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The pathogen *Batrachochytrium dendrobatidis* disturbs the frog skin microbiome during a natural epidemic and experimental infection

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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, whereas the laboratory and field results together strongly support Bd disturbance as a driver of bacterial community change during natural disease dynamics.

microbiome | disease ecology | *Batrachochytrium dendrobatidis* | chytridiomycosis | dysbiosis

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 (1–5). The microbiome can affect host health directly by influencing metabolism (6), development (7), inflammation (8), or behavior (9), but it may also influence host health indirectly through interactions with infectious pathogens. The microbiome may interact with pathogens through competition for resources, release of antimicrobial compounds, contact-dependent antagonism, or modulation of the host immune response (10), and an "imbalanced" microbiome may leave the host more susceptible to pathogen infection (11, 12). At the same time, an invading pathogen may disrupt the microbiome (10, 13–15). Thus, the microbiome may play a role in disease resistance, or may itself be disturbed or altered by invading pathogens. Although a wealth of recent research has described associations between microbiome composition and a variety of syndromes in both humans and animals (16–25), 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* (Bd). Bd is an aquatic fungus that infects the skin of amphibians and disrupts osmoregulation, a critical function of amphibian skin (26). 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 (27, 28). Bd has a broad host range spanning hundreds of amphibian species, and has been implicated in population extinctions and species declines worldwide (29–34). 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 (35, 36). Bacterial species isolated from the skin of amphibians have been shown to inhibit the growth of Bd and other fungal pathogens in culture (37–39), possibly by producing antifungal metabolites (40, 41). In a controlled laboratory experiment, inundation of *Rana muscosa* with the bacterium *Janthinobacterium lividium* protected frogs from subsequent Bd infection (42). 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, whereas very little is known about whether Bd infection alters the diverse skin microbiome. Examining

Significance

Animals are inhabited by communities of microbes (the microbiome) that potentially interact with pathogens. Detailed studies of microbiome–pathogen interactions in nature are rare, and even when correlations are observed, determining causal relationships is challenging. The microbiome–pathogen relationship is of particular interest in the case of *Batrachochytrium dendrobatidis*, a chytrid fungus that infects the skin of amphibians and is causing amphibian declines worldwide. We documented a strong correlation between pathogen load and skin bacterial communities of frogs during natural disease episodes. We then showed experimentally that infection alters the microbiome, with similar bacteria responding in both laboratory and field. The results indicate that the chytrid pathogen drives changes in the amphibian skin microbiome during disease episodes in wild frogs.

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Data deposition: The sequences reported in this paper have been deposited in the National Center for Biotechnology Information Sequence Read Archive, www.ncbi.nlm.nih.gov/sra (accession nos. SRR1598941, SRR1598942, SRR1598944).

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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 (43). Few studies have applied next-generation sequencing methods to characterize the microbial communities on amphibian skin (44–47), 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 the laboratory 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 the 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 (28, 48). 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.

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 rRNA gene amplicon pyrosequencing and quantitative PCR (qPCR), 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 laboratory, field, frogs, and water, are available in *SI Results* and Fig. 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, and 0.0012; proportion of variance explained: 0.37, 0.23, 0.30, and 0.26 for the Marmot, Mono, Unicorn, and Conness populations, respectively), and Mantel tests showed a significant correlation between the distance matrices of Bd loads and bacterial communities (P values: 0.0006, 0.0008, 0.0006, and 0.0023; Spearman's rank correlation coefficients: 0.49, 0.42, 0.37, and 0.31 for Marmot, Mono, Unicorn, and Conness populations, 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 nonmetric multidimensional scaling (NMDS) ordination axis 1 plotted against Bd load (Fig. 1). 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 OTUs 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 (Fig. 2). There was remarkable consistency in bacteria–Bd correlations among the four frog populations: Seven 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 seven cases (Fig. 2 *B* and *C*). In addition, examining both significant and nonsignificant 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 were available from multiple populations (Fig. 2*B*). There was also taxonomic consistency in the relationships between Bd and bacteria (Fig. 2*A*): OTUs that were negatively associated with Bd belonged primarily to the Betaproteobacteria, Gammaproteobacteria, or Actinobacteria, with a few representatives from the Acidobacteria and Alphaproteobacteria, whereas 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 laboratory studies (42), 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, and Conness) show enzootic Bd dynamics, characterized by moderate Bd loads and long-term frog population persistence despite Bd infection (27, 49). Census and infection data show these populations have been infected with Bd since 2004 or earlier, with no sign of population decline (27, 49). 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 mo) that resulted in catastrophic population decline, with no postmetamorphic 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 (Figs. 1 and 2).

