05
Rambaud	September 6	Marmot	August 30		0.422	0.0020	5.76E-05
Dusy-1	September 9	Unicorn	August 29		0.335	0.0030	7.92E-05
Marmot	September 14	Unicorn	August 29		0.465	0.0030	7.92E-05
Unicorn	August 29	Marmot	August 30		0.487	0.0030	7.92E-05
Unicorn	September 13	Mono	September 16		0.182	0.0030	7.92E-05
LeConte	September 4	Mono	September 16		0.305	0.0040	9.94E-05
Dusy-1	September 9	Kuna	August 27		0.247	0.0040	9.94E-05

survey 1		survey 2					
Conness	August 18	Unicorn	August 29		0.28	0.0040	9.94E-05
Dusy-1	September 9	Snowpole	August 31		0.255	0.0050	0.000119
Marmot	September 14	Dusy-2	August 23		0.317	0.0050	0.000119
Marmot	August 30	Snowpole	August 31		0.344	0.0080	0.000180
Mono	September 1	Unicorn	August 29		0.349	0.0080	0.000180
Conness	August 18	Kuna	August 27		0.243	0.0080	0.000180
Unicorn	September 13	Snowpole	August 31		0.276	0.0100	0.000222
Marmot	September 14	Mono	September 16		0.192	0.0130	0.000283
Mono	September 1	Marmot	September 14		0.233	0.0140	0.000300
Unicorn	September 13	Conness	August 18		0.12	0.0160	0.000337
Marmot	September 14	Snowpole	August 31		0.257	0.0180	0.000373
Rambaud	September 6	Dusy-1	September 9		0.18	0.0190	0.000388
LeConte	September 4	Unicorn	August 29		0.245	0.0200	0.000402
Mono	September 1	Marmot	August 30		0.269	0.0210	0.000409
Mono	September 16	Dusy-1	August 22		0.222	0.0210	0.000409
Rambaud	September 6	Mono	September 16		0.22	0.0250	0.000479
Rambaud	September 6	Kuna	August 27		0.139	0.0260	0.000491
Dusy-1	August 22	Unicorn	August 29		0.182	0.0270	0.000503
Mono	September 1	Mono	September 16	X	0.181	0.0330	0.000605
Dusy-1	August 22	Snowpole	August 31		0.143	0.0370	0.000669
Mono	September 1	LeConte	September 4		0.173	0.0380	0.000678
Rambaud	September 6	Unicorn	September 13		0.202	0.0390	0.000686
Unicorn	September 13	Unicorn	August 29	X	0.192	0.0450	0.000770
Unicorn	September 13	Kuna	August 27		0.17	0.0450	0.000770
Dusy-1	August 22	Kuna	August 27		0.124	0.0470	0.000793
Rambaud	September 6	Unicorn	August 29		0.12	0.0500	0.000833
Mono	September 16	Snowpole	August 31		0.154	0.0550	0.000904
Kuna	August 27	Snowpole	August 31		0.124	0.0620	0.001006
Mono	September 16	Kuna	August 27		0.146	0.0640	0.001026
Dusy-1	September 9	Mono	September 16		0.118	0.0710	0.001124
Mono	September 16	Unicorn	August 29		0.148	0.0720	0.001126
Dusy-1	September 9	Unicorn	September 13		0.117	0.0760	0.001174
LeConte	September 4	Rambaud	September 6		0.112	0.0810	0.001236
Rambaud	September 6	Snowpole	August 31		0.064	0.1320	0.001990
Conness	August 18	Dusy-1	August 22		0.089	0.1510	0.002250
LeConte	September 4	Marmot	August 30		0.104	0.1660	0.002445
Mono	September 1	Snowpole	August 31		0.059	0.2170	0.003159
Unicorn	September 13	Dusy-1	August 22		0.073	0.2210	0.003181
LeConte	September 4	Snowpole	August 31		0.034	0.2620	0.003729
Unicorn	August 29	Snowpole	August 31		0.027	0.2670	0.003758
Kuna	August 27	Unicorn	August 29		-0.005	0.4410	0.006139
LeConte	September 4	Conness	August 18		0.615	0.0001	7.92E-06

CHAPTER THREE

Innate host differences and environmental variation shape the frog skin microbiome and affect resistance to the fungal pathogen *Batrachochytrium dendrobatidis*

ABSTRACT

Symbiotic microbial communities play key roles in the health and development of their multicellular hosts. Understanding why microbial communities vary among different host species or individuals is an important step toward understanding the diversity and function of the microbiome. The amphibian skin microbiome may play a role in limiting infection of the host by the fungal pathogen *Batrachochytrium dendrobatidis* (Bd), but the factors that determine the phylogenetic composition of the amphibian skin microbiome, and therefore may ultimately contribute to disease resistance, are not well understood. We designed a two-phase laboratory experiment to quantify the contributions of host genetic background and the environment in shaping the microbiome of the Sierra Nevada yellow-legged frog, *Rana sierrae*. Two *R. sierrae* population sources, six types of natural lake water, and a sterile water habitat were tested for effects on the composition of frog skin bacterial communities using a fully crossed experimental design. Both frog source and aquatic habitat affected the *R. sierrae* skin microbiome. Notably, differences in the microbiomes of frogs housed in water collected from lakes in the Sierra Nevada mimicked patterns observed in wild populations: in nature, microbiomes differ between frog populations that persist with or decline due to Bd, and in the current experiment frog skin microbiomes differed depending on whether the frogs were housed in water from field sites inhabited by persisting or declining *R. sierrae* populations. We tested if the skin microbiomes established in the first phase of this experiment were linked to downstream resistance to infection by the pathogen *Bd*. Experimental treatments did not confer complete resistance to pathogen infection, but did affect infection trajectories. The rate of increase in pathogen loads differed between frogs

from the two source populations. The aquatic habitat also affected infection trajectories, with frogs housed in sterile water suffering more rapid increases in pathogen loads than frogs housed in natural lake water, although the specific geographic source of the natural lake water had no effect. Taken together, the data show that both host background and the environment affect the amphibian skin microbiome, and that some of these differences are associated with variation in the rate of increase in pathogen infection. We further analyzed associations between Bd infection severity and microbiome composition before and after Bd infection in order to distinguish effects of Bd on the microbiome from effects of the microbiome on Bd. Bd infection severity was significantly correlated with overall microbiome composition after Bd infection, but not before Bd infection, indicating that the effect of Bd on the microbiome was stronger than the effect of the microbiome on Bd.

INTRODUCTION

Symbiotic bacterial communities are ubiquitous inhabitants of multicellular organisms and play important roles in the health and development of their hosts (Dethlefsen et al., 2007; Engel and Moran, 2013; Grice and Segre, 2012; Philippot et al., 2013). Much research has focused on the microbiome of the human gut, but recent studies have begun to explore the microbiome of the skin, which in many animals is the largest organ in the body and a primary line of defense against pathogens. Shifts in the complex skin microflora of humans are linked to psoriasis, atopic dermatitis, acne, and *Dermodex*-associated rosacea, although it is not yet known whether microbiome shifts are a cause or effect of these diseases (Grice, 2014). In a mouse model, skin-associated bacteria influenced skin-specific immune responses and severity of infection by the protozoan parasite *Leishmania major* (Naik et al., 2012). The links between microbiome composition and host health highlight the importance of understanding the factors that shape symbiotic communities: Understanding why bacterial communities differ can help identify the causes of imbalances (dysbioses) in the microbiome, and advance our ability to mediate disease susceptibility by facilitating the maintenance of healthy microbial communities.

