

ORIGINAL ARTICLE

Environmental fluctuations and host skin bacteria shift survival advantage between frogs and their fungal pathogen

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Fluctuating environments can modulate host–pathogen interactions by providing a temporary advantage to one of the interacting organisms. However, we know very little about how environmental conditions facilitate beneficial interactions between hosts and their microbial communities, resulting in individual persistence with a particular pathogen. Here, we experimentally infected *Eleutherodactylus coqui* frogs with the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*) under environmental conditions known to confer the survival advantage to the host during the warm-wet season, or alternatively to the pathogen during the cool-dry season. We used 16S rRNA amplicon sequencing to quantify changes in bacterial richness and phylogenetic diversity, and identified operational taxonomic units (OTUs) that became overrepresented or suppressed as a consequence of *Bd* infection. During the warm-wet season, frogs limited *Bd* infections, recruited putatively beneficial bacteria and returned to pre-infection levels of richness and phylogenetic diversity. In contrast, during the cool-dry season, *Bd* infections kept increasing through time, and bacterial diversity remained constant. Our findings confirm that infection outcome not only depends on abiotic factors, but also on biotic interactions between hosts and their associated bacterial communities.

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Introduction

Fungal pathogens currently pose a global emerging threat to plants, animals, and ecosystems (Fisher *et al.*, 2012). These pathogens are extremely effective at colonizing external surfaces (for example, integuments, epidermis, skin), which are already inhabited by other microorganisms. Bacteria can affect fungal colonization, survival, growth, and virulence by altering physiological and chemical processes including inhibition of colonization sites (Franks *et al.*, 2006), regulation of nutrient availability, production of antifungal compounds (Brucker *et al.*, 2008), or by providing precursors for fungal virulence factors (Wargo and Hogan, 2006). Furthermore, host external surfaces are continuously exposed to changes in abiotic factors, providing an additional source of selection that may act differently for bacteria and

fungi. Therefore, quantifying fungal–bacterial interactions—and considering fluctuations in environmental conditions—has the potential to advance our understanding of infection outcomes that range from full disease resistance to mortality.

The relationship between the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) and skin microbial communities in amphibians provides an excellent system to test the strength and direction of fungal–bacterial interactions. *Bd* is a pathogenic fungus that infects the stratum corneum and deeper cell layers of amphibian skin (Greenspan *et al.*, 2012), disrupting osmotic balance, and often causing host mortality (Voyles *et al.*, 2009). Epibiotic bacteria also inhabit the outer layers of the skin, and often accumulate around secretory glands (Lauer *et al.*, 2007). Over the last few years, we have seen a rise in studies quantifying the effects of *Bd* infection on skin microbiomes (Jani and Briggs, 2014; Becker *et al.*, 2015; Longo *et al.*, 2015; Walke *et al.*, 2015). Fungal infection disrupts the stability of bacterial communities in susceptible amphibians in both field and lab (Jani and Briggs, 2014; Longo *et al.*, 2015). However, bacterial community structure before infection may also determine host survival; frogs with the ability to clear *Bd* infection carried a different bacterial community compared with individuals that died (Becker *et al.*, 2015). Microbial communities of these *Bd*-resistant frogs did not exhibit any variation in

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structure, suggesting that stability provide resistance against *Bd* (Becker *et al.*, 2015). However, unaccounted factors such as abiotic environmental fluctuations, seasonal environmental bacterial reservoirs and the standing host bacterial assemblage at time of infection, might also underlie differences in microbial communities. Therefore, to achieve a better understanding of disease as a disturbance process at the individual-level and how it translates into recovery at the population-level, we need to quantify *Bd*–microbiome interactions under variable and biologically realistic scenarios as would be experienced by wild amphibian populations.

In this study, we measured beneficial and antagonistic interactions between fungi and bacteria using field mesocosm experiments. Mesocosms offer the advantage of exposing hosts to natural conditions (that is, variable daily temperatures and precipitation, changes in shade, wind) that might differentially affect fungal infection and microbial communities. Considering the environmental heterogeneity that amphibians experience in their natural habitats, experimental inoculations in the lab may render an unrealistic turnover of microbial species. It is not surprising that skin microbiomes of wild-caught amphibians experience large shifts in community structure and composition when brought to the lab (Loudon *et al.*, 2013; Becker *et al.*, 2014), especially because in the lab they lack the environmental reservoirs that permit natural microbial recruitment (Loudon *et al.*, 2013; Walke *et al.*, 2014), thus promoting the establishment of skin bacterial communities very different to those originally found in nature.

We performed controlled inoculations in which hosts were exposed to *Bd* under natural field conditions, thereby minimizing potential artificial selection of bacteria. We challenged amphibians under conditions known to confer the advantage to the host—or alternatively to the pathogen (Longo *et al.*, 2010), by performing the experiment over two seasons. During the warm-wet season, host immune function is at its maximum (Maniero and Carey, 1997; Raffel *et al.*, 2006), beneficial symbionts are likely present and abundant on the amphibian skin and infection risk is low (Longo *et al.*, 2010). Thus, we predict that these three factors will promote resistance strategies (reduced *Bd* load), resulting in rapid recovery after infection. As both temperature and precipitation decrease during the cool-dry season, the advantage shifts to the pathogen. Cool temperatures promote the release of zoospores from sporangia (Woodhams *et al.*, 2008), host immune function is reduced (Maniero and Carey, 1997; Raffel *et al.*, 2006) and beneficial symbionts are less common or lost. At these stressful times, we predict that hosts will not be able to control infections, and consequently die with high infection intensities (Longo *et al.*, 2013). Understanding how the environment modulates these interactions is crucial to explain why some amphibians can persist with *Bd*

infection, yet cannot fully recover after disease-mediated declines.

Materials and methods

Experimental design

We captured a total of 98 adult *Eleutherodactylus coqui* at El Yunque National Forest (18.299°N, 65.779°W, 649 m) during the warm-wet and cool-dry season (see Supplementary Table S1 for sample sizes and dates). We placed frogs in individual plastic bags and determined sex, body size (snout-vent-length, SVL in mm) and weight (g). Using new pairs of gloves for each individual, we swabbed frogs using a standard number of strokes (five times per foot, each side of the belly, and drink patch, for a total of 40 strokes). Swabs remained frozen at -20°C until DNA extraction.

In the field, we housed each frog individually in a perforated 23-cm² plastic mesh basket (HydroFarm, Petaluma, CA, USA) that was bleached before the experiment. Lids consisted of tulle fabric and fastened with antimicrobial rubber bands (Alliance Rubber Company, Hot Springs, AR, USA; size 117B). Inside the plastic basket (Supplementary Figure S1), we added dried leaves of *Prestoea montana*, a common palm found in the forest that is used by frogs as a calling and retreat site (Beard *et al.*, 2003). We moistened the leaves in each unit by spraying with sterile distilled water. All leaves were collected from a single palm at the location of the experiment. Experimental containers were kept together under the tree canopy on the forest floor in a 10 m² area.