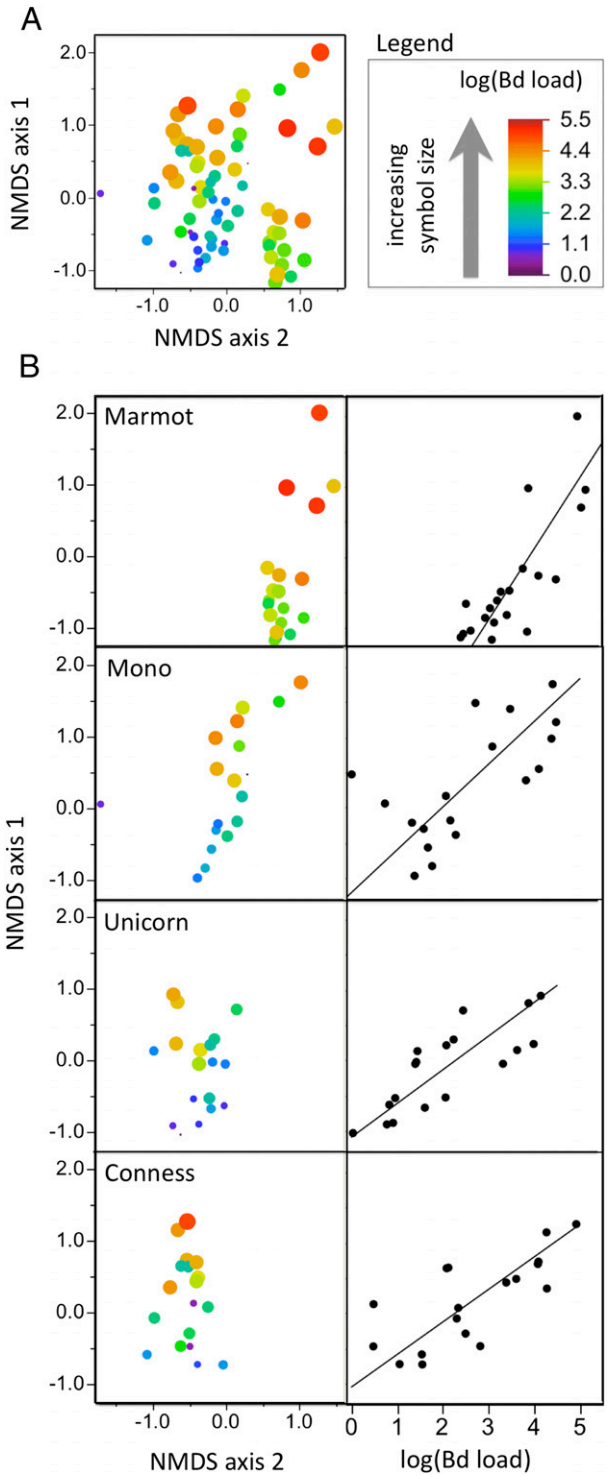

Fig. 1. Skin bacterial community composition covaries with Bd load in wild frogs. (*A*) NMDS ordination of bacterial communities from the four frog population visits (stress = 0.17, marker color and size indicate Bd load). (*B*) NMDS ordination from *A* separated by frog population visit (*Left*, labeled 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 and DISTLM) as reported in *Methods*.