The majority of our knowledge of skin microbiome assembly draws on studies of humans and standard laboratory models (e.g. mice). In humans, differentiation of microflora of different body sites (e.g. mouth, gut, skin) develops in the first 15 days after birth (Costello et al., 2013), and at least initially the human microbiome may be influenced by delivery (birthing) mode (Dominguez-Bello et al., 2010). Skin microbiomes of humans show microsite-specific differentiation as well as variation among individuals and with time

(Costello et al., 2009). Hand-associated microbes differ between human populations (Blaser et al., 2013; Hospodsky et al., 2014), suggesting that ethnic, cultural, behavioral/occupational, or environmental differences may influence the skin microbiome. Comparisons of healthy and immune-compromised patients suggest that the skin microbiome is regulated in part by the host immune system (Smeekens et al., 2013). Much less research has focused on skin microbiome assembly in non-human animals, but recent research, fueled in part by the need to manage a serious amphibian skin disease, has begun to explore the amphibian skin microbiome.

In amphibians, understanding the assembly and dynamics of the skin-associated microbiome has direct implications for management of *Batrachochytrium dendrobatidis* (Bd), a fungal pathogen that infects the skin of amphibians and causes the potentially lethal disease chytridiomycosis. Bd has been implicated in amphibian declines worldwide (Berger et al., 1998; Briggs et al., 2010; Crawford et al., 2010; Kilpatrick et al., 2010; Lips et al., 2006; Vredenburg et al., 2010), and currently there are no widely available, proven methods to control the disease in wild populations. Recent research has raised the possibility that symbiotic bacteria present on amphibian skin might affect resistance to chytridiomycosis. Differences in the skin microflora of wild amphibians coincide with differences in apparent disease resistance (Chapter 2; Woodhams et al., 2007, 2014). Incubating amphibians with isolates of the bacterial species *Janthinobacterium lividum* conferred resistance to Bd infection in two laboratory experiments (Harris et al., 2009; Muletz et al., 2012). However, in two other studies, augmentation with J. *lividum* or an antifungal isolate of *Pedobacter cryoconitis* failed to protect frogs from Bd infection and resulting disease (Becker et al., 2011; Woodhams et al., 2012). This variation in the efficacy of probiotic isolates is probably

the product of multiple factors, which may include ecological context (host condition, environmental parameters, microbial community composition, Bd inoculation dose) or genetic differences among hosts, bacterial isolates, or Bd isolates. Another likely important factor is the stability of the skin microbiome in the face of Bd infection. We previously showed that Bd infection alters the amphibian skin microbiome (Jani and Briggs, 2014), and it follows that the sensitivity of the microbiome to disturbance may affect its ability to limit Bd infection. Better understanding of the factors controlling the amphibian skin microbiome, in both the presence and absence of Bd, are critical to refining microbially-based approaches to mitigate the spread and impact of Bd.

A few recent studies have begun to examine the factors shaping the amphibian skin microbiome. Species-specific microbiome differences among amphibians sharing a common lake or pond environment have been clearly demonstrated (Kueneman et al., 2014; McKenzie et al., 2012; Walke et al., 2014). Microbiome differences among populations of a given amphibian species have also been observed (Chapter 2; Kueneman et al., 2014; Walke et al., 2014), although potential covariation between host genetic and environmental differences in these studies precludes firm conclusions regarding the cause (host or environmental) of among-population variation in the microbiome. The skin of amphibians appears to select for certain bacterial taxa (Jani and Briggs, 2014; Walke et al., 2014), but at the same time the microbiome is not entirely unaffected by environmental drivers. The skin microflora of salamanders differs between animals housed in sterile liquid medium compared with non-sterile soil (Loudon et al., 2013), indicating that substrate structure or the presence of bacteria, or both, affect the amphibian skin microbiome. In addition, our previous work showed that the composition of bacterial communities on the skin of amphibians was

correlated with the composition of aquatic environmental bacterial communities (Chapter 2). We also know that Bd infection alters the microbiome (Jani and Briggs, 2014). In summary, we know that the microbiome differs among host species and is affected by dramatic changes in the environment (e.g. field soil versus sterile liquid medium) or infection by Bd. But important questions remain. For example, we know nothing about the cellular or molecular mechanisms of microbiome assembly in amphibians. Even more basic is the question of whether the microbiome is affected by genetic variation within a given host species. The latter question is critical in understanding within-species variation in the microbiome and, potentially, variation in disease resistance. In addition, while we know that wholesale changes in the environment (such as different lakes, or a habitat made of sterile water compared with living soil) affect the microbiome, we do not know what properties of the environment drive those changes. We also do not know if microbiome disturbance by Bd alters or overrides host or environmental control of the microbiome. Finally, we lack understanding of the links between microbiome assembly and function, specifically how host and environmental effects on the microbiome may in turn influence subsequent resistance to disease.

In the current study, we use a fully crossed factorial experiment to simultaneously test the effects of host genetic background and the aquatic environment in shaping within-host-species variation in the microbiome. We then examine how microbiome variation shaped by host and environmental factors affects resistance to Bd. We focus here on microbiome assembly and function in the Sierra Nevada yellow-legged frog (*Rana sierrae*), a species that is severely threatened by Bd (Chapter 2; Rachowicz et al., 2006; Vredenburg et al., 2007, 2010). Notably, *R. sierrae* populations exhibit variation in their response to Bd infection:

many populations have rapidly been driven extinct by the pathogen ("declining populations"), but some populations appear to tolerate infection and have co-existed with the pathogen for years ("persisting populations") (Briggs et al., 2010; Chapter 2; Knapp et al., 2011; Vredenburg et al., 2010). Among wild populations of *R. sierrae* or its close relative *Rana muscosa*, differences in skin bacterial communities are correlated with differences in disease dynamics (host persistence or decline due to Bd, (Chapter 2; Woodhams et al., 2007)), suggesting that symbiotic bacteria may play a role in these frogs' response to infection. In the current study, we address several open questions regarding the assembly and function of the *R. sierrae* skin bacterial microbiome (hereafter simply "microbiome" for brevity). First, we tested if innate differences between *R. sierrae* populations shape differences in the microbiome. Second, we examined how differences in the aquatic environment drive variation in the microbiome. More specifically, we experimentally tested if differences in the lake water inhabited by frogs can explain why the microbiomes of *R. sierrae* differ between populations that persist with Bd and populations that are driven extinct by the pathogen. We then tested if the variation in the microbiome that is shaped by differences in the host and aquatic environment leads to differences in resistance to Bd infection or chytridiomycosis.

METHODS

Collection of R. sierrae *and lake water.* All equipment, including nets and shoes, that was likely to come into contact with frogs or lake water was disinfected (incubated with 0.1% quaternary ammonium solution 128 for at least 5 minutes) before beginning work in any field site. Small or sensitive equipment was disinfected with 70% ethanol. *R. sierrae* were collected as eggs or tadpoles from two populations in the Sierra Nevada. One population was

located in a lake of Humphrey's Basin (Sierra National Forest), and the other population was located in Dusy Basin (Kings Canyon National Park). All collections were made during the 2010 field season under research permits from the National Park Service and U.S. Forest Service, and subsequently housed at the University of California, Santa Barbara (UCSB) in facilities certified by the UCSB Institutional Animal Care and Use Committee (IACUC). We targeted "stranded" eggs for collection, meaning that the eggs were found in microhabitats where they were unlikely to survive; for example, ephemeral water bodies that were separated from the main lake body and would very likely desiccate before tadpoles hatching from eggs could metamorphose and move out of water to reach the main lake. (*R. sierrae* tadpoles require two to three years to reach metamorphosis in the wild.) Eggs and tadpoles were reared in UCSB animal care facilities and were at the subadult stage at the time of this experiment.

