Cutaneous bacteria, but not peptides, are associated with chytridiomycosis resistance in Peruvian marsupial frogs

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Abstract
Amphibians are a highly threatened vertebrate group, and populations of these animals have declined drastically. An important global threat to amphibians is the pathogenic fungus Batrachochytrium dendrobatidis (Bd), which causes the disease chytridiomycosis. However, not all species develop chytridiomycosis when exposed to Bd. We compared susceptibility to disease in two species of marsupial frogs and found that Gastrotheca nebulanastes is susceptible, whereas its congeneric G. excubitor is resistant. Since Bd is a skin pathogen, it is possible that cutaneous defenses like symbiotic bacteria and antimicrobial peptides protect the resistant species. We tested this hypothesis by comparing the anti-Bd abilities of cutaneous defenses between the two Gastrotheca species. Cultivable bacteria and peptides were isolated from the skin and tested for their abilities to inhibit Bd with in vitro co-culture assays. Twenty-six bacteria were identified by sequencing their 16S rRNA gene and 19 peptides were profiled by MALDI TOF mass spectrometry. We found that bacteria, but not peptides, differed between the two species in their ability to inhibit Bd growth. The resistant G. excubitor harbored more isolates of cultivable anti-Bd bacteria both in number and proportion (6/15 vs. 1/11). Also, the one anti-Bd isolate from G. nebulanastes demonstrated the weakest ability to inhibit Bd growth. Our results highlight the importance of anti-Bd skin bacteria in providing frog species with protection from Bd and can inform mitigation strategies for other wildlife diseases.

Introduction
Cutaneous defenses are important for amphibians because the permeability of their skin allows easy access for pathogens (Rollins-Smith et al., 2011). The pathogenic chytrid fungus Batrachochytrium dendrobatidis (Bd; Longcore, Pessier & Nichols, 1999) invades the amphibian skin with motile zoospores, which encyst and proliferate in keratinized dermal tissues (Berger et al., 2005a; Kilpatrick, Briggs & Daszak, 2010). Eventually, the functionality of the integument is interrupted with the onset of chytridiomycosis (Berger, Speare & Skerratt, 2005b; Voyles et al., 2009). This disease is of conservation concern because of its recent global spread and lethal effects (Daszak & Berger, 1999). Understanding the role of skin defenses may lead to the development of novel Bd mitigation strategies. Although the adaptive immune system also plays an important role in providing protection from disease (McMahon et al., 2014), here we only focus on skin defenses critical during the initial stages of inoculation and spread of Bd.

Amphibian skin is coated in a mucus consisting of a micro-environment of symbiotic bacteria and host defense compounds. These bacteria are transmitted from soil (Muletz et al., 2012) and water (Harris et al., 2009b) via sharing of refugia, and possibly via vertical transmission in species that provide parental care (Walke et al., 2011). This microbial community is often diverse (Lauer et al., 2008; Jani & Briggs, 2014) and host-specific (McKenzie et al., 2012), but community composition can vary among discrete populations of the same host species (Lam et al., 2010). Certain bacteria isolated from the skin of chytridiomycosis-resistant amphibians have demonstrated the ability to inhibit the growth of Bd in vitro (Harris et al., 2006; Flechas et al., 2012) because they produce anti-Bd metabolites (Brucker et al., 2008; Loudon et al., 2014). The experimental addition of anti-Bd bacteria to susceptible hosts has mitigated the effects of chytridiomycosis (Harris et al., 2009b), suggesting hope for bioaugmentation therapy with probiotics (Bletz et al., 2013).

Additionally, some frogs secrete antimicrobial peptides (AMPs) on their skin. Natural peptide mixtures collected from several species of frogs successfully inhibited the growth of Bd in vitro (Rollins-Smith et al., 2002a). A better understanding of how AMPs and skin bacteria contribute to chytridiomycosis resistance will inform strategies to protect...
vulnerable frogs in situ, allowing populations to persist in their natural environment.