Because we did not know the infection status of each individual frog on collection, our experimental design tests for the effect of *Bd* addition on natural skin bacterial communities. We designed a fully factorial mesocosm experiment with two factors (*Bd* and skin bacterial communities) and two levels each (manipulation and no manipulation), and assigned individuals randomly to experimental treatments. To manipulate skin bacterial communities, we applied a 1.5% hydrogen peroxide (HP) bath for 1 min, followed by rinsing with sterile distilled water. This procedure artificially reduces the abundance of skin microbes without hindering host health (Becker and Harris, 2010; Muletz *et al.*, 2012). To manipulate *Bd* infection rates, we cultured *Bd* JEL427 in 1% tryptone agar plates and collected zoospores for use in the inoculation experiments. This strain of *Bd* was isolated from an infected *E. coqui* collected at the same locality in 2004. We experimentally infected individuals with 10^6 zoospores in a total of 5 ml in sterile sample containers for 24hrs (Cole Parmer, Vernon Hills, IL, USA; EW-060490-40). Frogs in the *Bd*– treatments were sham-infected with sterile distilled water. Thus, our four experimental treatments consisted of: *Bd* addition with reduced bacterial community manipulation (*Bd*+/*HP*+), *Bd* addition with intact bacterial community (*Bd*+/*HP*–),

no *Bd* added but bacterial community manipulated (*Bd*–/HP+) and negative controls with no *Bd* infection or bacterial community manipulation (*Bd*–/HP–). We swabbed and weighed each frog every 2 days post-inoculation for 2 weeks using the same procedure described above. We recorded any instances of individuals showing the typical signs of chytridiomycosis, including lethargy, inappetence, changes in skin coloration or sloughing, abnormal postures and loss of righting reflex (Voyles *et al.*, 2009). Skin sloughing was the most obvious sign of infection when swabbing (Supplementary Figure S2), thus we considered it as a binary predictor in our statistical analyses.

Frogs remained in enclosures for 15 days and were fed crickets every 2 days after data collection and swabbing. Our experimental design captures the bacterial community in its entirety, and does not differentiate between transient and resident bacteria contributed by environmental sources. We performed the experiment two times: once during the warm-wet season (November 2014) and once during the cool-dry season (January 2015). During November 2014, the average temperature was 23.4 °C (min. 17.2 °C, max. 29.4 °C) and total precipitation was 349 mm. In contrast, the average temperature in January 2015 was 22 °C (min. 18.3 °C, max. 26.7 °C) and total precipitation was 163 mm (Palma Sola Station, NOAA Online Weather Data). The Institutional Animal Care and Use Committee of Cornell University (2013-0074), Puerto Rico's Department of Natural Resources (2014-IC-74), and the US Forest Service approved experimental animal use. Frogs were euthanized at the end of the experiment, and archived at the Cornell University Museum of Vertebrates.

Quantification of *Bd* infection intensity

We extracted DNA from swabs using the MO BIO Power Soil DNA isolation kit (MO BIO, Carlsbad, CA, USA). All DNA extraction plates contained negative controls (blanks). To determine each individual's infection intensity, we used standard methods of quantitative PCR (qPCR) for *Bd* diagnosis (Boyle *et al.*, 2004) and ran duplicate qPCR reactions for each sample using a Viiia7 qPCR machine (Life Technologies, Carlsbad, CA, USA). We used *Bd* isolate JEL427 to generate the standard curve of zoospores for the qPCR runs. Individual infection intensity was measured as the mean number of zoospore genomic equivalents estimated by qPCR from the two well reactions.

16S rRNA amplicon sequencing

We followed the Earth Microbiome Project protocol to quantify diversity of bacterial communities from skin swabs (Caporaso *et al.*, 2012). Briefly, we PCR-amplified the V4 region of the 16S rRNA gene using universal primers 515F and 806R. We ran reactions

in triplicate plus a negative water control. Amplicons were visualized in 2% agarose gel, pooled, and quantified using QuantiFluor (Promega, Madison, WI, USA). Equimolar quantities from each sample (100 ng) were combined to generate the amplicon library, and then cleaned using Agencourt AMPure XP beads (Beckman Coulter, Brea, CA, USA). We prepared three libraries for Illumina MiSeq (2 × 250 bp) sequencing at the Biotechnology Research Center (BRC) Genomics Facility at Cornell University.

Sequence processing and OTU assignment

We used Quantitative Insights into Microbial Ecology v 1.9.0 (QIIME) to split libraries and assign sequences into operational taxonomic units (OTUs) (Caporaso *et al.*, 2010b). We assigned OTUs using the subsampled open reference protocol in QIIME, which clusters sequences against the Greengenes database (May 2013) using uclust (DeSantis *et al.*, 2006; Edgar, 2010). Briefly, 0.1% of the reads that did not match the Greengenes database are subsampled and clustered *de novo* (Caporaso *et al.*, 2010a; Rideout *et al.*, 2014). We excluded OTUs with fewer than 100 reads (Bokulich *et al.*, 2013; Loudon *et al.*, 2013), removed samples with less than 10 000 reads in coverage and normalized the OTU table using cumulative sum scaling in QIIME (McMurdie and Holmes, 2014). The number of reads in the remaining samples ranged from 10 030 to 273 016 (average: 49 510). Raw reads have been deposited in the NCBI Sequence Read Archive (SRR4010786).

Statistical analyses of *Bd* infection intensity and alpha diversity of bacteria

We log-transformed *Bd* infection intensity [$\log_{10}(x+1)$] for all analyses performed in R version 3.2.3 (R Core Team, 2014). To determine differences in infection at baseline (day 0), we compared initial *Bd* prevalence and intensity between seasons by using a χ^2 test of independence and a *t*-test, respectively. We used three separate linear mixed models to estimate changes in *Bd* infection intensity, OTU richness and phylogenetic diversity over time for each treatment, and treated individual ID as a random effect. We used R package lme4 (Bates *et al.*, 2014) to design the mixed models with the general equation: $lmer(y \sim \text{treatment} + \text{day} + \text{season} + \text{skin_slough} + \text{weight} + \text{initial_load} + (y+1) + \text{treatment} * \text{day} + \text{treatment} * \text{season} + \text{day} * \text{season} + \text{treatment} * \text{slough} + \text{day} * \text{slough} + \text{season} * \text{slough} + (1 | \text{individual_ID}))$. For these models, we were interested in understanding the interactions of treatment, day and season effects, which could provide insights into the progression of the infection under different environmental scenarios. We used individual-level variables such as weight and evidence of skin sloughing (binary coded) to understand changes in host responses through time. We took into account previous exposure of each individual by

incorporating their initial load (infection intensity, richness, or phylogenetic diversity) at day 0 as a fixed effect. We calculated least squares means, confidence intervals and Tukey's comparisons for the fixed effects and interactions using package lsmeans in R (Lenth and Hervé, 2016).