Lake water to be used as aquatic habitat treatments in the experiment was collected from six Sierra Nevada lakes, all of which are or were either inhabited by persisting *R. sierrae* populations at the time of this study or had been previously inhabited by *R. sierrae* populations that had declined due to Bd one to three years prior. Water was collected in 2.5-gallon (approximately 10 liter) collapsible polyethylene jugs (Cubitainer), which were washed with 0.1% quaternary ammonium solution 128, triple-rinsed with tap water, soaked in tap water for at least 15 minutes, then rinsed again. Jugs were additionally triple-rinsed in lake water at the collection site before being used for lake water collection. Because most *R. sierrae* populations are located in back-country areas far from roads, frogs and water were transported from field sites on foot, with transport times ranging from approximately three to twelve hours. Lake water was stored at 4°C at the Sierra Nevada Aquatic Research

Laboratory for up to eight days, then transported, with water jugs held in insulated containers with ice, to UCSB where it was stored at 4°C until use. The experiment was initiated within four days after the arrival of the water at UCSB. Prior to being added to experimental frog tanks, all lake water was filtered (1.2 μm pore size) to remove larger particles, including any Bd cells that may naturally occur in lake water. Based on published size distributions of freshwater bacteria (Šimek and Chrzanowski, 1992), we estimate that at least 80% of planktonic bacteria in lake water should pass through 1.2 micron pores and remain in the water after filtration.

Preparation of Bd and sham inocula: Four Bd strains (TST75, CBJ4, CJB5, CJB7) were used in this experiment. Two of the Bd strains were isolated from persisting *R. sierrae* populations and the other two strains were isolated from declining populations. Bd cultures from frozen stocks were grown in 1% (w/v) tryptone liquid medium. Once viability was confirmed, cultures were passaged to agarose tryptone media in petri plates (10 g L^{-1} tryptone, 10 g L^{-1} agar). Tryptone plates without Bd added were prepared in parallel with cultures as a sham inoculum. Cultures and sham plates were harvested after four to six days of growth by flooding plates with sterile water for 45 minutes to induce release of zoospores from sporangia and then collecting the zoospore suspension. To avoid introducing Bd culture medium to frog tanks along with Bd inoculum, zoospores and sham inocula were rinsed three times by gently pelleting (500 G, 5 min) in 50 mL conical tubes, drawing off the supernatant, and resuspending cells (or sham inoculum) in 35 mL sterile water. After rinsing, cells were counted visually at 200X magnification on a compound light microscope using a hemocytometer. The four strains were pooled, with an equal concentration of each strain, and the cell suspension was diluted to 200,000 cells mL^{-1} and used immediately.

Experimental design. We conducted a two-phase experiment to simultaneously test determinants of microbiome composition and downstream effects on resistance to Bd infection:

Experiment phase 1 – microbiome assembly: The first phase of the experiment tested effects of aquatic environments and host genetic background on the *R. sierrae* skin microbiome. To vary host population, forty-two frogs from each of the two *R. sierrae* populations were included in the study. To vary the aquatic habitat, each frog was housed in an individual tank with water from one of seven Water Sources, which included water collected from six different lakes and one sterile water treatment. We chose lake water as our focus because *R. sierrae* are aquatic amphibians most often found either in lake water or basking on adjacent rocks. The high elevation Sierra Nevada lakes where the species is found are rocky, oligotrophic habitats with little soil or vegetation. Sterile water was prepared by autoclaving bottled drinking water (Arrowhead), and sterility was confirmed by plating cooled 100 uL aliquots onto R2A and LB agar plates. Lake water was collected from six Sierra Nevada lakes, three of which were inhabited by *R. sierrae* populations that persist with Bd, while the other three were inhabited by populations that declined due to Bd. We used a fully crossed design with 14 treatments (2 Frog Sources x 7 Water Sources), and six replicate frogs assigned to each treatment. For two weeks prior to beginning the experiment, animals were co-housed in large common-garden tanks to standardize any pre-experiment environmental effects (Jani and Briggs, 2014). Non-sterile, bottled drinking water was used to provide an aquatic habitat in the pre-experiment, common-garden tanks. Immediately before beginning the experiment, each frog was treated with 3% hydrogen peroxide (50 ml in a 100 ml container) for 30 s, and then rinsed thoroughly with sterile water (two 100-ml sterile water

baths lasting two and eight minutes, respectively), in an attempt to reduce and standardize the bacterial community present on the skin of frogs (Harris et al., 2009). To initiate the experiment, twelve frogs (six from each population) were randomly assigned to each of the seven Water Sources. All frogs were housed in individual tanks, each containing water from one of the seven Water Sources, for the duration of the experiment. Each frog was offered 7 crickets once per week, and tank water was changed after feeding to minimize the introduction of food-associated bacteria to experimental tanks. Tanks were randomly assigned positions in environmental chambers maintained at 17°C with a 12 hr photoperiod.

Experiment phase 2 - Bd challenge: Three weeks (21 days) after beginning the experiment, 42 of the frogs (3 frogs from each Frog Source x Water Source treatment) were challenged with Bd (200,000 zoospores per frog for three consecutive days). The remaining 42 frogs served as Bd-free controls and received a sham inoculum. Frogs were monitored daily throughout the experiment. At 60 days post-infection, the experiment was concluded and surviving frogs were cleared of Bd infection by treatment with Itraconazole. Infection status for all frogs was confirmed by quantitative PCR.

R. sierrae is a Federally listed endangered species, and was listed as threatened at the time when this experiment was conducted, in 2011. To minimize the use of *R. sierrae* for experiments while maximizing research progress, we designed this experiment to address three research questions: (1) What factors control microbiome composition? This question is addressed in the first 3-week phase of the experiment. (2) How does variation in the skin microbiome affect resistance to Bd? This question is addressed by examining how variation in the microbiome present at the end of phase 1 (just prior to Bd challenge) is correlated with downstream severity of Bd infection. (3) Does Bd infection alter the microbiome? This

question is addressed by comparing the microbiomes of Bd-infected and Bd-free (control) frogs after Bd challenge. The first two research questions are the focus of the current work, while the third question is addressed elsewhere (Jani and Briggs, 2014).

Data collection: Microbes present on frog skin (including bacteria and Bd) were sampled at least once weekly, (twice weekly during Phase 1, before Bd challenge). New nitrile gloves were worn for each frog handled, and frogs were rinsed twice with 60 ml sterile water before swabbing the skin using a sterile synthetic swab (Medical Wire and Equipment Company) following standard protocols (Briggs et al., 2010; Jani and Briggs, 2014). Swab buds were immediately placed in sterile microcentrifuge tubes on ice and frozen within one hour of collection. We monitored symptoms of chytridiomycosis, including weight loss, inappetence, and excessive shedding of skin. We recorded snout-to-vent length and weight of all frogs before infection and at six and eight weeks post-infection. We counted the number of crickets eaten by each frog weekly and scored the amount of shed skin present in tank water using a qualitative 3-level rating system: no shed skin, moderate amount, or copious amount of shed skin observed in tank water. Prior to adding lake water to frog tanks, bacteria present in lake water were sampled by filtering 250 ml from each lake Water Source through a 0.22 μm pore polyethersulfone filter (Sterivex-GP; Millipore). Filters with samples of aquatic bacteria were frozen immediately.

DNA extraction. Total DNA (including bacterial and Bd DNA) from frog skin swabs was prepared for PCR using Prepman Ultra as described previously (Jani and Briggs, 2014). DNA from aquatic bacterial samples was extracted following Nelson (2009).

Quantifying Bd load. We quantified Bd loads from all swabs collected immediately prior to Bd challenge and weekly thereafter until the end of the experiment. Bd load (also referred to as Bd infection intensity) was measured by quantitative PCR (qPCR) applied to skin swab DNA samples as described previously (Boyle et al., 2004; Jani and Briggs, 2014), with Bd standards provided by the laboratory of Alex Hyatt (CSIRO, Australia).