We tested the efficacy of cutaneous defenses from marsupial frogs (Hemiphractidae) in a community decimated by Bd in Peru (Catenazzi et al., 2011; Catenazzi, Lehr & Vredenburg, 2014). We first examined variation in disease susceptibility by comparing survivorship of Gastrotheca excubitor and G. nebulanastes after exposure to Bd zoospores. Following results of these experiments, we tested the hypothesis that variation in susceptibility could be explained by interspecific differences in cutaneous defenses, as previously suggested with bacteria (Rebollar et al., 2016) and peptides (Woodhams et al., 2006). We sampled frogs for cultivable skin bacteria, which were identified by DNA barcoding, and for natural peptide mixtures, whose compositions were determined by mass spectrometry. Finally, bacteria and peptide mixtures were each tested for their abilities to inhibit the growth of Bd in co-culture assays, and results were interpreted in light of our findings from the susceptibility experiments.

Materials and methods

Study species and Bd surveys

We collected marsupial frogs Gastrotheca excubitor and G. nebulanastes in montane scrub, cloud forest, and high elevation grassland habitats from 2000 to 3800 m a.s.l. near Manu National Park in southeastern Peru. Prevalence of Bd infection has been similar in these species in 2008 (no infections) and in 2009 (18% for G. excubitor and 25% for G. nebulanastes) (Catenazzi et al., 2011). At the time of capture we recorded each frog’s elevation, microhabitat use, and height above ground. Bacterial sampling and infection experiments occurred from May to August 2012 (using the same frogs), peptide sampling from May to August 2014, and Bd sampling from May 2012 to August 2014 (Supporting Information Table S1). Our research was approved by the Institutional Animal Care and Use Committees of Southern Illinois University and San Francisco State University.

Each captured frog was sexed, measured, weighed, and their skin swabbed for the presence and intensity of Bd infection following Hyatt et al. (2007). We extracted DNA from swabs using PrepMen Ultra and analyzed DNA using real-time quantitative PCR (qPCR) with an Applied BioSystems (Foster City, CA, USA) 7300 (2012 samples), and a Life Technologies (Carlsbad, CA, USA) StepOne Plus (2014 samples) according to the protocol of Boyle et al. (2004) and Hyatt et al. (2007), except extracts were analyzed once instead of three times. We calculated zoospore equivalent (ZE), the genomic equivalent for Bd zoospores, by comparing the qPCR results to a set of standards of known concentrations (extracts obtained from cultured Bd strain GPL 1; 100, 10, 1.0 and 0.1 zoospore genomic equivalents).

Disease resistance

We exposed wild frogs to live zoospores shed by highly infected hosts, and compared their survivorship with survivorship of non-exposed frogs. The conditions at our remote field station did not allow us to use pure Bd cultures, nor to use hosts of known infection status history. Before our experiments, we cleared any pre-existing infection by immersing frogs in a 1% itraconazole solution for 5 min a day for 7 days (Garner et al., 2009). Each animal was then randomly assigned into either the treatment group (Bd-exposed, \( n = 5 \) G. excubitor and 6 G. nebulanastes) or the control group (non-Bd-exposed, \( n = 5 \) G. excubitor and 7 G. nebulanastes). We used 14 locally sourced and Bd-infected Telmatobius marmoratus (Catenazzi, Vredenburg & Lehr, 2010; Warne et al., 2016) to expose the treatment group to Bd (Supporting Information Appendix S1).

Each frog in the treatment group was placed in a two-chambered exposure container with a single Bd-infected T. marmoratus for 5 h per day for five consecutive days from 30 June to 4 July 2012. Each container had two parallel mesh barriers affixed across the middle, preventing direct contact between frogs, but allowing mixing of a thin layer of water across the two sides. Control group frogs were maintained in similar containers but without T. marmoratus.

After infection, starting on 5 July we monitored Bd infection by swabbing frogs at weekly intervals until the death of the animal, or until the end of the experiment on 26 August (52 days for surviving frogs). All frogs were fed daily and were housed individually in 1.2 L plastic containers with vented lids, where they experienced natural variations in temperature and photoperiod (Supporting Information Appendix S1, Fig. S1). Bd-infection status and body weight were recorded at the time of capture, after the anti-Bd treatment, and at weekly intervals.