To determine if host ability to respond to infection changed between seasons, we compared the relative change in infection intensity between two stages (early vs. late). We considered their average infection intensity during days 0 to 3 as the early stage, and days 6 to 15 as the late stage. Individuals showing a positive response (early–late > 0), possibly developed the infection, and were able to clear or reduce it. In contrast, individuals showing a negative response (early–late < 0) were unable to control infections by the end of the experiment. We used a χ^2 test to determine whether seasons were associated to the ability to clear/reduce infection, remain infected or uninfected.

Differential abundance of bacteria

For the analyses of differential bacterial abundance, we filtered our OTU table to include only samples for which individual frogs persisted throughout the entire experiment (73 frogs \times 6 sampling days (1 initial + 5 post-inoculation) = 438 samples). By doing this filtering step, we avoided including effects related to host mortality. We used R packages phyloseq and DESeq2 (McMurdie and Holmes, 2013; Love *et al.*, 2014) to import the OTU table to the R environment and identify differences in abundance of bacterial taxa between treatment groups with respect to the negative controls (*Bd*–/HP–). We calculated the geometric means of bacterial abundance counts before estimating size factors. Our design in DESeq2 tested the effects of day, treatment, season, *Bd* infection intensity, interactions between treatment*day, season*day and treatment*season on the abundance of each bacterial OTU. Specifically, our objective was to answer three questions about the most common host-associated bacteria: (1) which bacterial taxa (if any) were significantly over abundant (or suppressed) when comparing between wet and dry seasons? (2) which OTUs increased or decreased in abundance after the addition of *Bd*, clearing of bacteria (HP+) or both and (3) do these responses depend on the season? For these analyses, we used an alpha of 0.01 to decrease Type I error. We filtered taxa using this cutoff in the adjusted *P*-values, and narrowed our focus to the top 30 most abundant taxa based on mean number of reads.

Changes in beta diversity of bacteria

We measured changes in beta diversity by comparing skin bacterial communities using unweighted and weighted Unifrac distances, as well as Bray–Curtis distances. We used function *adonis* in

R package *vegan* (Oksanen *et al.*, 2015) to perform permutational multivariate analyses of variance (PERMANOVA). These analyses tested for differences in bacterial community structure across seasons, treatments and *Bd* infection status at three time points (initial: Day 0, after inoculation: Day 3, and end of experiment: Day 15). We report changes in beta diversity over time using a detrended correspondence analysis plot of unweighted-Unifrac distances.

Results

Interactions between time, season and, skin sloughing determined *Bd* infection intensity

At capture, *Bd* prevalence among individuals was similar across seasons (wet: 50% vs dry: 46%, $\chi^2 = 0.157$, *df* = 1, *P* = 0.692, Supplementary Table S1), but *Bd* infection intensity was significantly higher during the cool-dry season (*t* = 2.67, *df* = 93, *P* < 0.01, Supplementary Table S1), confirming previous findings of seasonal patterns of infection (Longo *et al.*, 2010). Our mixed model of *Bd* infection intensity revealed significant effects of all interaction terms except treatment*season, which indicated that treatment effects did not vary between the two experimental trials (Supplementary Table S2; Supplementary Figure S3). As expected after inoculation, individuals in *Bd*+ treatments had higher *Bd* infection intensities than individuals in *Bd*– treatments (Figure 1a; Supplementary Table S3). However, inoculation with *Bd* and application of the HP bath (*Bd*+/HP+ treatment) did not augment *Bd* infection intensity as predicted if bacterial communities buffered pathogen growth compared with *Bd*+/HP– treatments (day 0 to day 3: $\beta = 0.20$, *df* = 113.33, *t* = 0.822, *P* = 0.84, Figure 1a). Furthermore, HP did not increase or decrease average infection intensity in control treatments (*Bd*–/HP+ vs *Bd*–/HP–, *P* > 0.05, Supplementary Table S3). The interaction term day*season revealed that average *Bd* infection intensity showed fluctuations through time that depended on seasons (Figure 2a). More specifically, frogs maintained relatively constant infection intensities through time only during the warm-wet season, as shown by the lack of significance among daily pairwise comparisons (Figure 2a; Supplementary Table S3). In contrast, during the cool-dry season *Bd* infection intensity increased significantly over time and peaked at day 12 (Figure 2a; Supplementary Table S3).

Changes in *Bd* infection intensity were also correlated with the magnitude of skin sloughing, which varied within treatments, seasons, and with time (all interactions *P* < 0.04, Supplementary Figure S4; Supplementary Table S2). Consistent with the signs of chytridiomycosis, the proportion of individuals with skin sloughing was significantly higher in *Bd*+ treatments and during the cool-dry season (Supplementary Figure S4). However, skin

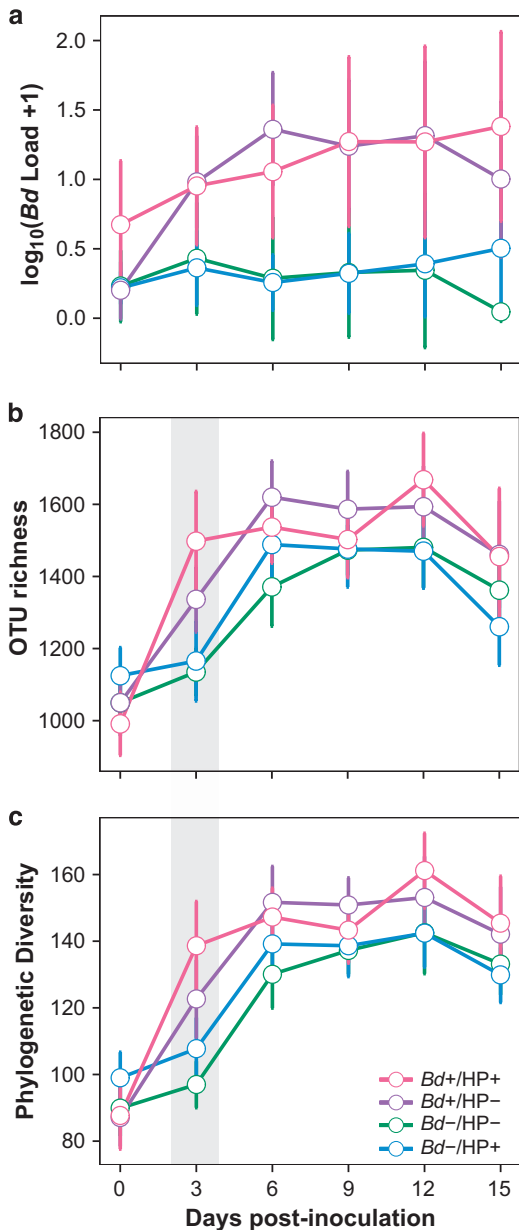


Figure 1 Significant interactions between treatment and time in: (a) *Bd* infection intensity, (b) OTU richness and (c) phylogenetic diversity of skin bacterial communities. Circles represent the mean of each treatment by day, lines across the mean represent the 95% confidence intervals. Significance for the interaction terms is available in Supplementary Tables S2–S5. Gray shading indicates significant differences between *Bd*+ and *Bd*- treatments.