Selection of samples for bacterial 16S sequencing. Due to the labor requirements and cost of sequencing, it was not feasible to analyze bacterial communities of all samples (>1,000) collected in the experiment. Instead, we analyzed bacterial communities at three time points: (1) immediately before beginning the experiment; (these samples serve as a control to confirm that no differences exist between experimental water treatments before beginning the experiment); (2) after 3 weeks (21 days) of exposure to the various water treatments, but immediately before Bd challenge; (3) three weeks after Bd challenge (i.e. 21 days post-infection, PI). The 21 days PI time point was chosen for two reasons. First, Bd loads at three weeks PI were representative of loads observed during epidemics in the wild (Jani and Briggs, 2014). Second, because all frogs in the experiment were still surviving at three weeks post-infection, analyses conducted at this time point maintain a balanced experimental design. Samples of aquatic bacteria in stored lake water were also analyzed on three dates roughly corresponding to the dates on which frog skin bacteria were analyzed.

Bacterial community sequencing and bioinformatic processing. The bacterial communities present on frog skin and in lake water were characterized by sequencing of a portion of the 16S gene, as described in detail in Jani and Briggs (2014). Briefly, the V1-V2 region of the 16S gene was amplified using barcoded primers with sequencing adapters, and PCR products were purified, pooled in equimolar quantities, and sequenced on a Roche/454 GS FLX

instrument using Titanium chemistry. The program mothur v 1.30 (Schloss et al., 2009) was used to quality-filter sequences, align them to a non-redundant representative subset of the SILVA v111 SSU Ref 16S curated alignment database (Nelson et al., 2014), and cluster sequences into 95% sequence identity operational taxonomic units (OTUs) and phylotypes. Sequences were classified using the Bayesian classifier of Wang *et al.* (2007) and each OTU was assigned a consensus taxonomy from SILVA v111 (Pruesse et al., 2007; Quast et al., 2013). Weighted Unifrac distance (Lozupone and Knight, 2005) was calculated from OTU relative abundance data to quantify the degree of phylogenetic difference among bacterial communities from different samples. To estimate bacterial community richness and diversity, the number of observed OTUs (S_{OBS}), Chao's richness estimate (Chao, 1984), Shannon diversity, and Shannon evenness were calculated after subsampling to 500 sequences per sample.

Testing effects of frog source and aquatic environment on the microbiome. We tested for effects of Water Source and Frog Source on the *R. sierrae* microbiome in the absence of Bd by examining bacterial communities from frogs sampled immediately before Bd challenge. This time point is designated 0 days post-infection (0 days PI), and at this point each frog had been exposed to one of the 7 Water Source treatments for three weeks, providing ample time for the Water Source treatments to take effect. Our response variable was Unifrac distance, a measure of phylogenetic dissimilarity between bacterial communities. We used permutational multivariate ANOVA (PERMANOVA) with Frog Source (Dusy or Humphreys) and Water Source (7 sources listed in Table 1) as fixed factors. The Frog x Water interaction was not significant and was dropped from the model. In addition to testing effects of the seven Water Sources, we tested two specific hypotheses about how the aquatic

environment might affect the *R. sierrae* microbiome. Our primary hypothesis was that the aquatic habitat helps shape differences in the microbiome between *R. sierrae* populations that persist or decline due to Bd. Based on this hypothesis, we predicted that frogs housed in water from lakes inhabited by persistent populations would harbor different bacterial communities than frogs housed in water from lakes inhabited by declining populations. We used PERMANOVA with Frog Source and Site Persistence (water from a persistent or declining population) as main effects and Water Source nested in Habitat Persistence to account for the fact that each class of water (persistent or declining) encompasses three different lake Water Sources. We also tested if the frog skin microbiome differed between frogs housed in sterile water and frogs housed in non-sterile lake water. For this test we used PERMANOVA with Frog Source and Water Sterility ("sterile" or "live") as main effects and Water Source nested in Water Sterility to account for the fact that the live water treatment encompasses six distinct Water Sources.

We repeated all analyses (tests of effects of Frog Source, Water Source, Water Sterility, and Site Persistence on the microbiome) using data collected 21 days PI to test if host and aquatic environment effects are detectable among frogs once Bd is introduced. Bd infection affected bacterial communities in this experiment, as presented in (Jani and Briggs, 2014), therefore, for analyses of bacterial communities 21 days PI, Bd infection (Bd+, Bd-) was also included in ANOVA models to account for the effect of Bd on microbiome composition.

To confirm that no effect of water treatments existed before the water treatments were applied, ANOVA to test the effects of Water Source and Frog Source on the microbiome were also conducted at the beginning of the experiment (21 days pre-infection, immediately before placing frogs in their respective experimental tanks with lake water).

Testing effects of Frog Source and Water Source on Bd infection severity. Bd load data collected from swabs before Bd challenge and weekly thereafter were used to examine infection trajectories through time. All frogs in the Bd- treatment remained free of Bd, with the exception of one frog, which became contaminated with Bd and was excluded from analyses. Only Bd load data from the 42 frogs in the Bd+ treatment were analyzed for Bd infection trajectories because we were interested in effects of experimental treatments on variation in Bd trajectories given that frogs were exposed to Bd. We used repeated measures ANOVA (RM-ANOVA) to test for differences among experimental treatments in the rate of increase of Bd load through time. Data for RM-ANOVA were restricted to swabs collected between 0 and 21 days PI because 21 days PI was the latest time point for which all frogs were still alive. As with tests for treatment effects on the microbiome described above, we tested effects of Frog Source and Water Source and then followed up with specific hypothesis tests for effects of Water Sterility and Site Persistence.

Testing direct links between bacterial communities and Bd infection severity. An objective of this study was to tease apart the effects of Bd on the microbiome from effects of the microbiome on Bd infection. For this analysis we included only frogs in the Bd+ treatment, since we are interested in how the microbiome affects the severity of infection given that a frog is exposed to the pathogen. We used Mantel tests to calculate Spearman rank correlation coefficients between the distance matrices based on the microbiome (before Bd challenge and 3 weeks PI) and the distance matrix based on Bd load (3 weeks PI). The strength of the two effects (Bd effect on microbiome and vice versa) was examined by comparing the Bd-microbiome correlation coefficients.

Statistical details. Parametric statistical analyses (ANOVA, RM-ANOVA) were performed in JMP v.10 (SAS Institute Inc., Cary, NC, USA, 1989-1212). Non-metric multidimensional scaling (NMDS) ordination and permutation-based analyses of bacterial community composition, including PERMANOVA and permutational Mantel tests, were conducted in Primer-e v6 (Clarke and Gorley, 2006). Bd load data were $\log_{10}(X+1)$ transformed for all analyses.

Pooling of data for graphical display. We used NMDS ordination to visualize multidimensional data (e.g. Unifrac distances). In NMDS, the stress associated with an ordination provides a measure of the distortion of the data incurred when multidimensional data are represented in fewer (usually 2) dimensions. In this study, NMDS stress for most ordinations was unacceptably high, generally between 0.15 and 0.22. This may be due to very high variability in the data. To reduce stress in the ordinations, we pooled data across replicates within each treatment and day. Ordination plots therefore display data pooled within treatment and day, while all statistical analyses were conducted on the unpooled data, as specified in descriptions of the statistical models. Ordinations are for data visualization only and do not affect statistical results.

RESULTS

Frog Source and Water Source contribute to microbiome composition. For clarity, an outline of the variables tested is provided in Table 2. At 0 days PI (before Bd challenge, but 21 days after initiation of Water Source and Frog Source experimental treatments), microbiome composition was significantly affected by both Water Source and Frog Source. Frogs from the two source populations harbored significantly different bacterial communities, and this

was true regardless whether all treatments or only lake water treatments were considered (PERMANOVA: P=0.0166, P=0.0040 for all treatments or only lake water treatments, respectively, Figure 1a). Bacterial diversity did not differ between the two frog populations (P>0.05 for all richness and diversity metrics examined). *R. sierrae* microbiome composition differed based on Site Persistence (population persistence or decline of the field site from which water was collected, P=0.0002, Figure 1b). In addition to differing in microbiome composition, frogs housed in water from persistent field sites also harbored slightly but significantly greater bacterial diversity than frogs housed in water from field sites associated with epidemic declines (S_{OBS} P=0.0083, Chao's richness P=0.0021, Shannon diversity P=0.0054, Shannon evenness P=0.0243; Figure 2). In analyses focused on water sterility, frogs housed in sterile water harbored different bacterial communities than frogs housed in live water (P=0.0009, Figure 3a), but there was no difference in bacterial diversity between the two groups.