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 were compared with 42 uninfected control animals both preinfection and 3 wk postinfection (PI). All animals in the Bd^+ treatment became infected, with Bd loads at 3 wk PI comparable to those Bd loads observed in field surveys [mean $\log_{10}$(Bd load) = 3.35, SD = 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, SE = 2.09; infected: mean = 33.5 OTUs, SE = 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 frogs relative to uninfected frogs (Table S2). There was strong concordance in the taxa affected by Bd infection in the experiment and the 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 eight genera showed significant effects in both the laboratory and field, and for all eight 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, whereas genera that were negatively correlated with Bd load in the field were more abundant on uninfected frogs in the experiment (Fig. 3 and Fig. 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 2-wk period (Table S1). We assessed the magnitude of temporal change in the microbiomes of frogs in the three populations over the same 2-wk 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 ($P = 0.0017$; Fig. 4*A*), whereas the two enzootic frog populations (Mono and Unicorn) showed no significant change in Bd loads over the same time period ($P = 0.7210$ and $P = 0.7841$, respectively). Bacterial

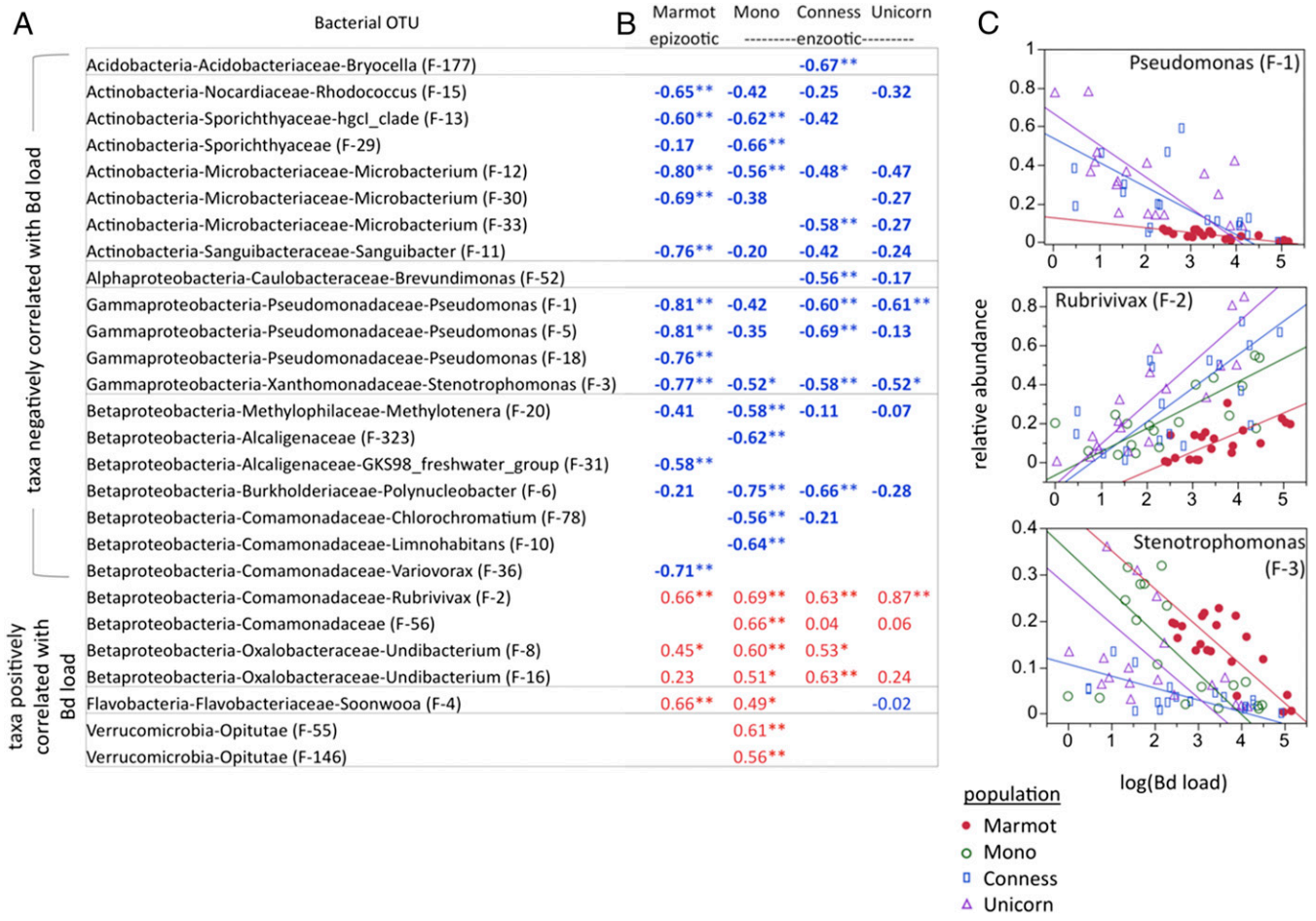

Fig. 2. Bacterial taxa show consistent associations with Bd load across frog populations. (*A*) Bacterial OTUs with negative (*Top*) vs. positive (*Bottom*) associations with Bd fall into distinct taxonomic groups (listed, with OTU identifiers in parentheses). (*B*) Bacterial associations with Bd are 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 one population. (*C*) Representative scatter plots 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. Scatter plots 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.