sloughing did not reduce pathogen growth, because individuals with this clinical sign had significantly higher infection intensities in *Bd*+ treatments ($P < 0.001$, Figure 3; Supplementary Table S2) and in particular on day 12 ($\beta = -1.12$, $df = 313.67$, $t = -3.428$, $P < 0.001$).

Our data showed that previous exposure to *Bd* was positively associated with the average *Bd* infection intensities during the experiment (Figure 4; Supplementary Table S2). Therefore, infection status at the beginning of the experiment predicted host

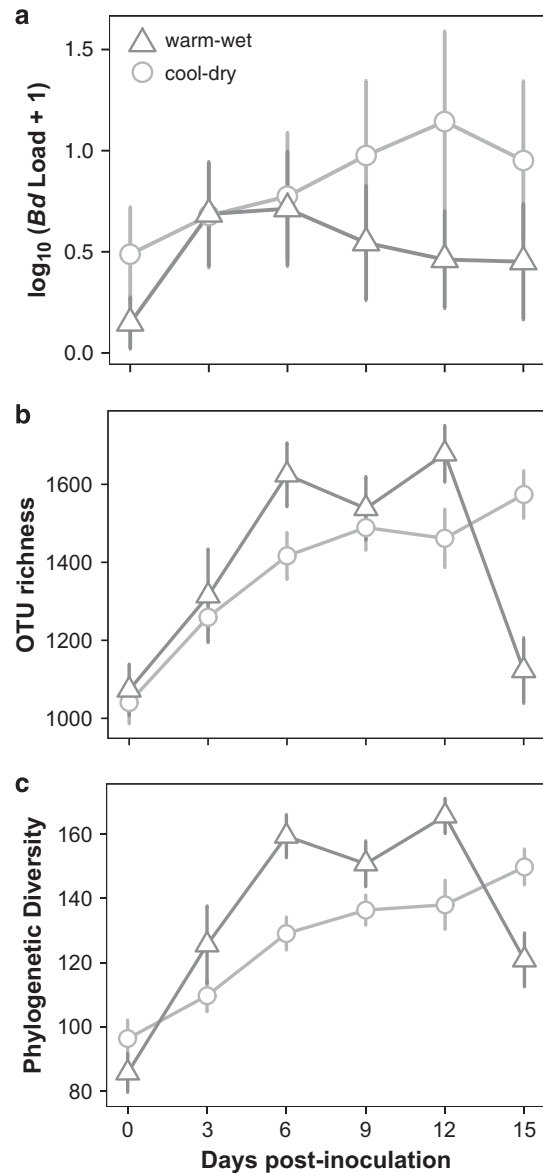


Figure 2 Significant interactions between season and time in: (a) *Bd* infection intensity, (b) OTU richness and (c) phylogenetic diversity of skin bacterial communities. Each circle represents the mean (across all treatments) for the cool-dry season. Triangles denote the mean for the warm-wet season. Lines represent the 95% confidence intervals. Significance for the interaction terms is available in Supplementary Tables S2–S5.

responses to infection. During the warm-wet season, a significantly higher proportion of frogs cleared or reduced the infection at earlier stages of the experiment (Figure 5), indicating that hosts were favored during this season. In contrast, this advantage shifted to the pathogen during the cool-dry season, and 64% of frogs showed negative responses to infection (Figure 5).

Only six individuals died during the course of the experiment (1 in warm-wet season and 5 in cool-dry season, Supplementary Table S4). All of them were infected during the experiment, including several individuals in the *Bd*-treatments (Supplementary

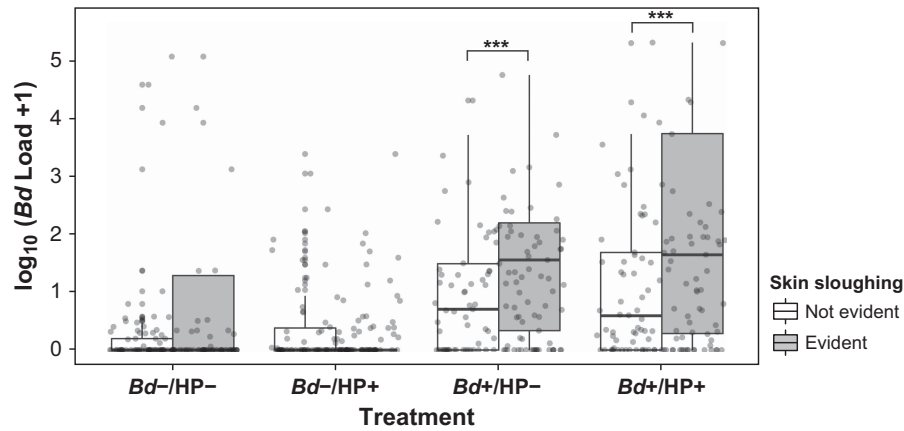


Figure 3 Boxplot of $\log_{10}(\text{Bd load} + 1)$ classified by treatment and skin sloughing. Points represent the raw data. Asterisks denote significance of Tukey's pairwise comparisons from the mixed model term Treatment \times Slough (Supplementary Table S2). An example of skin sloughing is shown in Supplementary Figure S2.