Microbiome composition of samples collected at the start of the experiment (immediately before placing frogs in their respective water treatments) showed no effect of Water Source (P>0.05), confirming that the effects of Water Source observed after application of experimental treatments was indeed due to the treatments. In contrast, the composition of microbial communities did differ between the two Frog Sources at the start of the experiment (P=0.0035), indicating that effects of Frog Source on the microbiome are at least partially robust to normalizing forces such as shared aquatic environments created by the common-garden pre-experiment tanks or the pre-experiment hydrogen peroxide treatments employed in this study.

Three weeks after Bd infection, skin bacterial communities still differed based on Frog Source and Water Source. Bacterial community composition differed between frogs from the two source populations (P=0.0007, Figure 1c) as well as between frogs housed in water from field sites with different disease dynamics, i.e., population persistence or decline (P=0.0001, Figure 1d). Microbiomes also differed between sterile and live water treatments (P=0.0007, Figure 3b).

Bd infection trajectories and frog survival are affected by Water Source and Frog Source. Frogs from Humphreys Basin showed more rapid rates of Bd load increase than frogs from Dusy Basin (repeated measures ANOVA: P_{Frog}=0.0006, $P_{FrogxTime}$=0.0150, Figure 4a). Water Source also affected Bd load trajectories ($P_{WaterSource}$=0.0010, $P_{WaterSource\ x\ Time}$=0.0120). However, the disease dynamics of field sites (persistent or declining) from which water was collected had no affect on Bd load trajectories (P>0.05, Figure 4b). Instead, the effect of Water Source appeared to be primarily due to differences between Bd load trajectories of frogs housed in sterile water compared with frogs housed in live lake water ($P_{WaterSterility}$=0.0010, $P_{WaterSterility\ x\ Time}$=0.0120, Figure 4c). Patterns in frog survival were consistent with Bd load trajectories: Kaplan Meier survival curves differed based on Frog Source (Chi-square: log rank test, P=0.0461; Wilcoxon test, P=0.0108, Figure 4d) but not Site Persistence (P>0.05, Figure 4e). Water Sterility affected survival curves (log rank, P<0.0001; Wilcoxon, P<0.0001, Figure 4f).

Limited direct evidence for microbiome effects on Bd infection. Differences in skin bacterial communities prior to Bd challenge were not correlated with Bd infection intensity at 21 days PI (Mantel test: P<0.05). However, differences in frog skin microbiomes at 21 days PI were correlated with Bd infection intensity measured on the same day (21 days PI, P=0.0037,

Spearman's rank correlation coefficient=0.15), consistent with Bd disturbing bacterial communities.

Symptoms caused by Bd infection. All frogs in the Bd+ treatment became infected with Bd, and Bd loads increased rapidly with time (Figure S1a). Increases in Bd load were accompanied by weight loss: Frogs in the Bd- group appeared to gain weight over the course of the experiment, while frogs in the Bd+ group lost weight (repeated measures ANOVA with Bd treatment, Frog Source, and Water Source as explanatory variables; $P_{BdxTime}<0.0001$, Figure S1b). All frogs in the Bd- group survived the experiment, but the Bd+ group experienced considerable mortality (Figure S1c). We used ANOVA models to formally test effects of Bd infection on frog appetite (number of crickets eaten per week) and skin shedding measured (based on an ordinal 3-level rating system) at 6 and 8 weeks post infection. Frog Source and Water Source were included as additional explanatory variables in the model. Bd infection led to reduced appetite ($P<0.0001$ at 6 and 8 weeks PI, Figure S2a) and increased skin sloughing ($P<0.0001$ at both 6 and 8 weeks PI, Figure S2b).

DISCUSSION

Summary. Wild *R. sierrae* populations exhibit distinct disease dynamics, either persisting or declining in response to Bd infection. These differences in disease dynamics in wild populations coincide with differences in the composition of skin-associated bacterial communities (Chapter 2), suggesting that the *R. sierrae* microbiome may play a role in response to Bd infection. Here, we aimed to identify factors that shape the *R. sierrae* skin microbiome. Our second objective was to clarify causal Bd-bacteria relationships by directly testing if microbial community composition affects Bd loads. Our results demonstrate that

both host background and the aquatic environment affect the skin microbiome. We found that innate differences between conspecific populations led to differences in the skin microbiome. We also found that lake water to which frogs are exposed can shape differences in the skin microbiome, even in the absence of additional environmental factors such as sediment, vegetation, or contact with other amphibians. Bd infection trajectories also differed between experimental treatments, covarying with both host and environmental treatments. However, because experimental treatments such as varying host population source or water source likely affect factors other than the microbiome, further research is required to definitively test a direct chain of events linking host and environmentally-induced differences in the microbiome with downstream resistance to disease.

Frog population affects skin microbiome and Bd infection trajectory. Previous work showed differences between amphibian species sharing a common lake environment (Kueneman et al., 2014; McKenzie et al., 2012; Walke et al., 2014). Here, we show that within-species variation in the microbiome is at least partly controlled by innate differences between host populations. Furthermore, the effect of Frog Source was significant both in the absence of Bd and after Bd infection, indicating that the disturbance to the microbiome caused by Bd infection does not completely override host effects on the microbiome. We also showed that the rate of increase in Bd loads in the experiment differed between frogs from the two populations, demonstrating that host-controlled microbiome variation is associated with variation in host response to pathogen infection. Thus there is potential for a cascade of effects in which host background influences the microbiome, which in turn affects infection dynamics. However, it is also possible that frog population background affects disease dynamics through unknown mechanisms independent of the skin microbiome. Further

research is needed to clearly determine whether the association we observed between frog population, frog skin microbiome, and infection dynamics indicates a causal link between symbiotic bacteria and rates of pathogen growth on the host.

In this study, rates of Bd load increase varied, but no frogs fully resisted or cleared Bd infection, and all frogs challenged with Bd eventually developed high Bd loads. Thus, none of the experimental treatments conferred true resistance to infection or disease. It is not surprising that neither the Dusy Basin nor Humphreys Basin frog populations exhibited resistance to disease, since wild populations in both of these locales have collapsed due to Bd. (This study used frogs in existing laboratory colonies to avoid unnecessary collection of wild *R. sierrae*. No existing colonies from populations persisting with Bd were available.) However, the Bd epidemic and resulting population decline were extremely rapid in Humphreys Basin compared with Dusy Basin. In Humphreys Basin, Bd was detected late in the 2010 field season, and by the time field sites became accessible after snow-melt the following spring, the entire metapopulation had declined to near extinction. In Dusy Basin, Bd was first detected in 2008, but the resulting decline was more gradual, with postmetamorphic *R. sierrae* observed through the 2010 field season (R.A. Knapp, personal communication). There are reports of *R. sierrae* observed in Dusy Basin even later than 2010, but because intervention treatments were applied in 2010 it is possible that survival beyond 2010 is due to those interventions in addition to or instead of innate population differences. However, regardless of dynamics after 2010, the metapopulation decline in Dusy basin spanned at least two full years, whereas the metapopulation crash in Humphreys basin spanned less than one year. Increased metapopulation connectivity in Humphreys Basin may have contributed to the increased rate of Bd spread in that region. Our results suggest innate

differences between host populations may also have played a role, although we present this possibility as a hypothesis rather than a conclusion since we tested only one population from each basin and frogs were not matched for age or size.