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 [Fig. 4 *B* and *C*; 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 ($P_{population} < 0.0001$, $P_{time} < 0.0001$), the interaction between time and frog population was significant ($P_{population \times time} = 0.0007$). 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 previously discussed field analyses, 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 were consistent with Bd-driven change. Specifically, OTUs that were negatively correlated with Bd (in analyses restricted to single sampling visits; Fig. 2) tended to decrease with time in the analysis of temporal change across two sampling visits within the outbreak population. Similarly, OTUs that were positively correlated with Bd (within sampling visits) tended to increase in time across two sampling visits [ANOVA with Tukey honestly significant difference (HSD) post hoc tests: $P < 0.05$; Fig. 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 (50), usually under laboratory

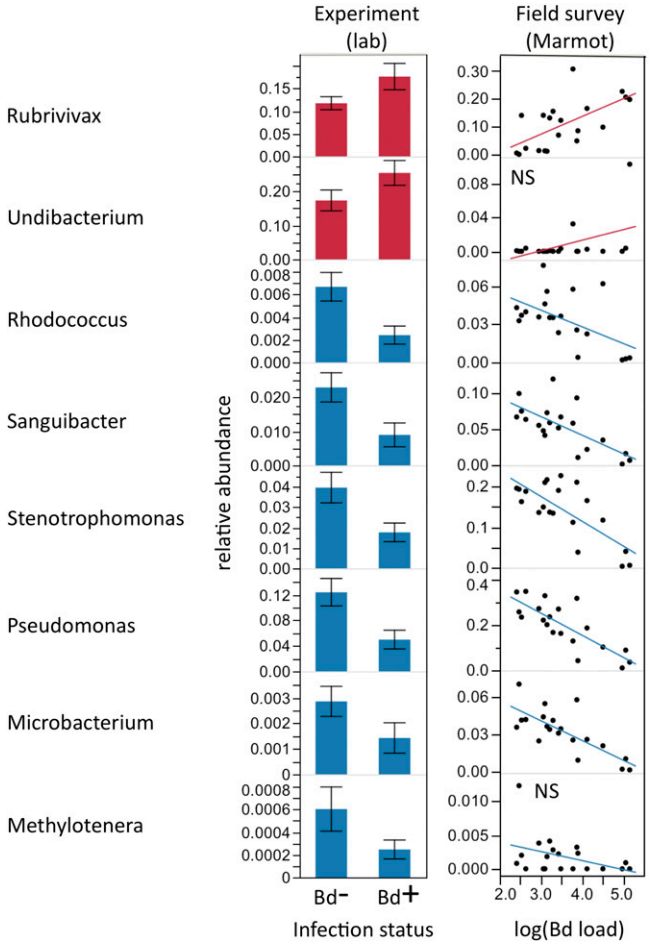

Fig. 3. Bd–bacterial relationships are consistent across the laboratory experiment and field surveys. (*Left*) Comparison of bacterial OTU mean relative abundances between Bd-infected and uninfected frogs in the laboratory experiment. (*Right*) Correlations between OTU relative abundance and Bd load in the 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 [not significant (NS)], all relationships are significant ($P < 0.05$ and $Q < 0.05$). For clarity, for each genus, only one representative OTU from the laboratory and one field population visit (Marmot sampled on August 30) is shown (complete results are provided in Fig. S2). The eight genera shown are those from which OTUs were significantly associated with Bd infection in the laboratory experiment and at least one field population. Relative abundances are proportions of the total sampled community. Error bars are SE.

