Cool Temperatures Reduce Antifungal Activity of Symbiotic Bacteria of Threatened Amphibians – Implications for Disease Management and Patterns of Decline

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Abstract

Chytridiomycosis, caused by the fungus Batrachochytrium dendrobatidis (Bd), is a widespread disease of amphibians responsible for population declines and extinctions. Some bacteria from amphibians’ skins produce antimicrobial substances active against Bd. Supplementing populations of these cutaneous antifungal bacteria might help manage chytridiomycosis in wild amphibians. However, the activity of protective bacteria may depend upon environmental conditions. Biocontrol of Bd in nature thus requires knowledge of how environmental conditions affect their anti-Bd activity. For example, Bd-driven amphibian declines have often occurred at temperatures below Bd’s optimum range. It is possible these declines occurred due to reduced anti-Bd activity of bacterial symbionts at cool temperatures. Better understanding of the effects of temperature on chytridiomycosis development could also improve risk evaluation for amphibian populations yet to encounter Bd. We characterized, at a range of temperatures approximating natural seasonal variation, the anti-Bd activity of bacterial symbionts from the skins of three species of rainforest tree frogs (Litoria nannotis, Litoria rheocola, and Litoria serrata). All three species declined during chytridiomycosis outbreaks in the late 1980s and early 1990s and have subsequently recovered to differing extents. We collected anti-Bd bacterial symbionts from frogs and cultured the bacteria at constant temperatures from 8°C to 33°C. Using a spectrophotometric assay, we monitored Bd growth in cell-free supernatants (CFSs) from each temperature treatment. CFSs from 11 of 24 bacteria showed reduced anti-Bd activity in vitro when they were produced at cool temperatures similar to those encountered by the host species during population declines. Reduced anti-Bd activity of metabolites produced at low temperatures may, therefore, partially explain the association between Bd-driven declines and cool temperatures. We show that to avoid inconsistent antifungal activity, bacteria evaluated for use in chytridiomycosis biocontrol should be tested over a range of environmental temperatures spanning those likely to be encountered in the field.

Introduction

Emerging wildlife diseases can cause species declines and extinction [1], and disease emergence and pathogenicity may depend on environmental context [2]. Therefore, patterns of decline and disease management strategies may be understood by examining the effects of environmental context on disease emergence. One way environmental context might affect disease dynamics is by altering species interactions in the complex assemblage of microbiota inhabiting wildlife [3]. Chytridiomycosis, a disease caused by the fungal pathogen Batrachochytrium dendrobatidis (Bd) and responsible for rapid and extensive population declines in over 200 amphibian species since the late 1970s [4,5], serves as a model system for understanding other wildlife diseases [6]. Twenty years of research on Bd and its interaction with amphibians and their environment can provide insight for anyone interested in the effects of diseases on the conservation of wildlife [5,7].

Currently, no effective treatments or preventative actions are available to manage chytridiomycosis in wild populations of threatened amphibians [8]. However, bioaugmentation, by supplementing populations of anti-Bd bacteria, has proved effective in laboratory trials and may be a viable option for disease management if it also provides increased protection in nature [9–11]. Previous experimental work on anti-Bd bacteria was conducted under constant laboratory conditions [11,12]. However, naturally-occurring symbiotic bacteria, including antibiotic producers, vary in abundance and physiological activity with environmental context [13–15]. This variation can affect the success of biocontrol programs [16–18], and can have strong
evolutionary and ecological impacts [3]. Bacteria chosen for probiotic use based on high levels of anti-\(Bd\) activity under constant conditions in the laboratory could have lower levels of anti-\(Bd\) activity in the more variable conditions occurring in nature. If this occurs, then choosing bacteria for use in the management of chytridiomycosis, will require knowledge of how candidate bacteria are affected by a range of environmental conditions [9].