Differences in rates of Bd load increase may have practical importance even when no true disease resistance exists. For example, the slower Bd-induced declines in Dusy Basin enabled researchers to mobilize intervention efforts (treatment of frogs with the antifungal drug Itraconazole as well as a bacterial isolate identified as *Janthinobacterium*; coordinated by R.A. Knapp, V.T. Vredenburg, C.J. Briggs). In contrast the rapidity of the *R. sierrae* decline in Humphreys basin precluded any attempt at intervention. Thus, even without complete resistance, variation in the rate of increase in Bd infection intensity can have practical conservation implications. In the current study we found that even in captivity, frogs from Dusy and Humphreys populations harbor different bacterial communities, and also exhibit different rates of increase in Bd loads and host survival that mirror disease dynamics in the field. These results highlight the importance of further research to understand host population level differences, both in terms of host genetics and associated symbiotic microbes. That we observed differences even among closely related *R. sierrae* populations indicates that future studies comparing *R. sierrae* populations with qualitative differences in response to infection (persistence versus decline) will likely be even more revealing.

Aquatic environment affects skin microbiome and Bd infection trajectory. A key finding of our study is that housing frogs in water from different lakes was sufficient to mimic microbiome patterns in the field: namely, differences between persisting and declining *R. sierrae* populations. In the field, *R. sierrae* populations that persist with Bd harbor different bacterial communities than populations that declined due to Bd (Chapter 2). Similarly, in the

current laboratory experiment, frogs developed different bacterial communities depending on whether they were housed in water collected from field sites inhabited by persisting populations or declining populations. This result is remarkable given the limitations of our mesocosms, which consisted simply of tanks with water from different lakes. No vegetation or sediment from field sites was added to the mesocosms. Lake water in bottles and tanks differs from water in the lake from which it was collected, and we previously showed that wild *R. sierrae* have different microbiomes from captive *R. sierrae* (Jani and Briggs, 2014). However, even our limited mesocosms composed of different types of lake water conferred differences in the bacterial communities on *R. sierrae* skin. Further analyses will be required to determine the extent to which the specific bacterial taxa that differed between lake water treatments in the current laboratory experiment are related to the taxa that differed between persistent and declining populations in the field (Chapter 2). Notably, all frogs in this experiment were from declining populations, which is important because frog source and water source are not confounded and we can conclude that the differences observed between different water treatments are indeed due to those water treatments. In contrast, in field surveys we cannot conclude whether differences in the microbiomes of persisting and declining populations is due to the environment or frog genetic background, or both. Notably, the difference between microflora of persisting and declining populations was more dramatic in field surveys (Chapter 2) than in the current experiment, indicating that not all of the variation observed in the field is captured by our experiment. We think it is most likely that, in addition to the aquatic environment, host genetic background contributes to differences between declining and persisting populations in the field, although additional environmental variables not tested in our experiment (such as lake sediment) may also play a role. Also of

note is the fact that Water Source (as well as Water Sterility and Habitat Persistence) affected the *R. sierrae* microbiome in both the presence and absence of Bd. Together, these results demonstrate that water is an important environmental driver of variation in the microbiome, and differences in water sources alone can explain some of the variation in microbiomes between persisting and declining *R. sierrae* populations in the field. However, these differences in skin microflora did not translate to any difference in the rate of Bd load increases. Thus, to the extent that our mesocosms represent environmental variation, we found no evidence supporting the hypothesis that environmentally-mediated differences in the microbiome determine *R. sierrae* populations persistence or decline due to chytridiomycosis. We speculate that host genetic differences may be more important than environmental drivers in determining *R. sierrae* population response to Bd infection.

This experiment also revealed differences in the overall composition of microbiomes of frogs housed in sterile compared with non-sterile aquatic environments. These results are consistent with a study of terrestrial salamanders (Loudon et al., 2013), in which maintaining salamanders in non-sterile soil compared with sterile liquid medium led to differences in skin bacterial communities. However, while Loudon *et al.* observed higher bacterial diversity on animals kept in non-sterile soil than those kept in sterile liquid medium, we found no difference in diversity between sterile and non-sterile aquatic environments. This divergence of our results from those of Loudon may reflect host species differences. It is also possible that differences in the Loudon *et al.* study reflect effects of both environment sterility and substrate structure (liquid medium compared with soil). In addition, in our experience microbiome diversity exhibits less consistent patterns than microbiome composition. For example, we found an effect of Bd infection on diversity during a controlled laboratory Bd

challenge experiment, but no correlation between diversity and Bd load among frogs in a given lake in the field (Jani and Briggs, 2014). Furthermore, the choice of diversity index can affect results: We found that the observed number of OTUs is affected by Bd infection, but other metrics of richness and diversity were unaffected. It is therefore difficult to draw clear conclusions from the difference in results regarding bacterial diversity in the current study compared with work in other systems.

In addition to affecting the *R. sierrae* microbiome, the experimental aquatic environment affected Bd infection trajectories. Increases in Bd loads were more rapid in frogs housed in sterile water than frogs housed in live lake water. Thus, the species pool present in the aquatic environment is linked to differences in the frog skin microbiome as well as downstream disease dynamics. None of the Water Source treatments conferred complete resistance to infection, but it is possible that under some conditions factors delaying the rate of increase of infection burdens may provide time for the host immune system to respond to the pathogen. Adaptive immune responses to Bd have been observed in Cuban treefrogs (*Osteopilus septentrionalis*, (McMahon et al., 2014), and current studies are examining adaptive immunity in *R. sierrae* (Toothman and Briggs in prep). As with differences in frog genetic background, the tentative conclusion of a cascade of effects from aquatic environment to skin microbiome to disease dynamics is only one possible interpretation of the data, and it is important to consider alternative explanations. For example, in addition to harboring different bacterial communities, water sources may vary in water chemistry, which may affect the *R. sierrae* microbiome. Another possible explanation for why Bd dynamics differed between live and sterile water treatments is that Bd survival in the aquatic habitat is directly affected by Water Sterility, which could lead to differences in the density of infective

zoospores in the aquatic environment, affecting the rate of Bd load increase on frogs. In the latter case, Water Sterility affects the frog microbiome and also independently affects disease dynamics, without there necessarily being a causal link between the frog microbiome and disease dynamics. Studies have found that grazing by aquatic crustaceans affects Bd zoospore densities (Hamilton et al., 2012; Kagami et al., 2014; Searle et al., 2013). In the current study, we filtered all macro-organisms from lake water, but bacteria present in the water may interact with Bd, and presence of organic matter, albeit minimal in these oligotrophic lakes, may provide resources for Bd growth or survival. Thus we cannot conclude with certainty that the differences in disease trajectories between live and sterile water treatments were caused by a cascade of effects from aquatic bacteria to frog microbiome to disease dynamics. Additional studies examining growth and survival of Bd in different aquatic environments in the absence of macrofauna may help clarify interpretation of our results.

Teasing apart cause and effect in correlations between Bd and the microbiome. Surveys of wild populations of *R. sierrae* and its close relative *Rana muscosa* have found correlations between population response to Bd (persistence or decline) and skin-associated bacterial communities (Chapter 2; Woodhams et al., 2007). However, Bd has also been shown to disturb the microbiome (Jani and Briggs, 2014). It is therefore impossible to determine from field surveys of infected populations whether correlations between bacteria and Bd load are due to variation in protective effects of bacteria or Bd-induced disturbance of the microbiome. In the current study, we used a controlled experiment to tease apart cause and effect. We found that the severity of Bd infection is significantly correlated with overall composition of the microbiome after Bd infection, but not before Bd infection. These results

indicate that in this experiment the effect of Bd infection on the microbiome was stronger than the effect of the microbiome on Bd infection. This result is consistent with the fact that, despite variation in rates of disease progression, all frogs in this study were susceptible to chytridiomycosis. Similar analyses comparing populations or species that show greater distinction in resistance to Bd would provide valuable additional insight to the role of the amphibian microbiome in disease resistance.

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TABLES AND FIGURES

Table 1. Sources of water used as treatments in the experiment. "Population response to Bd" indicates whether the *R. sierrae* population inhabiting each lake declined due to Bd or persists by co-existing with the pathogen.