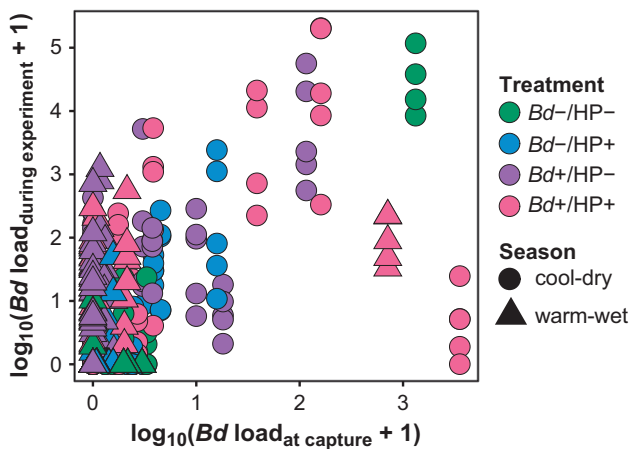


Figure 4 Relationship between initial *Bd* infection intensity (at capture) and *Bd* infection intensity during the experiment (GLM (initial infection intensity): $\beta = 0.76 \pm 0.12$, $F = 77.2$, $t = 6.316$, $P < 0.001$, Supplementary Table S2).

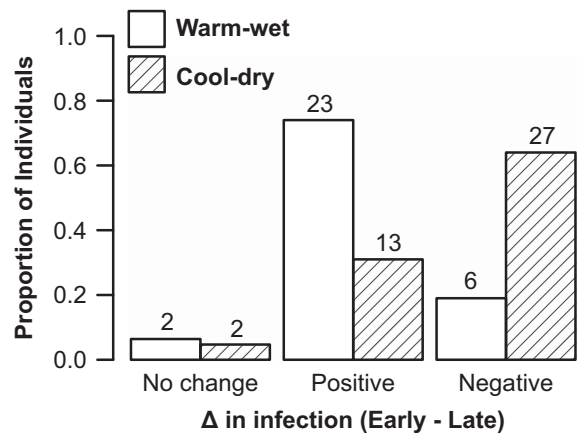


Figure 5 Proportion of individual frogs categorized by their ability to recover after experimental inoculation with *Bd*. At the end of the experiment, an individual frog could either remain uninfected, clear or reduce the infection (positive responses) or remain infected (negative responses). Number of frogs is reported on top of the bars.

Figure S3). Some natural infections also resulted in host mortality. For example, one individual from the *Bd* - /HP - treatment in cool-dry season died 13 days after collection (initial load: 1323 zoospores, load at death: 117 846 zoospores; Supplementary Figure S3).

Interactions between time, treatment, season, and skin sloughing also modulated alpha diversity of skin microbes

With respect to alpha diversity of skin bacterial communities, the mixed models showed significant effects of interactions between treatment*day, day*season and day*skin sloughing. Despite overall increase in OTU richness and phylogenetic diversity through time (Figures 1b and c; Supplementary Tables S5 and S6), we found delayed responses dependent on the addition of *Bd*. Individuals in *Bd*+ treatments experienced an increase in bacterial

diversity three days post-infection, whereas individuals in *Bd*- treatments only exhibited a comparable increase after six days (Figures 1b and c). The application of both *Bd* and HP resulted in a higher rate of change in richness and phylogenetic diversity from day 0 to day 3 (Figures 1b and c), which stabilized after day 6. On day 3, OTU richness of individuals in the *Bd*+ /HP - treatment tended to be lower than *Bd*+ /HP + treatments (Figure 1b), but the comparison fell short of significance ($\beta = -205.15$, $df = 373.90$, $t = -2.381$, $P = 0.08$). The interaction between season and day revealed a steady increase in OTU richness and phylogenetic diversity during the cool-dry season (Figures 2b and c; Supplementary Tables S5 and S6). Remarkably, skin bacterial communities returned to their initial OTU richness before inoculation only during the warm-wet season (Figure 2b, day 3 to 15: $\beta = 78.7$, $df = 365.76$, $t = 0.717$, $P = 0.95$). Phylogenetic diversity

also showed a similar pattern from day 3 to day 15 (Figure 2c, $\beta = -10.7$, $df = 362.76$, $t = -1.11$, $P = 0.80$).

Although we did not detect a significant interaction between season and sloughing that could help explain this reduction in diversity, we found a significant effect of the interaction between day and skin sloughing on bacterial diversity ($F = 3.32$, $P = 0.01$, Supplementary Table S5; Supplementary Figure S5), indicating that the few individuals shedding skin significantly reduced OTU richness from day 9 to day 12. This change was mostly driven by individuals from the cool-dry season, because during the warm-wet season only one individual showed skin sloughing during day 9 (Supplementary Figure S5). In addition, phylogenetic diversity also dropped significantly from day 9 to day 12 for individuals that were experiencing skin sloughing ($diff = -48.8$, $df = 368.52$, $t = -2.898$, $P = 0.03$), independent of season.

Seasons and treatments differentially affected particular members of skin bacterial communities

The analyses of fold change abundance of bacteria from the cool-dry season to the warm-wet season revealed strong shifts in at least 420 bacterial taxa after FDR correction (Supplementary Tables S7 and S8). In general, 241 taxa exhibited positive fold changes during the cool-dry season (above the '0' \log_2 fold change threshold), the season in which frogs carried higher *Bd* infection intensities (Supplementary Table S7). By ordering all OTUs with significant fold changes (241+179 OTUs) with respect to their average counts (Figure 6; Supplementary Table S8), we found that 63% of the top 30 OTUs were suppressed during the cool-dry season. For example, *Acinetobacter rhizosphaerae*, a coccobacilli with putative anti-*Bd* function (Woodhams *et al.*, 2015), was overrepresented during the wet-warm season and decreased during