Environmentally induced variation in the protection afforded to amphibian hosts by bacterial symbionts might also partially explain patterns of past chytridiomycosis-driven declines. In the tropics, where the impacts of chytridiomycosis have been most severe, higher elevations and cooler seasons have been associated with higher prevalences of infection, more intense infections, and more frequent declines [19–24]. \(Bd\)’s relatively cool thermal optimum (17–25°C) [25] may partially explain this pattern [21], but many declines have occurred at temperatures well below this window, where one might expect chytridiomycosis to be less severe [26].

There are a number of reasons why declines might occur at very low temperatures. In natural environments, \(Bd\) may respond to low temperatures by increasing fecundity [27], and amphibian hosts’ immune defenses may be weaker [28]. Another possible contributor to the high incidence of \(Bd\)-driven declines at temperatures below \(Bd\)’s in vitro thermal optimum is that symbiotic cutaneous bacteria, which would otherwise reduce the severity of chytridiomycosis, may have reduced activity or population density at cooler temperatures. At present, there is no published information on how the composition or anti-fungal activity of assemblages of anti-\(Bd\) bacteria responds to changes in environmental context.

To examine the effect of temperature on the production of anti-\(Bd\) metabolites by bacteria, we sampled bacterial symbionts from the Australian hydrid frogs \(Litoria serrata\) and \(Litoria nannotis\) in 2010. We also examined bacteria isolated by Bell [29] and Bell et al. [30] from \(Litoria rheocola, L. serrata\), and \(L. nannotis\) in 2009. All three species experienced population declines following chytridiomycosis outbreaks, although some populations have since recovered or recolonized [23,31]. We identified the bacteria and characterized their anti-fungal activity across a range of the temperatures experienced by their amphibian hosts.

**Materials and Methods**

**Ethics Statement**

All procedures involving animals received clearance from the James Cook University Animal Ethics Review Committee (approval number A1316) and all field sampling was permitted by the Queensland Department of Environment and Resource Management (permit WITK05922209).

**Collection and Isolation of Bacteria**

To screen for anti-\(Bd\) activity, we collected bacteria from twelve frogs (six \(L. serrata\), six \(L. nannotis\)) caught at Windin Creek in Wooroonooran National Park, Queensland, Australia (~750 m a.s.l., S 17°21′57″ E 145°42′54″) in February 2010. We rinsed each frog with a stream of sterile distilled water to remove non-resident bacteria [32,33], then swabbed its dorsal and ventral surfaces and legs twice, using a sterile rayon swab (MW112, MWandE, Bath UK). We streaked each frog’s swab onto a low nutrient agar plate (R2A, Becton, Dickinson and Company, New Jersey, US). We used new gloves and plastic bags to catch, handle, and hold each animal to prevent disease transmission or contamination of samples. We released frogs at their point of capture immediately after swabbing. After returning our samples to the laboratory, we isolated each morphologically-distinct bacterium into axenic (pure) culture using standard microbiological techniques, and checked Gram stains of each isolate using light microscopy to ensure purity [34]. Axenic isolates were stored on ceramic Microbank microbeads (Microbank, Pro-Lab Diagnostics U.K.). The beads are stored dry at ~80°C. When a live culture is required, a bead can be dropped into liquid media or streaked across solid agar.

**Identification of Anti-\(Bd\) Bacteria**

To determine whether bacterial isolates inhibited growth of \(Bd\) in vitro, we performed challenge assays using a method developed by Bell et al. [30] with slight modifications described here. We inoculated bacteria from Microbank microbeads into one mL TGHb broth (eight g tryptone, one g gelatine, two g lactose per liter of water) in 24-well plates (Costar 3524, Corning, New York, US) and incubated them for 48-hours at 23°C. We then centrifuged each liquid culture for five minutes at 7500 xg, and filtered the supernatant through a 0.22 μm syringe filter (Millex GV, Millipore, Massachusetts, US). This left a cell-free supernatant (CFS) containing bacterial metabolites.