conditions (13, 14, 42, 51, 52), but are rarely documented during natural outbreaks of known infectious pathogens in the wild. A few studies have demonstrated associations between pathogen infection and bacterial diversity (53) or the abundance of certain cultured isolates in the field (39), 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, as well as by 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 has 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, nonrandom 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 the 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 5 wk 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* is thought to succumb to chytridiomycosis (28), and by the following season, a complete population crash had ensued, with a census finding no postmetamorphic 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 the 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 (Fig. 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. Although 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 reconstruct accurately 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

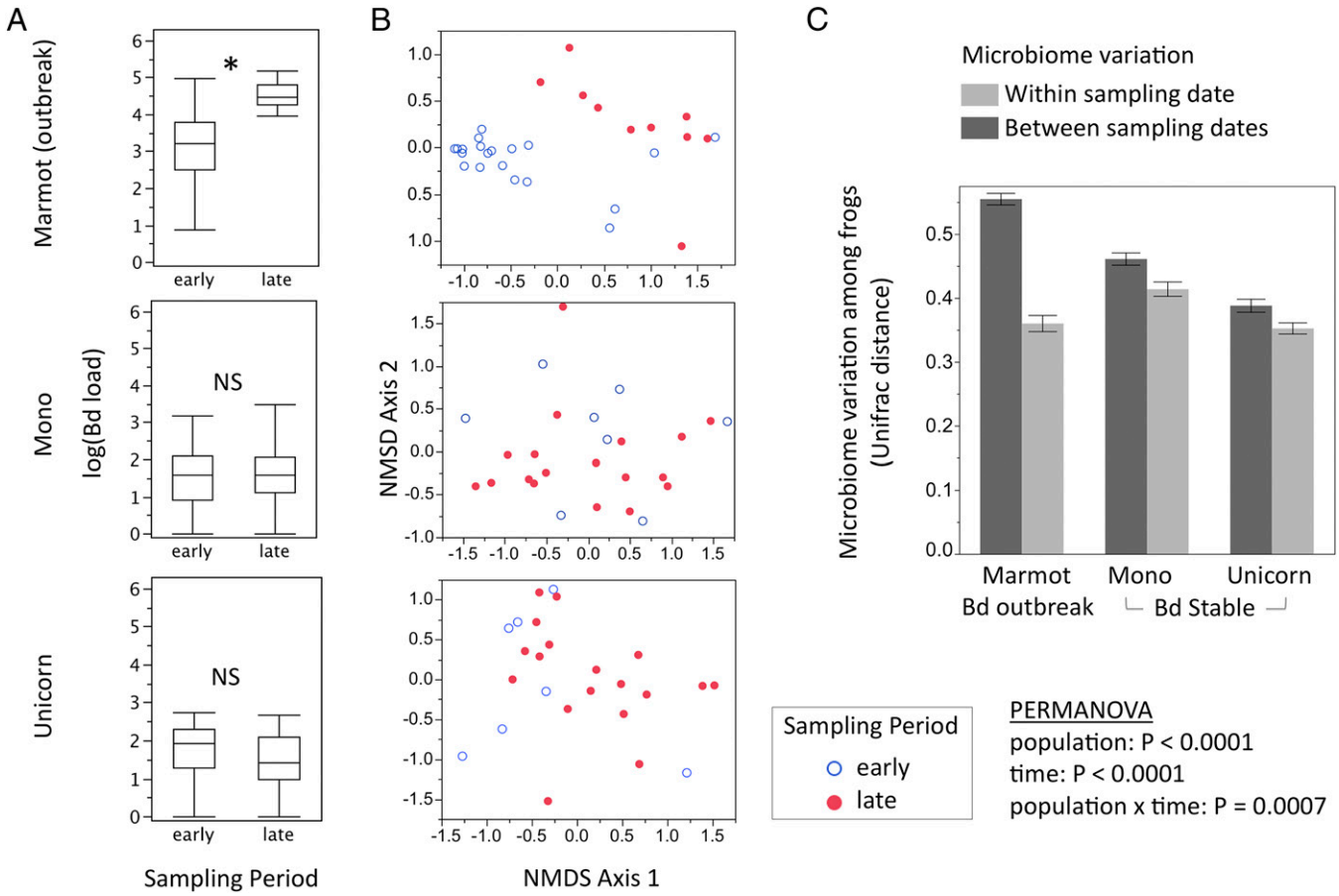

Fig. 4. Increased temporal change in skin microbiome during a Bd outbreak population relative to enzootic populations. (*A*) Bd loads increased through time in the epizootic population experiencing a Bd outbreak (Marmot; $P < 0.0001$; asterisk indicates statistical significance) but were stable in the enzootic populations (Mono and 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 population 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 d apart in each population, with all samples collected 29 August through 16 September 2010.

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 (Fig. 3). This consistency is remarkable given that overall microbiome composition differed between frogs in the laboratory and field (*SI Results* and Fig. 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 genera responded to Bd infection in both the field survey and laboratory experiment (Fig. 3). Bacteria in the genera *Rubrivivax* and *Undibacterium* responded positively to Bd infection in the laboratory and field. *Rubrivivax* are purple nonsulfur Betaproteobacteria in the widespread freshwater family Comamonadaceae that have been isolated from several environmental sources (54). Whereas 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 (55) and documented in shrimp intestines (56). *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 the relative abundance of *Janthinobacterium* and its relative

Undibacterium in response to Bd infection are interesting, given the previously documented protective effects of *Janthinobacterium* (42, 57), 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, and commensals, which have been found on the skin of amphibians in previous studies (39), sometimes at very high relative abundances comparable with the relative abundances observed in the current study (58). *Pseudomonas* species are commonly used in biological control in agriculture (4) and have been shown to have anti-Bd activity in laboratory tests (59), although isolates from *Rana cascadae*, a close relative of *R. sierrae*, showed no anti-Bd activity (58). 