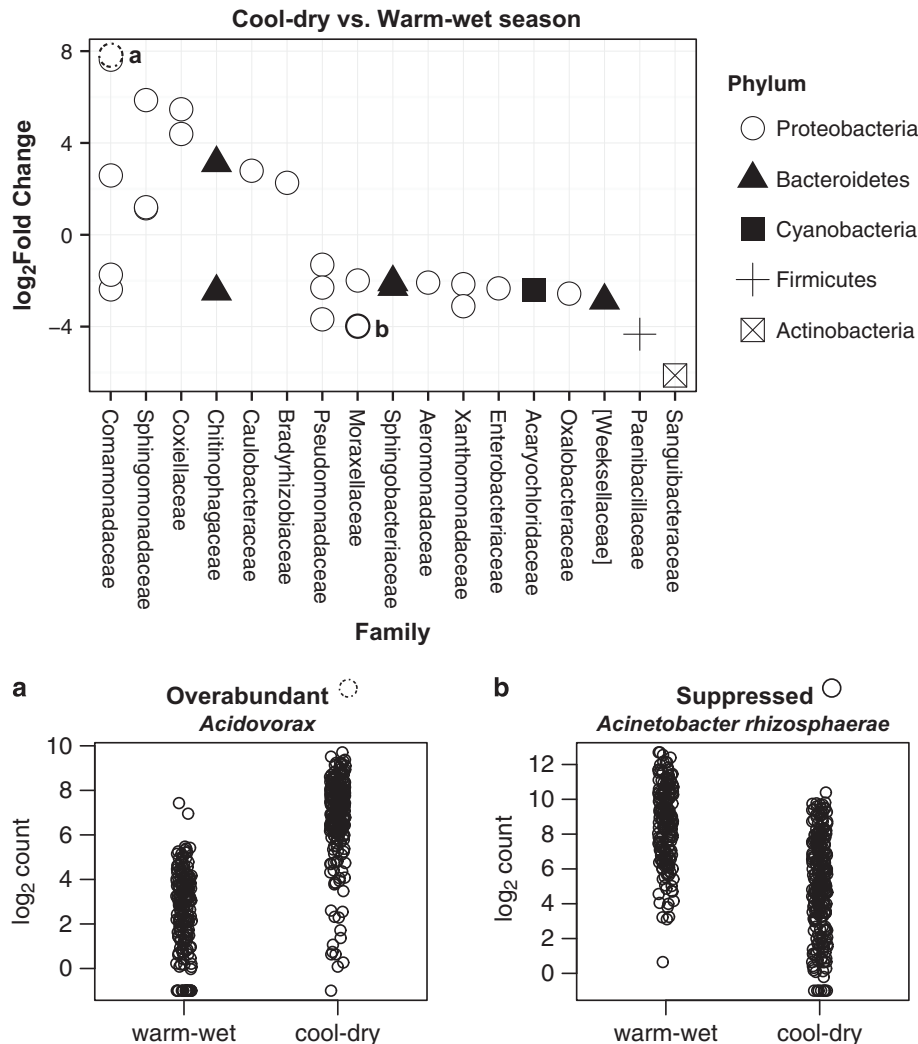


Figure 6 Differential abundance of bacteria between the cool-dry season and the warm-wet season. Taxa above the zero line are significantly over abundant during the cool-dry season (a). In contrast, taxa below the zero line are significantly suppressed during the cool-dry season (b). We show two examples to illustrate the changes. Identities of each OTU can be found in Supplementary Table S7.

the cool-dry season (Figure 6). In contrast, we detected OTUs such as *Acidovorax* that were abundant during the cool-dry season, but less common during the wet-warm season (Figure 6).

The addition of *Bd* changed the abundance of 82-1638 bacterial taxa, depending on whether or not season was considered (Supplementary Tables S7–S9). For the most abundant OTUs, *Bd* typically resulted in the increase of Proteobacteria and Bacteroidetes (Figure 7a). *Myroides odoratimimus*, an opportunistic pathogen member of the family Flavobacteriaceae (Maraki *et al.*, 2012), was the most abundant bacterium enhanced by the introduction of *Bd* (Figure 7a; Supplementary Table S9). Interestingly, *A. rhizosphaerae* (family Moraxellaceae, \log_2 fold change -6.97) was the least common bacterial taxon overall (Figure 7a; Supplementary Table S9).

The application of HP caused an overall reduction in bacterial abundance as expected, with 999 taxa significantly suppressed compared with 754 that became over abundant (Supplementary Table S7). HP reduced 77% of the top 30 most abundant taxa (Supplementary Table S9), in particular the members of the phylum Proteobacteria (Figure 7b). Similar to the application of *Bd*, *M. odoratimimus* was the most abundant taxa followed by *Acidovorax* (family Comamonadaceae, Figure 7b), indicating the possible role of *M. odoratimimus* as an opportunistic pathogen. Finally, the addition of both *Bd* and HP doubled the \log_2 fold change of *M. odoratimimus* when compared with *Bd* or HP alone (Supplementary Table S9).

Skin bacterial community structure differed by seasons and treatments

At capture (Day 0), frogs collected during the warm-wet season carried a different bacterial community structure than those collected during the cool-dry season (Figure 8, PERMANOVA Unweighted Unifrac: $F_{1,72} = 4.03$, $R^2 = 0.054$, $P < 0.001$; Weighted Unifrac: $F_{1,72} = 8.81$, $R^2 = 0.113$, $P < 0.001$; and Bray–Curtis: $F_{1,72} = 5.89$, $R^2 = 0.077$, $P < 0.001$). As expected, treatment, *Bd* infection status, and the interaction between seasons and treatments were not significant during Day 0. However, 3 days after inoculation, we detected significant changes in community structure by seasons (Unweighted Unifrac: $F_{1,85} = 9.66$, $R^2 = 0.096$, $P < 0.001$), treatments ($F_{1,85} = 2.33$, $R^2 = 0.069$, $P < 0.001$), their interaction (Unweighted Unifrac: $F_{1,85} = 1.91$, $R^2 = 0.056$, $P < 0.001$), but these were unrelated to *Bd* infection status (Unweighted Unifrac: $F_{1,85} = 1.18$, $R^2 = 0.012$, $P = 0.178$). The same permutational tests using weighted Unifrac and Bray–Curtis distances matrices showed similar results, thus are not presented here. By the end of the experiment, we recovered very similar patterns to Day 3, in which seasons (Unweighted Unifrac: $F_{1,77} = 10.25$, $R^2 = 0.114$, $P < 0.001$), treatments ($F_{1,77} = 1.64$, $R^2 = 0.055$, $P < 0.001$), their

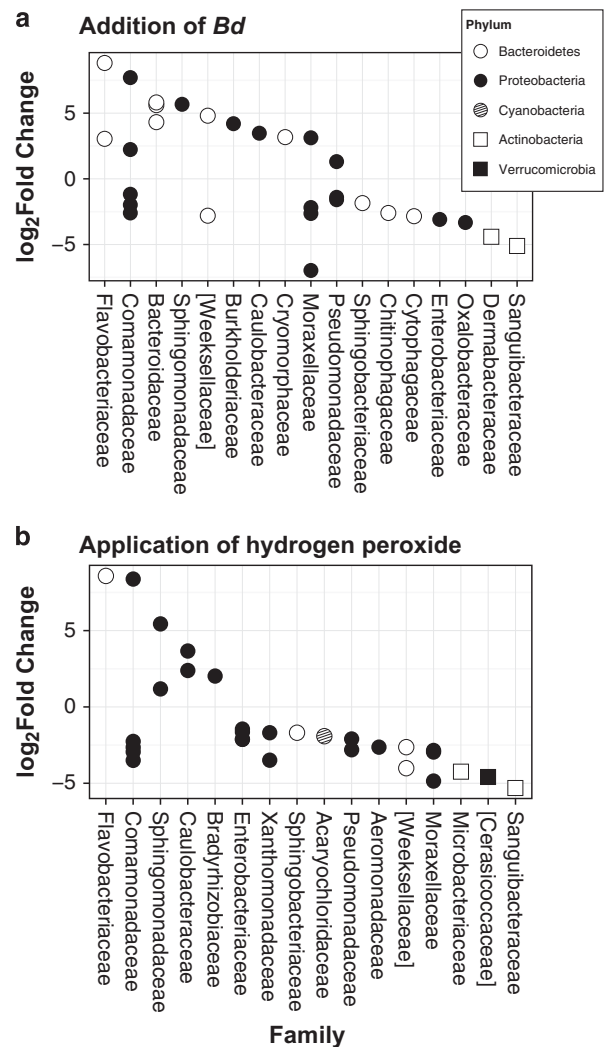


Figure 7 Differential abundance of bacteria between (a) *Bd* +/HP- treatment and *Bd*-/HP+ treatment, and (b) *Bd*-/HP+ treatment and *Bd*-/HP- treatment. Taxa above the zero line are significantly over abundant, whereas taxa below the zero line are significantly suppressed. Identities of each OTU can be found in Supplementary Table S8.