In 96-well assay plates (Costar 3595, Corning, New York, US), we inoculated 1.0 × 10⁵ live \(Bd\) zoospores (isolate Gibbo River, L. Les, 06-LB-1 isolated by L. Berger in 2006 from a \(Litoria lecointei\) and passaged weekly) suspended in 50 μL of fresh TGHb into each of five replicates containing 50 μL of each CFS. We included replicates of positive and negative controls consisting of 1.0 × 10⁵ live and heat-killed \(Bd\) zoospores, respectively, suspended in 100 μL TGHb. We incubated the plates at 23°C and monitored progress of \(Bd\) growth in each well with daily spectrophotometric readings at 492 nm [35]. We continued monitoring until maximum growth was observed in the positive control and in the majority of wells containing CFS (four days). This is the most conservative point in the assay at which to determine anti-\(Bd\) activity; i.e., this is the point at which the ratio of \(Bd\) growth in CFSs to \(Bd\) growth in the positive control is maximized. We examined 96-well plates using light microscopy and excluded from our analyses any replicates that had become contaminated.

We transformed mean optical densities at 492 nm (OD₄₉₂) for each isolate-temperature combination on each day, to correct for initial coloration of CFSs (by subtracting the mean initial OD₄₉₂) and background absorbance of inoculated zoospores on that day (by subtracting the mean negative control OD₄₉₂) [30]. Using the corrected value on the maximum growth day for both the positive control and for each CFS by temperature combination, we standardized isolate-specific values against the similarly corrected value for the positive control. This produced a measure of \(Bd\) growth in each CFS by temperature combination as a proportion of that in the positive control. Finally, we multiplied the corrected and standardized values by 100 percent. This returned the following values: 100% for the positive control; 0 for total inhibition of \(Bd\) growth; >0 but <100% for partial inhibition, and >100% for CFSs in which \(Bd\) grew better than in the positive control. Thus, the values are the percent \(Bd\) growth relative to that in the positive control. Following Bell et al. [30] we considered CFSs in which \(Bd\) growth was reduced on average at least 63.5% below that in the positive control to be strongly inhibitory. This value is a conservative correction for the maximum observed effects of nutrient depletion in the media carried into challenge assay wells along with inoculated CFSs. When \(Bd\) is inhibited more than 63.5%, it is assumed to be true CFS-driven inhibition, and not due to nutrient depletion [30].
We extracted DNA from each isolate that strongly inhibited Bd, first by three freeze-thaw cycles of 10 minutes each at −80°C and 70°C, and then, if freeze-thaw cycles alone did not yield sufficient DNA for successful PCR, using a Qiagen (Hilden, Germany) DNeasy blood and tissue kit with pretreatment for Gram-negative bacteria, as per the manufacturer’s protocol. DNA was amplified using universal bacterial 8F and 1492R primers [36], and sequenced by Macrogen, Inc. (Seoul, South Korea). We aligned forward and reverse sequences in Geneious [37] and matched to sequences in the NCBI GenBank database (http://ncbi.nlm.nih.gov) to identify bacteria. We have submitted genetic sequence data to GenBank.

Experimental Challenge Assays

To test for temperature-induced changes in bacterial anti-Bd activity we performed additional challenge assays and quantitative analysis using bacteria identified in the initial screening assay as strongly inhibitory. In addition to the bacteria isolated from frogs sampled in February 2010, we included strongly inhibitory bacteria isolated from Litoria nannotis, L. serrata, and L. theocola at Windin Creek in the Austral winter of 2009 and tested using the methods described above [29]. In total, we tested 24 isolates in the experimental challenge assays, all of which inhibited Bd growth by more than the 63.5% threshold in initial challenge assays (Table 1).

We inoculated each bacterium from a Microbank microbead into a 25 cm² flask (TPP, Trasadingen, Switzerland) containing 10 mL TGHbL broth and incubated it at 23°C for at least 48 hours until growth was observed. We then added 500 μL of each bacterial inoculum to 1 mL TGHbL in each of six, 24-well plates. One plate was placed in each of six incubators set at 8, 13, 18, 23, 28, and 33°C, respectively. We chose temperatures to approximate the range of conditions experienced by Litoria spp. in the Australian Wet Tropics [38].