Bacterial sampling and identification

Immediately following capture, we handled frogs (12 G. excubitor and 8 G. nebulanastes) using sterile nitrile gloves and rinsed them twice with dechlorinated water (Lauer et al., 2008) before swabbing them on their left, right, ventral surfaces, hindlimbs, and interdigital membranes (Flechas et al., 2012). Each swab was streaked directly onto nutritive agar and incubated for 2 weeks. Each bacterial morphotype was then streaked onto a new nutritive agar plate, until obtaining pure colonies. We identified bacterial isolates by sequencing the 16S rRNA gene (Supporting Information Table S2). We extracted DNA using Prepman Ultra, and amplified it by PCR with GoTaq Green Master Mix (Promega Corporation, Madison, WI, USA) and 16S primers 27F and 1492R (Lane, 1991). PCR products were verified by gel electrophoresis and sequenced (MCLAB, San Francisco, CA, USA). We used GENEIOUS (Biomatters Limited, Auckland, New Zealand) to assemble sequences consisting of at least 60% high quality base pairs, and to compare consensus sequences to known microbial DNA sequences using the default parameters of BLAST search with the NCBI database. Identities of nearest relatives were approximated to genus or species level based upon the identity grades of the BLAST results. Isolates were considered to be the same bacterium if their 16S consensus sequences were >99% similar.
Bacteria Bd-growth inhibition assays

We tested the ability of bacteria to inhibit Bd-growth in triplicate via a co-culture assay (Harris et al., 2006). Bd was isolated from the mouthparts of a Hyla arborea (Hylidae) tadpole captured near our study site in June 2012, and maintained in culture on TgH medium (10 g tryptone, 10 g agar, 4 g gelatin hydrolysate, 1 L distilled water) at 23°C (Flechas et al., 2012). We harvested zoospores and combined them with 16 mL of water. One mL of this zoospore rich solution was spread on to a Petri dish containing TgH medium and allowed to dry. Each isolate of Gastrotheca bacteria was streaked in a line across one side of the Petri dish, and the negative control Escherichia coli (strain DH5α without anti-Bd ability) was streaked across the other side of the dish. Each Petri dish was incubated at 23°C for 3 days, and then checked for a zone of inhibited Bd growth around the isolate. The strength of an isolate’s anti-Bd ability was determined by quantifying the relative distance from the isolate to where 50% Bd growth occurred (Supporting Information Appendix S2), so that isolates with stronger inhibitory abilities had greater distances.

AMP sampling

We stimulated peptide secretion by injecting 40 nmol norepinephrine per g body weight (Nutkins & Williams, 1989; Rollins-Smith et al., 2002b; Holden et al., 2015) into the dorsal lymph sacks of G. excubitor (n = 47) and G. nebulanastes (n = 36), then placed frogs in plastic bags with 24 mL of HPLC water to collect peptides. After 15 min we removed frogs, added 1 mL of 50% HCl (Woodhams et al., 2006), and partially filtered samples through Sep Pak C-18 cartridges (Waters Corporation, Milford, MA, USA) (Goraya, Knoop & Conlon, 1998). Peptides were eluted from cartridges with a 70% acetonitrile and 0.1% HCl solution and vacuum centrifuged to dryness. We measured peptide concentrations with a Pierce microBCA protein assay kit (Thermo Scientific, Rockford, IL, USA) using bradykinin as the standard (Smith et al., 1985; Rollins-Smith et al., 2002b).

Bd-growth inhibition assays for peptide mixtures

We tested dilutions (500, 250, 100, 50, 25, 12.5, 6.25 and 3.125 µg mL⁻¹) of whole peptide mixtures of G. excubitor (n = 9) and G. nebulanastes (n = 8). We obtained zoospores from Bd cultures (JEL 197; J. E. Longcore, University of Maine, Supporting Information Appendix S3) on agar plates flooded with 1% tryptone broth containing penicillin and streptomycin by filtering (20 µm filter) the broth under vacuum. Growth inhibition assays of 5 × 10⁵ zoospores were conducted on 96-well microtiter plates and consisted of five replicates of: each peptide dilution, positive control (Bd without peptides), and negative control of heat-killed (10 min at 60°C) zoospores. The absorbance at 490 nm (A₄₉₀; Rollins-Smith et al., 2002b) was recorded using an accuSkran GO UV/VIS spectrophotometer (Fisher Scientific, Hampton, NH, USA). Plates were then covered by a lid, sealed, and incubated at 19–20°C for 7 days, when A₄₉₀ was recorded again. Assays with more than three contaminated wells within a treatment were removed from analysis. The mean relative Bd growth (pΔA₄₉₀) was determined for each peptide dilution (Supporting Information Appendix S3). pΔA₀₉₀ < 1 represented Bd-growth inhibition, while pΔA₀₉₀ > 1 represented enhanced Bd-growth.