interaction (Unweighted Unifrac: $F_{1,77} = 1.50$, $R^2 = 0.050$, $P < 0.001$) still influenced bacterial community structure, but not individual *Bd* infection status (Unweighted Unifrac: $F_{1,77} = 1.08$, $R^2 = 0.012$, $P = 0.269$).

Discussion

By accounting for the variability in environmental conditions influencing host responses, not only our findings provide evidence that fungal diseases disrupt host health and skin bacterial communities, but also that amphibians show contrasting recovery patterns. We quantified, for the first time, the progression of fungal–bacterial interactions and how these correlated with disease outcome under natural environmental conditions, providing a novel

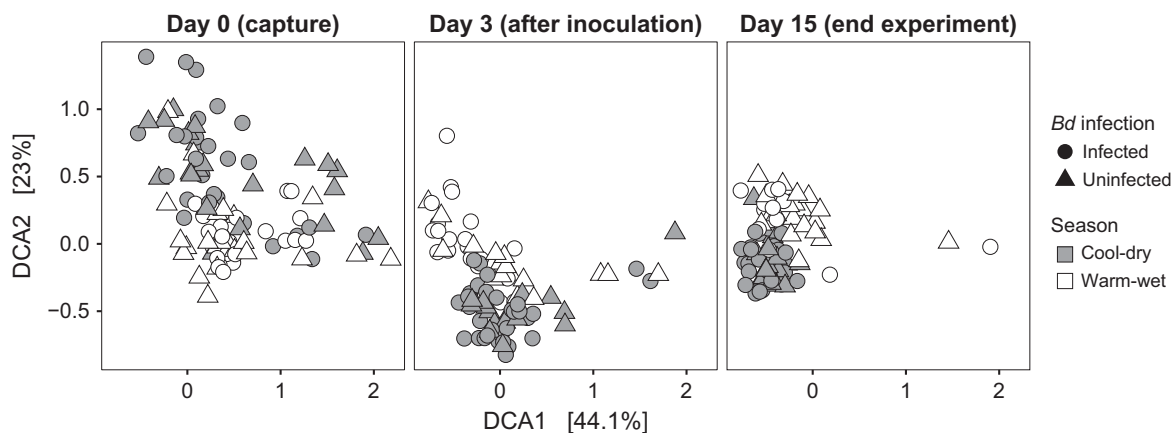


Figure 8 Detrended correspondence analysis plot of unweighted-Unifrac distances of skin bacterial communities at three different time points (capture, 3 days after inoculation, and at the end of the experiment). To facilitate visualization of points, we show each day in a separate panel.

perspective on the function of skin microbiome in amphibians. Challenging frogs with the same dose of pathogen provoked strikingly different host responses depending on environmental conditions, confirming our previous hypothesis of lability in host defenses (Longo *et al.*, 2010, 2013). During the warm-wet season, frogs were able to control infections significantly better than during the cool-dry season (Figures 2a and 5; Supplementary Figure S4) possibly due to increased temperatures and high humidity, resulting in elevated immune function (Maniero and Carey, 1997; Raffel *et al.*, 2006) and different symbiotic community (Figure 8). All frogs in both seasonal experiments showed an increase in bacterial diversity independent of *Bd* treatment. However, in the warm-wet season, this increase in bacterial richness and phylogenetic diversity was followed by a sudden drop at the end of experiment (Figures 2b and c). This finding indicates that the increase in diversity resulted from recruitment of new taxa to the skin microbiome, either through colonization of beneficial or opportunistic bacteria, or both. Only in the warm-wet season, did *E. coqui* frogs limit *Bd*, return to initial bacterial diversity found when captured, and benefit from the availability of putatively beneficial microbes such as *A. rhizosphaerae* (Figure 6). In contrast, as we predicted, frogs could not recover during the cool-dry season because beneficial microbes were suppressed, and the skin was colonized by putative opportunistic microbes such as *Acidovorax* and *M. odoratimimus*, which became more abundant as a secondary result of infection. Our results from the simultaneous application of both *Bd* and HP showed additive effects on *M. odoratimimus*, indicating that this OTU required special conditions to increase its abundance. Whether or not this OTU is an opportunistic pathogens of frogs still needs to be determined. Combined, our results confirm that natural fluctuations in environmental conditions shift the survival advantage from the pathogen to the host, and back

again, and that this shift includes interactions with host bacterial communities.

Coping with Bd under optimal environmental conditions

As ectotherms, amphibians rely on environmental temperatures to achieve essential physiological processes including digestion, immunity, metabolism and reproduction. Our findings showed that just a slight increase in temperatures experienced during the warm-wet season provides frogs with a significant advantage that helps minimize the impact of infections (Figure 5). Our results corroborate many others that show that higher temperatures decrease the severity of infection (for example, Woodhams *et al.*, 2008; Murphy *et al.*, 2011; Becker *et al.*, 2012; Rowley and Alford, 2013). In many of these cases, behavioral fever (that is, the process by which ectotherms seek areas of warmer temperatures to increase body temperatures) was the mechanism providing resistance to hosts. Behavioral fever did not occur during our experiment, because our mesocosm setting did not provide frogs the opportunity to achieve higher body temperatures and clear infection (Woodhams *et al.*, 2003; Rowley and Alford, 2013). Instead, in our experiment the combination of higher overall environmental temperatures and downstream processes, such as recruitment of bacterial diversity and enhanced immune capacity, may have modulated the observed infection outcomes.