We grew bacterial cultures for two to five days, based on the time to maximal absorbance at 492 nm (a surrogate for maximal bacterial concentration) and used these cultures to produce the CFSs included in the next round of challenge assays. Growing cultures to maximum absorbance minimized any differences among treatments that might have arisen if metabolite production was triggered by quorum sensing, a mechanism of detection and response in bacterial colonies based on population density [39]. When OD₄₉₂ stopped increasing (qualitatively determined from absorbance graphs of absorbance values) we produced bacterial CFSs as described for the initial screening assay. We harvested each individual bacterium as it reached maximum growth; i.e., we did not wait for absorbance of all bacteria in a plate to plateau before harvesting. This method does not account for possible differences in the maximum achievable concentration of any given bacterium at different temperatures. CFSs were held at 8°C for at least 48 hours until growth was observed. We then added 500 μL of each bacterial inoculum to 1 mL TGHbL in each of six, 24-well plates. One plate was placed in each of six incubators set at 8, 13, 18, 23, 28, and 33°C, respectively. We chose temperatures to approximate the range of conditions experienced by Litoria spp. in the Australian Wet Tropics [38].

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Experimental Challenge Assay

The experimental challenge assay continued for six days, at which point Bd in the positive control and in 136 of 157 CFSs had reached its maximum growth. All the 21 remaining CFSs had Bd growth and absorbances already well above the point at which they would have been considered inhibitory. Forty-one of 785 replicates (5.2%) became contaminated and were subsequently removed from analysis of the initial challenge assay. Bacterial responses to temperature included all of increased, decreased, and non-directional changes to CFS anti-Bd activity (Figure 3).

The temperature at which bacteria were cultured significantly affected the antifungal activity of their CFSs (Kruskal-Wallis test, \(\chi^2 = 15.35, df = 5, p<0.01\)). Antifungal activity tended to be lowest in CFSs produced at 8°C (Figure 4). Using the cutoff from the initial screening assay of 63.5% or greater inhibition of Bd growth relative to the positive control, 46% of tested CFSs had no strong antifungal activity when produced at 8°C, whereas no more than 28% produced at any of the warmer temperatures lost strong antifungal activity.

Bell et al. [30] observed various fungicidal (leading to the breakdown of Bd cells) and fungistatic (arresting Bd’s life cycle) effects of CFSs on Bd. We observed similar effects in the cultures we monitored visually by light microscopy. For example, in the CFSs of Pseudomonas fluorescens strain 1408 grown at 8°C, the zoospores of Bd appeared dead and deformed, whereas in the CFSs of Chryseobacterium sp. CH33 grown at 8°C, zoospores developed into sporangia without producing viable zoospores.
Variation in Antifungal Activity

The metabolites of many of the defensive bacterial symbionts of amphibians we tested had reduced antifungal activity when produced at 8°C. Such reductions in antifungal activity were likely caused by changes in the quantity, identity, or both of bacterially-produced substances, and could have contributed to chytridiomycosis-driven declines that occurred in high elevation populations of the three *Litoria* species sampled here [23,31,40]. Winter air temperatures in the high elevation habitats of these species can often be lower than 8°C [26,41]. Therefore, frogs may experience decreased bacterial protection from *Bd* in winter, which in Australia and elsewhere is when chytridiomycosis causes greater morbidity and mortality [19,23,42]. While *Bd* physiology may also be altered under variable temperature regimes and it will be important to test bacteria and *Bd* exposed together to a range of temperatures, our design and the in vitro assay we used did not allow for testing such effects. Nonetheless, our experiment is an incremental step towards understanding context-dependency in amphibian-*Bd*-bacteria interactions.