Peptide composition analysis

Peptide composition profiles were obtained for 36 G. nebulanastes and 47 G. excubitor via matrix-assisted laser desorption ionization time-of-flight mass spectrometry (Rollins-Smith et al., 2006; Woodhams et al., 2006) using a Thermo Scientific TraceGC PolarisQ Mass Spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) in linear mode, calibrated using the following standards (Sigma Aldrich, St. Louis, Missouri USA): bradykinin (m/z 904.468), angiotensin I (m/z 1296.685), glu-fibrinopeptide B (m/z 1570.677), ACTH 1-17 (m/z 2093.085), ACTH 16-39 (m/z 2465.199), and insulin oxidized B chain (m/z 3494.6513) (Holden et al., 2015). Combined standards were mixed with a matrix (10 mg mL⁻¹ α-cyano-4-hydroxycinnamic acid, 50% acetonitrile, and 0.1% trifluoroacetic acid in MilliQ water) at a ratio of 1:25 and samples were mixed with matrix at a ratio of 1:1.

Statistical analyses

All statistical analyses were completed using R v3.0.2 (R Core Team, 2008). We analyzed elevation data by two-way ANOVA, microhabitat use by a chi-squared test, and height above ground with a t-test. For Bd prevalence we considered swabs as Bd-positive if ZE > 0 and Bd-negative if ZE = 0. We log-transformed values [log(ZE + 1), henceforth ‘zoospores’] for infection levels analyses. We calculated prevalence data using Bayesian inference with Jeffrey’s non-informative priors in the ‘binom’ package (Dorai-Raj, 2014). We used the ‘survival’ package ( Therneau & Grambsch, 2000; Therneau, 2012) to compare survivorship of control and treatment groups, adding Cox’s proportional hazards model with censoring (Cox, 1972) to assess the risk of dying for Bd-exposed frogs. We performed separate analyses of covariance with initial weight as the continuous explanatory variable and treatment group as the categorical explanatory variable since initial weight could have affected survival (it did not and was removed from the models).

Peptide amounts were log-transformed to meet the requirements of normality and compared between species and sex/life stage (males, females, or juveniles) using a two-way ANOVA with Tukey’s post hoc test. Data from inhibition assays were normally distributed (indicated by plotting quantiles), and we removed outliers using Dixon’s Q test (Holden et al., 2015). We compared pΔA₀₉₀ from peptide-Bd inhibition assays between species by a split-plot ANOVA nesting individual as a random effect within species. The model was
adjusted with a Kenward-Roger degrees of freedom approximation and paired with a Tukey’s post hoc test.

Peptide profiles generated by mass spectrometry were analyzed using Flex Analysis (Bruker, Billerica, MA, USA). Peptide signals were compared between species using all samples and among samples from individuals used for growth inhibition assays. We analyzed only signals with frequency of detection of a peptide within a sample >10% and mass to charge ratios within the range of the standards. Signals +C6±2 Da were considered the same peptide, and those that were +22±2 Da were considered the sodium adducts of the same peptide. The effect of common peptide signals (shared by two or more assayed samples) on Bd inhibition was determined with one-way ANOVA.