Water Source	**Location**	**Population response to Bd**
Ebbetts Pass	Alpine County, CA	persisting
Mono Pass	Yosemite National Park, CA	persisting
Unicorn Pond	Yosemite National Park, CA	persisting
Barrett Lakes Basin	Kings Canyon National Park, CA	declined
Dusy Basin	Kings Canyon National Park, CA	declined
Humphreys Basin	Sierra National Forest, CA	declined
Sterile Water	NA	NA

Table 2. Description of variables included in statistical models. A given model included Frog Source and either Site Persistence or Water Sterility. Water Source was nested in Site Persistence or Water Sterility. Bd Treatment (Bd-infected or unexposed) was included in analyses of data collected after Bd infection.

Variable Name	Number of Levels	Description
Frog Source	2	Source population from which frogs in the experiment originated.
Water Source	7	Sterile water or one of six Sierra Nevada lakes from which water for experimental treatments was collected.
Site Persistence	2	Response to Bd (decline or persist) of the population that inhabited the lake from which water for this experiment was collected. The two levels each encompass three Water Sources (3 persisting lakes and 3 declining lakes).
Water Sterility	2	Sterile water or Lake Water. (Sterile Water is a single Water Source and Lake water encompasses 6 Water Sources)

Figure 1. Skin bacterial communities were significantly different between frogs from the two source populations prior to Bd infection (a) and differences persistend after Bd infection (b). Housing frogs in water collected from lakes inhabited by persisting or declining frog populations also led to differences in the microbiome, (c) and these differences persisted after bd infection (d). Plots are NMDS ordination of R. sierrae skin-associated bacterial communities sampled after 3 weeks exposure to experimental water treatments (left), and 3 weeks after Bd infection (right). Marker colors indicate Frog Source (top) or Site Persistence (bottom). NMDS stress: (a,b) 0.12; (c,d) 0.16.

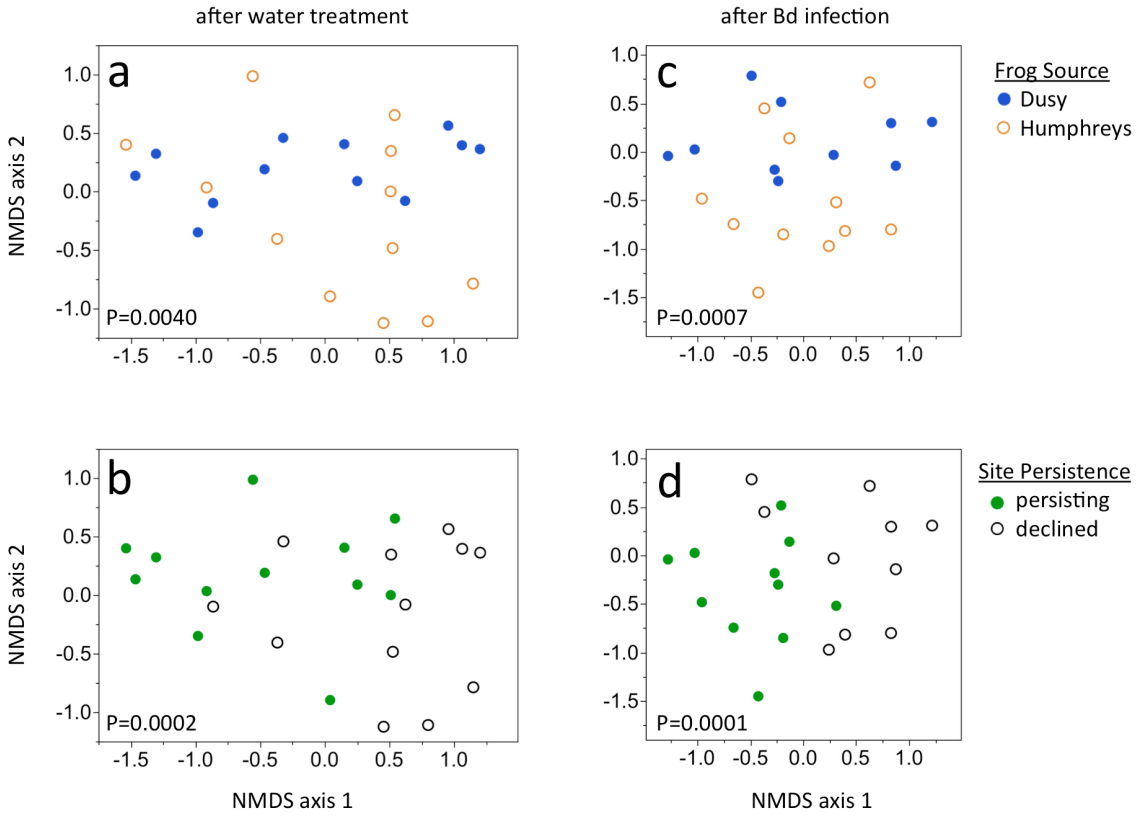


Figure 2. After 3 weeks of exposure to water treatments, bacterial diversity was slightly higher on *R. sierrae* housed in water that had been collected from lakes inhabited by persisting frog populations, compared with frogs housed in water collected from lakes inhabited by declining populations. $P<0.05$ for all four richness or diversity metrics.

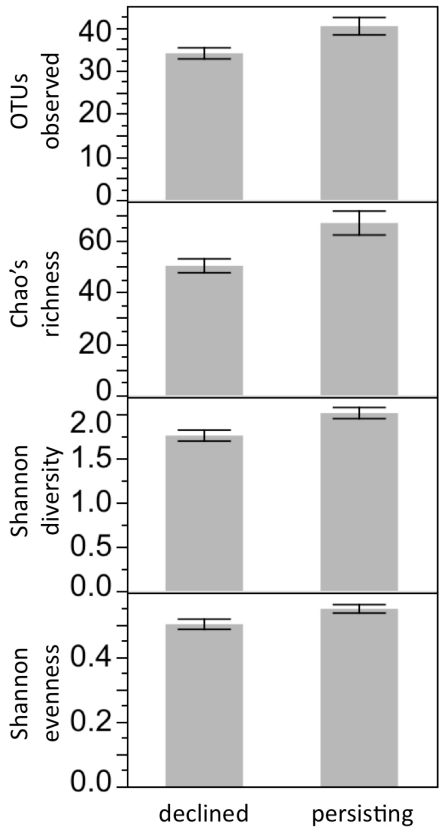


Figure 3. Skin bacterial communities differed between frogs housed in sterile water compared with frogs housed in live lake water after three weeks exposure to experimental water treatments (a). Differences persisted after Bd infection (b). Marker color indicates water treatmente (sterile or lake water). NMDS stress: (a) 0.09; (b) 0.16.