Anti-*Bd* bacteria occur on a wide geographic and phylogenetic range of amphibians [12,43,44], and the pattern that chytridiomycosis is more virulent at cool temperatures is also widespread [19–21,23]. Many bacteria can alter their rates of antibiotic production in response to environmental temperatures [17,45,46].

### Table 1. Bacteria identified as strongly inhibitory of *Batrachochytrium dendrobatidis* and used in the experimental challenge assay.

| Taxonomic name                      | Frog species†, Year | GenBank Accession ID, % Matching* | GenBank Accession Number |
|-------------------------------------|---------------------|----------------------------------|--------------------------|
| Actinobacteria                      |                     |                                  |                          |
| *Microbacterium* sp. HY14(2010)     | LN, 2010            | HM579805, 99.5                   | KJ191412                 |
| Bacilli                             |                     |                                  |                          |
| *Bacillus thuringiensis* isolate CCM15B | LN, 2010      | FN433029, 99.8                   | KJ191418                 |
| β-Proteobacteria                    |                     |                                  |                          |
| Uncultured *Silvomonas* sp. clone ntu63 | LS, 2010        | EU159476, 98.5                   | KJ191396                 |
| Bacterium H2                        | LR, 2009            | AY345552, 99.1                   | KJ191380                 |
| *Iodobacter* sp. CdM7               |                     |                                  |                          |
| Flavobacteria                       |                     |                                  |                          |
| *Chryseobacterium* sp. CH33         | LN, 2009            | GU353129, 99.1                   | KJ191375                 |
| Uncultured bacterium clone nbw1150f04c1 | LS, 2010        | GQ082309, 99.1                   | KJ191421                 |
| *Chryseobacterium hispanicum* type strain VP48 |             | AM159183, 98.7                   |                            |
| γ-Proteobacteria                    |                     |                                  |                          |
| *Halof_increase*                     | LN, 2009            | AB519795, 99.9                   | KJ191378                 |
| *Pseudomonas fluorescens* strain 1408 | LS, 2009        | GU726880, 99.9                   | KJ191384                 |
| *Pseudomonas fluorescens* strain d3_16s | LS, 2010        | HQ166099, 99.7                   | KJ191426                 |
| *Pseudomonas fluorescens* strain Ku-7 | LR, 2009        | AB266613, 98.9                   | KJ191386                 |
| *Pseudomonas koreensis* strain Ps 9–14 | LR, 2009       | NR025228, 99.9                   | KJ191376                 |
| *Pseudomonas koreensis* strain SSG10 | LN(3), 2010   | HM367598, 98.8; 99.8; 99.7       | KJ191409                 |
| *Pseudomonas koreensis* strain SSG5 | LN, 2010            | HM367599, 99.9                   | KJ191405                 |
| *Pseudomonas mosselii* strain WAB1873 | LN, 2010      | AM184215, 99.7                   | KJ191414                 |
| *Pseudomonas mosselii* strain R10   | LN, 2010            | DQ073452, 99.6                   | KJ191411                 |
| *Pseudomonas putida* strain PASS3-tqmb | LR, 2009        | EU043325, 99.7                   | KJ191420                 |
| *Pseudomonas* sp. SBR3-slima        | LN, 2010            | EU043328, 99.3                   | KJ191420                 |
| *Pseudomonas tolassi* strain NCPPB 2325 | LR, 2009      | AF320990, 100                    |                            |
| *Serratia marcescens* strain C1     | LS(2), 2010         | GU220796, 99.9; 99.7             | KJ191397                 |
| *Stenotrophomonas maltophilia* strain 682-1 | LR, 2009       | AB306288, 99.9                   | KJ191382                 |
| *Stenotrophomonas maltophilia* strain YLZZ-2 | LS, 2009     | EU022689, 99.6                   | KJ191387                 |
| *Stenotrophomonas* sp. 7-3          | LR, 2009            | EU054384, 99.6                   | KJ191381                 |
| Uncultured bacterium clone nbw969a06c1 | LN, 2010       | GQ043359, 98.4                   | KJ191393                 |
| *Xanthomonas* sp. CC-AFH5            |                     | DQ490979, 98.1%                  |                            |
| Uncultured bacterium clone P7D82-747 | LN, 2010       | EF509545, 99.6                   | KJ191417                 |
| *Stenotrophomonas* sp. TSG4          |                     | HM135101, 99.6%                  |                            |

Where the closest GenBank match was an unnamed bacterium, the closest named match is included immediately below (smaller, bold text) to give the best possible sense of phylogeny.