Results

Frog distribution and Bd infection

The Gastrotheca species differed significantly in their elevational distributions (d.f. = 1205, and \( P < 0.001 \)). Gastrotheca nebulastases were encountered from 2200 to 3000 m whereas G. excubitor was found from 3400 to 3800 m (Fig. 1b). Moreover, G. nebulastases inhabited montane forests and perched on leaves or branches on average 83.21 ± 15.04 cm above the ground, whereas G. excubitor was exclusively terrestrial (student’s \( T \) test, d.f. = 25, \( P < 0.001 \)) and was found in grasslands under rocks and soil (\( \chi^2 = 79, \) d.f. = 6, \( P < 0.001 \), Fig. 1a and c). Bd prevalence was similar among species but differed among years (Fisher’s exact test, \( P = 0.005 \), Table 1), and when removing the outlying prevalence for G. excubitor in 2012 there was no effect (Fisher’s exact test, \( P = 0.214 \)). Infection intensities were low in 2012 (<350 zoospores, except for one G. excubitor with 26 209 zoospores) and 2013 (all with <50 zoospores). In 2014, infection intensities varied from 1 to 126 zoospores in G. nebulastases and from 1 to 1223 zoospores in G. excubitor.

Resistance to chytridiomycosis

While all G. excubitor survived the experiment (Supporting Information Fig. S3a), Bd exposure increased mortality in G. nebulastases (Fig. 2a, \( P = 0.003 \)), despite the itraconazole bath not completely clearing Bd infection in control G. excubitor (all but one control frog remained infected, with

![Figure 1](image_url) Distribution of Gastrotheca excubitor (black, brown online) and G. nebulastases (gray, green online) differ by (a) the type of substrate, (b) their elevational ranges (puna = high–Andean grasslands), (c) the height above the ground when encountered, and (d) the compositions of their skin bacterial communities (* indicates anti-Bd bacteria). Inset photographs by D. Burkart.

| Table 1 Variation in Bd prevalence (confidence intervals in parentheses) of Gastrotheca excubitor and G. nebulastases |
|-----------------|---------|---------|---------|---------|---------|
| Species         | 2012    | 2013    | 2014    | 2014    | 2014    |
| G. excubitor    | 46% (29–64%) | 28 | 4% (0–14%) | 27 | 17% (8–29%) | 46 |
| G. nebulastases | 25% (8–47%) | 16 | 23% (5–47%) | 13 | 14% (5–26%) | 36 |
mean infection 1.23 ± 0.30 zoospores), but clearing infection in *G. nebulanastes*. Among Bd-exposed frogs, levels of infection 25 days after exposure were lower in *G. excubitor* (0.18 ± 0.11 zoospores) than they were in *G. nebulanastes* (2.42 ± 0.67 zoospores; \( t = 3.31, \) d.f. = 8, \( P = 0.01 \)). Furthermore, zoospores increased with time in Bd-exposed *G. nebulanastes* (\( R^2 = 0.86, F_{1,7} = 41.72, P < 0.001 \)), but not in Bd-exposed *G. excubitor* (\( R^2 = 0.01, F_{1,3} = 0.03, P = 0.88 \)).

**Bacteria Bd-growth inhibition assays**

Six out of 15 *G. excubitor* isolates, but only one out of 11 *G. nebulanastes* isolates were labeled as anti-Bd (Fig. 1d). Among isolates from *G. excubitor*, *Pseudomonas* sp. 9, was the strongest anti-Bd bacterium, with 50% Bd growth occurring at 75% of the distance from the isolate, whereas *Janthinobacterium lividum* was the weakest with 50% Bd growth occurring at 40% of the distance (Fig. 3). The one inhibitory isolate from *G. nebulanastes*, *Pseudomonas* sp. 3, was the weakest among all anti-Bd isolates, and 50% of Bd growth occurred at 38% of the distance.

**AMP Bd-growth inhibition assays**

Four out of eight peptide mixtures from *G. nebulanastes* and five out of nine from *G. excubitor* inhibited Bd. The two species’ peptides did not differ in their abilities to inhibit Bd growth (Fig. 4, split-plot ANOVA, d.f. = 1,15, \( P = 0.96 \)). There was no interaction between species and peptide mixture concentration (d.f. = 7,105, \( P = 0.86 \)). However, there was a significant effect of peptide concentration (disregarding species) (d.f. = 7,105, \( P = 0.046 \)): inhibition was higher at 500 \( \mu \)g mL\(^{-1}\) than at 6.25 \( \mu \)g mL\(^{-1}\) (Tukey’s post hoc test, d.f. = 7,105, \( P = 0.016 \)).