Host immunity and pathogen infectivity likely interacted with temperature to lower *Bd* infection intensities during the warm-wet season. On one hand, natural variation in temperature impacts the expression of amphibian immunity and influences susceptibility to disease (Maniero and Carey, 1997; Raffel *et al.*, 2006). On the other hand, warmer temperatures decrease the activity period and lifespan of *Bd* zoospores (Woodhams *et al.*, 2008; Voyles

et al., 2012), possibly affecting their dispersal and transmission. In terms of the possible immune mechanisms increasing resistance to infection during the warm-wet season, coqui frogs lack antimicrobial peptides against *Bd* (Rollins-Smith *et al.*, 2015), but they may rely on adaptive immunity, as has been shown for other frogs (Savage and Zamudio, 2011; Ellison *et al.*, 2014; McMahan *et al.*, 2014; Savage *et al.*, 2016). The fact that we found a positive relationship between previous exposure and *Bd* infection intensity during the experiment indicates that acquired immunity may not confer full resistance to coqui frogs (Figure 4), because individuals still get infected when challenged with the pathogen at high doses. Nonetheless, previous exposure may still offer partial protection, and that protection is enhanced during the warm-wet season by both abiotic variables and beneficial symbionts, allowing for seasonal population recovery.

In our experiment, a very small percentage (four frogs, Figure 5) of frogs remained uninfected throughout the experiment, regardless of treatment (Supplementary Table S4). Once inoculated, frogs had a high probability of clearing or reducing *Bd* infection intensity only during the warm-wet season (Figure 5). This finding suggests that pre-inoculation status does not reflect a naive state and could be considered an honest indicator of pathogen resistance across seasons, because frogs were significantly less infected and carried lower infection intensities than during the cool-dry season. In contrast, the likelihood of showing positive responses after infection decreased by half (from 74% in the warm-wet season to 31% in the cool-dry season, Figure 5), revealing that previously infected individuals lost any potential immune protection when environmental conditions became cooler and drier. Our data suggest that host immune function may be at maximum performance during the warm-wet season because frogs were able to reduce *Bd*, OTU richness and phylogenetic diversity in a period of 2 weeks. This result corroborates earlier findings that some amphibian immune factors might become ineffective at cooler temperatures (Maniero and Carey, 1997; Ribas *et al.*, 2009). Additional research on the identity of immune factors, and exactly how they are environmentally mediated, is clearly necessary.

Advantages of diverse skin microbiota during the warm-wet season

During the warm-wet season, harboring a diverse pool of putatively beneficial taxa may offer frogs an additional advantage to decrease infection intensity. Frogs at this time point had a different community structure than frogs collected during the cool-dry season (Figure 8). Our data demonstrated that many OTUs from families Xanthomonadaceae, Oxalobacteraceae, Pseudomonadaceae, among others, were

significantly more abundant during the warm-wet season (Figure 6). However, during the cool-dry season some of these bacterial taxa remained present, albeit at lower levels, as part of the normal microbiota (Figure 6, Supplementary Table S6). One such example is *A. rhizosphaerae*, an OTU that was suppressed during the cool-dry season in our experiment (Figure 8). Non-susceptible species have bacterial communities enriched with *Acinetobacter* (Rebollar *et al.*, 2016). Over the last few years, researchers have tested 27 isolates of *A. rhizosphaerae* against *Bd*, yet only 60% of them were able to inhibit the pathogen (Woodhams *et al.*, 2015). By testing candidate probiotics against several *Bd* isolates, Antwis *et al.* (2015) demonstrated that only a small proportion of bacteria have a broad-spectrum capacity. Thus, our results extend these recent findings by indicating that some candidate probiotics such as *A. rhizosphaerae* might also be inefficient at lower temperatures (Daskin *et al.*, 2014).

In vitro and *in vivo* antifungal activity of skin bacteria should differ as a consequence of environmental conditions and host immune factors. So far, most cases of *in vitro* anti-*Bd* activity has been quantified using bacterial metabolites and cell factors found in cell-free supernatants at a single temperature (Bell *et al.*, 2013). However, the quantity and identity of these metabolites may depend on temperature and bacterial density (Daskin *et al.*, 2014; Yasumiba *et al.*, 2016). Therefore, understanding environmental limits to *Bd* inhibition will be important if we are to consider any bacteria as potential probiotics for conservation management.

Skin sloughing as a tolerance strategy under suboptimal environmental conditions

Our results demonstrated that frogs could not limit *Bd* infections or colonization by new bacterial taxa during the cool-dry season (Figure 2). Nonetheless, only 5 of the 50 individuals in the experiment died during this season (Supplementary Table S4) and most frogs persisted despite higher infections (Supplementary Figure S3). Recent studies have demonstrated that increased skin sloughing does not have a measurable effect on *Bd* infection intensity in Australian green tree frogs (Ohmer *et al.*, 2014), yet reduces the amount of culturable microbes (Meyer *et al.*, 2012; Cramp *et al.*, 2014), and is more common at higher temperatures (Cramp *et al.*, 2014). Taken together, these results suggest a potential adaptive strategy for reducing opportunistic bacteria and other pathogens.

Our findings significantly differ from those studies in that we detected higher skin sloughing in the cool-dry season (Supplementary Figure S4). In addition, sloughing in coqui frogs did not measurably reduce overall *Bd* infection intensity and bacterial diversity (Figure 4; Supplementary Figure S5). The skin sloughing that we document here is not a natural skin shedding process, but part of the pathology

associated with chytridiomycosis (Voyles *et al.*, 2009). Whether sloughing was a cause or a consequence of *Bd* infection dynamics still needs to be determined. Because skin sloughing is a costly potential mechanism of defense, and we saw no reduction in pathogen load, we hypothesize that this mechanism contributes to tolerance in a different way. If sloughing in coqui frogs serves to maintain infections at bay for a fixed amount of time (cool-dry season), then this strategy could protect the host during a time that immune responses are slow, ineffective, or potentially damaging (Ellison *et al.*, 2015). Sloughing should be further explored using microscopy or molecular techniques to determine the viability of bacteria and *Bd*. In this way, we will be able to understand the functional consequences and the timing of this response to infection.

Conclusions

After a pathogen invades naive hosts, interacting species need to find a balance to persist. Limiting the severity of the damage caused by a pathogen, rather than eliminating them, is an advantageous strategy (Roy and Kirchner, 2000) not only for the host but also for the pathogen. In this paper, we described how a frog species, that persists in populations where *Bd* is enzootic, survives despite relatively high infection prevalence and intensity. Our results indicate that when the environment offers a survival advantage to the host, both increased temperatures and host-associated bacteria contribute to resistance. In contrast, during the cool-dry season, the environment favors the pathogen, thus frogs shift their defense strategy to tolerance. We propose that an important avenue for future research will be to obtain comparative and quantitative data on defense strategies that allow other species to persist with this infectious disease. Quantifying defense mechanisms and identifying the ones that provide the highest fitness benefit can significantly improve our management strategies for the reintroduction of susceptible and threatened species.

Conflict of Interest

The authors declare no conflict of interest.

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