*Where more than one inhibitory isolate most closely matched the same OTU, the percent matching is listed for both. We used the isolate with the first listed percent matching for the experimental challenge assay.

†LN = *Litoria nannotis*, LR = *Litoria rheocola*, LS = *Litoria serrata* (number of individuals from which the bacteria was isolated, if >1).

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### Discussion

**Variation in Antifungal Activity**

The metabolites of many of the defensive bacterial symbionts of amphibians we tested had reduced antifungal activity when produced at 8°C. Such reductions in anti-*Bd* activity were likely caused by changes in the quantity, identity, or both of bacterially-produced substances, and could have contributed to chytridiomycosis-driven declines that occurred in high elevation populations of the three *Litoria* species sampled here [23,31,40]. Winter air temperatures in the high elevation habitats of these species can often be lower than 8°C [26,41]. Therefore, frogs may experience decreased bacterial protection from *Bd* in winter, which in
Like the *Litoria* spp. we sampled, other amphibians may be more vulnerable to chytridiomycosis at cool temperatures if their bacterial protection from *Bd* is reduced. Larger scale, longitudinal studies characterizing the diversity and abundance of amphibian bacterial symbionts and their metabolites, *Bd* infection loads, and chytridiomycosis severity across environmental gradients and among seasons, are required to test this hypothesis.

Although the general trend was towards decreased anti-*Bd* activity when CFSs were produced at cooler temperatures, some bacteria did not show unidirectional responses to temperature (e.g., *Pseudomonas* sp. SBR3-slima, Figure 3); they produced metabolites without anti-*Bd* activity at the lowest temperature and at moderate temperatures. These complex temperature responses may have been caused by production of different antibiotics at different temperatures. Many bacteria produce more...
than one antibiotic [45], and some regulate their production through multiple genes [47] that could possess different temperature thresholds.

We selected the bacteria we tested in the experimental challenge assay because their supernatants were inhibitory when produced at 23°C in the initial screening assay. It is possible that a different subset of the entire sampled bacterial community could have been classed as inhibitory if the CFSs for the initial screening assay had been produced at different temperatures. One of our aims, however, was to document context-dependency in bioaugmentation candidates identified as inhibitory in ‘standard’ challenge assays conducted at 23°C [12,30]. Conducting this sort of laboratory assay under ecologically-relevant conditions has been identified as a necessary step towards selecting effective bioaugmentation strategies [9]. However, it is entirely possible that some bacteria that live on the skin of the *Litoria* species we sampled are effectively antifungal at low temperature and were not selected because they are not inhibitory at 23°C. Equally, the possibility of density-dependent responses of *Bd* to CFSs, if for example zoospores can degrade some bacterial products in the CFSs, means that had we conducted the initial challenge assay using more or fewer zoospores, different bacteria may have been identified as inhibitory and subsequently included in the experimental challenge assay. We used slightly different concentrations of *Bd* zoospores in the initial and experimental challenge assays due to temporal variation in the productivity of laboratory *Bd* cultures. However, all CFSs tested in the experimental challenge assay were inoculated with the same concentration of *Bd* zoospores and therefore any possible density-dependent responses of *Bd* to CFSs could not have affected the temperature-driven effects on CFS anti-*Bd* activity reported here.