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 (60); are also known to cause nosocomial infections in human patients; and have been found to be resistant to broad-spectrum antibiotics (61). *Stenotrophomonas* species have been isolated from amphibian skin (39, 59), and an isolate from harlequin toads (*Atelopus elegans*) inhibited Bd growth in laboratory tests (59). *Sanguibacter*, *Rhodococcus*, and *Microbacterium* belong to the Actinomycetales, a group with important applications in human medicine and long appreciated for broad antimicrobial activity (62), often through the production of secondary metabolites, including polyketides, alkaloids, peptides, and terpenes (63). *Rhodococcus* and *Microbacterium* species isolated from marine sponges showed activity against diverse pathogens, including bacteria and trypanosomes (*Microbacterium*) and viruses and fungi (*Rhodococcus*) (63).

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 antimicrobial, 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 finding may indicate that the sensitivity of these taxa to Bd renders them ineffective at mediating Bd infection, 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 (64). Furthermore, expression of phenotypic traits of a given species varies with ecological contexts, such as the single-strain vs. whole-community context, culture media vs. amphibian host, or laboratory vs. field. This diversity and context dependence may help explain why augmentation with *Janthinobacterium* isolates has been found to increase amphibian resistance to Bd infection in some contexts (42, 57) but failed to protect amphibians in another study (65).

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 (44–46), 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 et al. (58) tested for differences in cultured bacterial isolates between infected and uninfected *R. cascadae* and found no effect, although differences between the findings of Roth et al. (58) and the current study may be affected by methods (culture-based vs. pyrosequencing) or, potentially, differences in severity of infection. [Roth et al. (58) report binary Bd infection rather than Bd load, but another study found relatively low loads in *R. cascadae* (66), 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 (67), 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, whereas enzootic populations remain stable despite infection (27, 28). We found that many of the same bacterial taxa are correlated with Bd load during enzootic and epizootic Bd dynamics (Fig. 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 finding 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 preexisting 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 (Mantel test, $R = 0.42$, 0.37, and 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 finding 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.