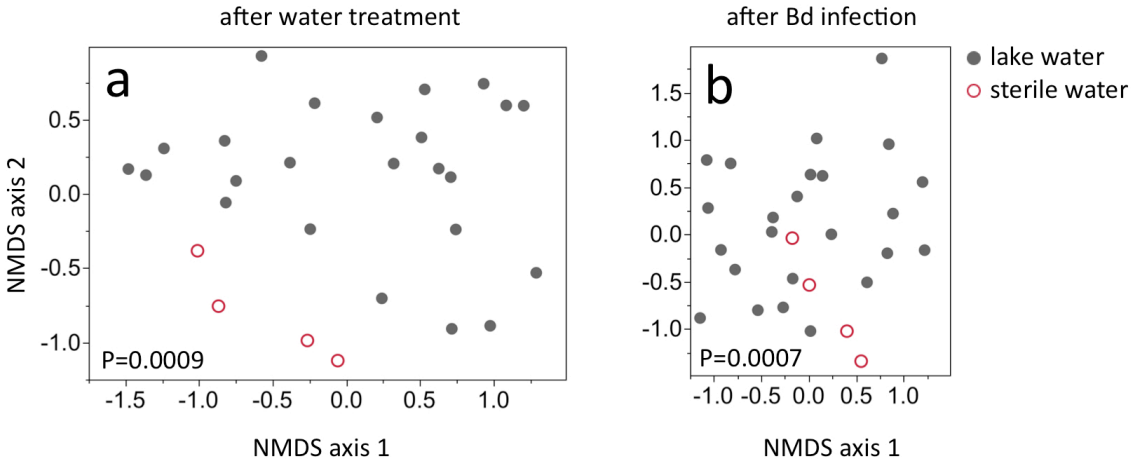
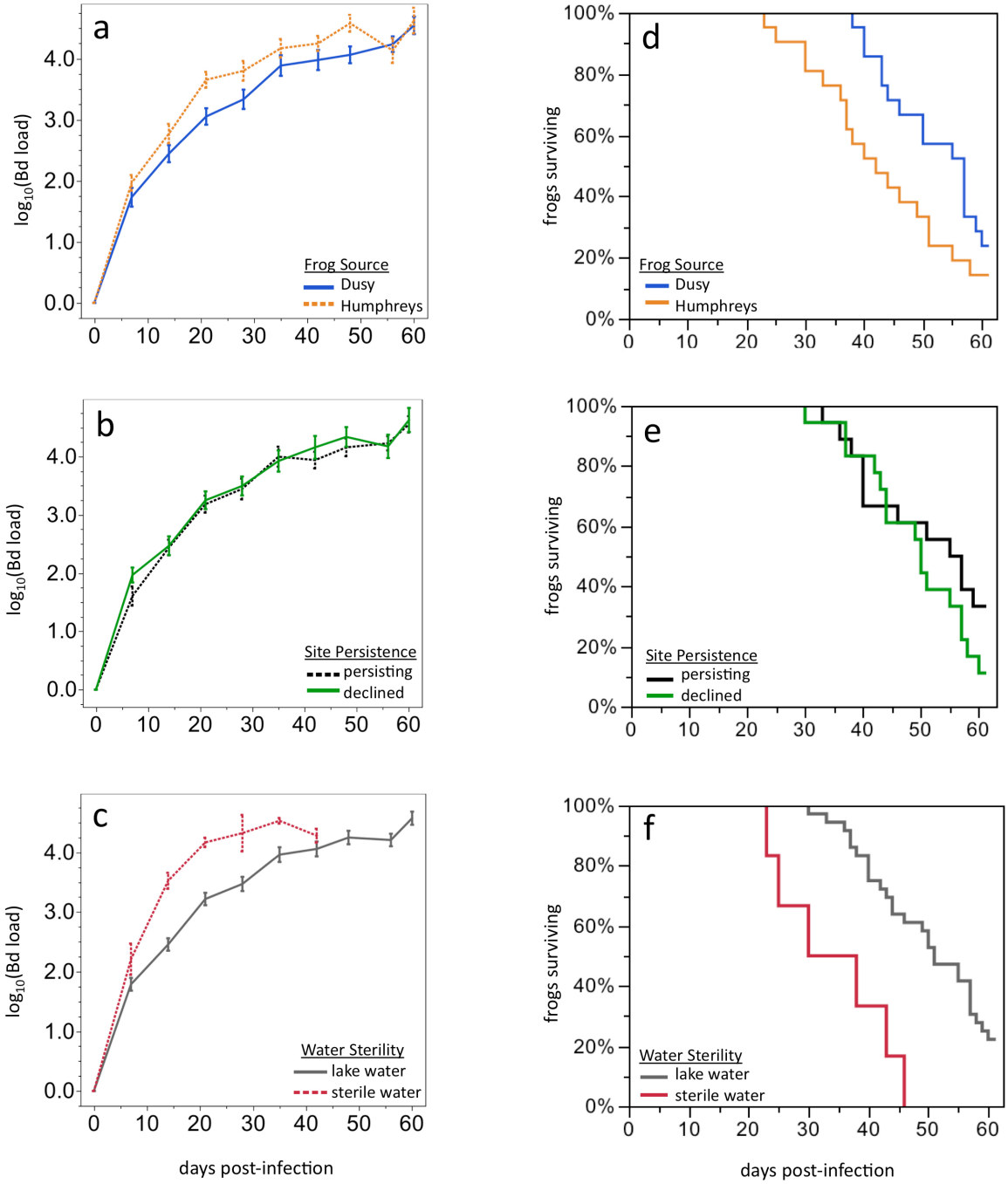


Figure 4. Bd load trajectories and survival of frogs, grouped by Frog Source (top), Site Persistence (middle), or Water Sterility (bottom). Rates of Bd load increase and survival curves were affected by Frog Source (a,d) and Water Sterility (c,f) but not Site Persistence (b,c). However, all frogs eventually developed high Bd loads.



SUPPLEMENTARY FIGURES

Figure S1. (a) Bd infection trajectories averaged across all Bd-exposed frogs compared with Bd-free control group. (b) Weight loss caused by Bd infection. (c) Survival curve for all Bd-exposed frogs compared with Bd-free control group.

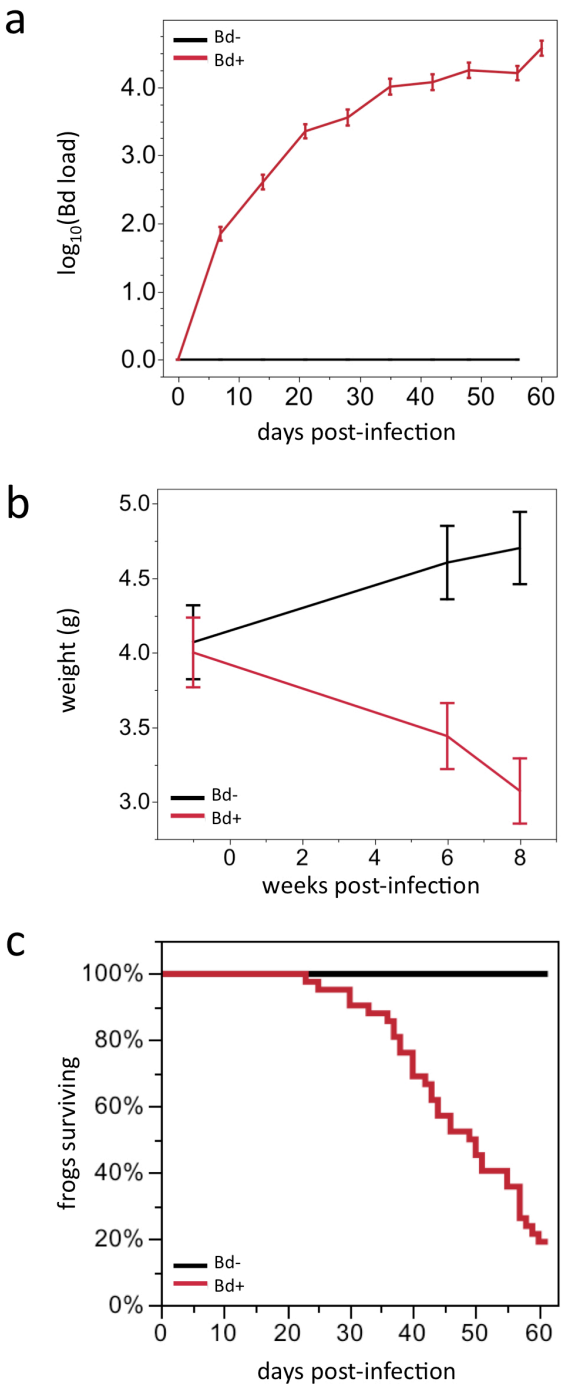


Figure S2. (a) Bd infection trajectories averaged across all Bd-exposed frogs compared with Bd-free control group. (b) Weight loss caused by Bd infection. (c) Survival curve for all Bd-exposed frogs compared with Bd-free control group.