To produce CFSs for the experimental challenge assay, we grew bacterial cultures to maximum absorbance in each temperature treatment. Because some bacteria may have been dying and their cells breaking apart at this stage, it is possible that these by-products, in addition to anti-fungal metabolites produced by live bacteria, could be partially responsible for observed anti-*Bd* activity. If bacteria died and broke apart to a greater extent in the higher temperature treatments, it could explain some of the reduced anti-*Bd* activity at 8°C. Further experiments employing chemical methods will be needed to definitively identify and evaluate the products responsible for differential anti-*Bd* activity of CFSs.

Our present study is the latest to find a substantial number of *Pseudomonas* species, a well-known group of antibiotic producers [45,48], among the antifungal cutaneous microbiota of amphibians [12,30,40,49]. Walke et al. [44] offered several ecological and physiological reasons for the prevalence of *Pseudomonads* among the defensive microbiota of amphibians. Our results show that the anti-*Bd* activity of at least some *Pseudomonads* depends on
environmental context (e.g., *Pseudomonas* sp. SBR3-slima, Figure 3). Only the most robustly antifungal isolates should be used in bioaugmentation, regardless of how common they may be.

### Management Implications and Future Research

Bacterial antifungal activity observed under a narrow spectrum of laboratory conditions could be lost on exposure to variable field environments. Using antifungal bacteria with inconsistent activity in bioaugmentation efforts could cost managers time and resources, and could create the illusion that bioaugmentation is less effective than if more appropriate isolates were used. Even closely related bacteria may respond differently to environmental variations, as did the Pseudomonads in Figure 3. One produced strongly antifungal metabolites across the entire 8–33°C range, whereas at 8°C others produced metabolites with no antifungal activity.

Only a few studies have characterized the metabolic products of amphibian symbionts [50–52]. Based on our observations of varied responses of *Bd* to CFSs from different bacteria, and the phylogenetic range represented in addition to the many Pseudomonads found, it is likely that a variety of different antifungal compounds were produced by the bacteria we tested. As mentioned above, future workers should seek to identify these compounds, so that it is possible to measure their concentrations on amphibian skin.

Our study focused on environmental context-dependent changes in the bacterial production of anti-*Bd* metabolites, but not on possible context-dependent changes in the fungus itself, or in the direct interaction between bacteria and *Bd* on the skins of live frogs. This study constitutes a step towards understanding environmentally induced variation in the amphibian-*Bd*-bacteria symbiosis, but no study has yet simultaneously assessed the responses of both *Bd* and bacteria to varying temperatures. Additionally, no mesocosm or field study of bioaugmentation in a natural environment has been published to date, and a host of questions remain surrounding the best methods of application of beneficial bacteria, non-target effects, and the term of protection afforded. Carrying out *Bd*-bacteria research *in vivo*, and ultimately in natural systems, will be necessary preliminary steps for bioaugmentation application, even if interpretation of specific experimental treatments is complicated. Given the opportunity to apply bioaugmentation for restoration and protection of many amphibian species globally, it is most important to develop effective management protocols for use with robustly-antifungal bacteria commonly found on target species' skin.

Research on the drivers of other wildlife diseases should also consider the possible effects of environmental context dependence. Some coral disease is exacerbated by warmer environmental temperatures [53]. White-nose syndrome in bats is driven by changes in the temperature of bat hibernacula, which may allow management by artificial temperature regulation in caves [54]. In the case of chytridiomycosis, seasonal and local temperature variation are important, both for their direct effects on the host-pathogen relationship [3,55] and because they modify relationships within the skin microbe assemblage as described here. The effects of environmental context can thus occur through multiple pathways and can determine the extent to which a disease threatens biodiversity. A thorough understanding of environmental context dependence must therefore be a priority when designing disease management strategies.

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Author Contributions
Conceived and designed the experiments: JHD SCB. Analyzed the data: JHD SCB LS RAA. Performed the experiments: JHD SCB. Contributed reagents/materials/analysis tools: RAA. Wrote the paper: JHD SCB LS RAA.

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