Methods

Field Surveys. This study was conducted in four *R. sierrae* populations in the Sierra Nevada of California (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 (27, 49). 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, and 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 postmetamorphic (i.e., subadult, 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 (27). 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 = 1,198 mL, range: 560–2,000 mL) through 0.22-μm pore polyethersulfone filters (Sterivex-GP; Millipore), which were immediately purged of remaining water and amended with 1.5 mL of sucrose lysis buffer [40 mmol·L^{-1} EDTA, 50 mmol·L^{-1} Tris·HCl, and 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 (68). 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 to 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 = 8–10 per population; Table S1).

DNA Extraction. Swab DNA was prepared for PCR using Prepman Ultra (Life Technologies) as described previously (27, 69). Briefly, each swab was incubated with 40 μL of Prepman Ultra at 95 °C for 10 min and centrifuged (3 min, 16,000 × g), and the supernatant was collected for use in PCR. Genomic DNA was extracted from water filter cartridges following the method of Nelson (68).

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 (27), and was measured by qPCR following the methods of Boyle et al. (69). 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 rRNA gene amplicon pyrosequencing as detailed in *SI 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 instrument using Titanium chemistry. The program mothur (version 1.30) (70) was used to quality-filter (denoise and screen for short, potentially low-quality, or chimeric reads) sequences, align them to a curated 16S alignment database (71), and cluster them into OTUs. Sequences were classified using the Bayesian classifier of Wang et al. (72), and each OTU was assigned a consensus taxonomy from SILVA version 111. Pairwise phylogenetic community distances [weighted UniFrac (73)] among all samples were calculated based on OTU relative abundances. Measures of bacterial richness and diversity [observed number of OTUs, Chao's estimated richness (74), and Shannon diversity and evenness] were calculated after randomly subsampling to 500 sequences per sample. Pyrosequencing runs have been deposited in the National Center for Biotechnology Information Sequence Read Archive (www.ncbi.nlm.nih.gov/sra) under accession numbers SRR1598941, SRR1598942, and SRR1598944; primer barcodes are listed in Tables S4 and S5.

Statistical Analyses. Multivariate analyses, including NMDS, ANOSIM, PERMANOVA, DISTLM, and Mantel tests, were conducted in Primer-E version 6 (PRIMER-E) (75). Data were transformed as needed to approximate a Gaussian distribution for parametric statistical analyses: $\log_{10}(X+1)$ for Bd load data and arcsine of square root for bacterial relative abundance data. In the figures, OTU relative abundance data are shown untransformed to provide an 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 (76) and applying a maximum threshold of Q = 0.05 (77). All other statistical analyses were performed using JMP version 10 (SAS Institute, Inc.).

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 2D space using 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 for categorical variables. To provide visual representation of multivariate relationships, where relevant, we fit orthogonal regression lines to scatter plots 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 the 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 homogeneously 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 d 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, although 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 *SI 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 were 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, whereas 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 Institutional Animal Care and Use Committee of the University of California, Santa Barbara, before beginning the study. For 2 wk before 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 Plexiglas 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 (one 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 nonsterile 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. Before 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, before adding lake water to tanks, bacteria present in lake water were sampled by filtration of 250 mL through a 0.22-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 3-wk acclimation period, half of the frogs were challenged with Bd (three doses of 200,000 zoospores, consisting of an equal mixture of four Bd strains isolated from wild *R. sierrae*: TST75, CJB4, CJB5, and 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 d postinfection (PI). A new pair of nitrile gloves was used to handle each frog, and frogs were rinsed twice with 60 mL of 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 h of collection. Bd loads were quantified from swab extracts by qPCR, 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 before Bd inoculation and 3 wk PI; the 3-wk PI time point was chosen because loads at that time point were comparable to Bd loads observed in the field). qPCR, 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 wk PI, 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 preinfection 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 among *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 min (78) or, in the case of small or sensitive equipment, disinfected with 70% ethanol.

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