We found 19 different peptides, 14 in *G. excubitor*, 10 in *G. nebulanastes*, and five shared (Supporting Information Appendix S4, Fig. S4). The most common shared peptide occurred in 27/46 *G. excubitor* and in 1/36 *G. nebulanastes* and had a mass to charge ratio of 1430 ± 2 Da; also present was its sodium adduct at 1452 ± 2 Da (Supporting Information Figs S4–S5). The other peptides were not shared. There was no correlation between presence of a peptide type and Bd growth effects, except for a *G. excubitor* peptide at 1298 ± 2 Da that
enhanced Bd growth (ANOVA, d.f. = 1,7, P = 0.011). Peptide amounts did not vary between species (d.f. = 1, 77; P = 0.363; Supporting Information Table S1).

**Discussion**

The two host species differed in resistance to chytridiomycosis because all of the *G. excubitor* survived the disease resistance experiment, whereas only one of the Bd-exposed *G. nebulanastes* survived. In Bd-exposed *G. nebulanastes* the infection levels increased with time since infection, whereas Bd-exposed *G. excubitor* maintained low infection levels throughout the experiment. The resistant *G. excubitor* harbors a greater proportion of anti-Bd skin bacteria than *G. nebulanastes* (40% vs. 9%, respectively). The anti-Bd abilities of peptide mixtures between the species did not differ and none completely inhibited the growth of Bd. Therefore, the difference in susceptibility between the *Gastrotheca* species is more likely attributed to differences in their cutaneous bacterial compositions, or other factors. These results demonstrate that chytridiomycosis resistance correlates with the number and proportion of a frog species’ anti-Bd skin bacteria (Lam et al., 2010; Flechas et al., 2012). The presence of *J. lividum* on *G. excubitor* is of particular interest. Anti-Bd strains of this bacterium have been isolated from multiple amphibian species (Woodhams et al., 2015), and have been applied successfully as a probiotic in laboratory experiments (Harris et al., 2009a,b). Despite *J. lividum* being well-known for the strong anti-Bd properties of its metabolites violacein and indole-3-carboxaldehyde (Brucker et al., 2008), our strain was the second weakest for anti-Bd capacity (Fig. 3). We did not observe the distinctive purple coloration characteristic of *J. lividum* in culture, attributed to the production of violacein. It is possible that our strain does not produce violacein but still produces indole-3-carboxaldehyde.

Although not previously known for its anti-Bd ability, we found that *Rahnella aquatilis* was a relatively strong inhibitor (Fig. 2). This bacterium is an antifungal symbiont of the Chinese beetle *Dendroctonus valens* (Winder, Macey & Cortese, 2010). Two isolates from *G. excubitor* in the genus *Pseudomonas* were both closely related to *Pseudomonas poae*, an antifungal symbiont of the sugar beet (Müller et al., 2013). Although both the isolates *Pseudomonas* sp. 1 and *Pseudomonas* sp. 2 had similar sequences, they differed in their abilities to inhibit Bd (Fig. 2). Additionally, some non-anti-Bd isolates had either identical or similar sequences to anti-Bd *Pseudomonas* strains. These findings suggest that functional variance can occur between closely related strains, however, *Pseudomonas* species are difficult to differentiate by their 16S rRNA sequence alone (Janda & Abbott, 2007) so further genetic analysis is necessary to determine if these strains are indeed separate species. The similarities of these *Pseudomonas* isolates could be due to the low quality of many of their 16S sequences. The one anti-Bd strain from *G. nebulanastes*, *Pseudomonas* sp. 3, is closely related to a bacterium known for having antifungal properties (Godfrey

![Figure 4](Fraction of maximum Bd growth (see Appendix S3) for each peptide sample assayed (mean, n=5) per concentration.)
et al., 2001). Although labeled as anti-Bd on the basis of its inhibition zone in one replicate, the removal of a replicate (see Supporting Information Appendix S2) questions the integrity of its inhibitory abilities. Therefore, we labeled Pseudomonas sp. 3 as potentially anti-Bd and suggest further testing to verify this ability.

Our results fail to support the hypothesis that AMPs explain the difference in susceptibility between these Gastrotheca species, and confirms previous findings that the expression of AMPs does not consistently correlate with resistance to Bd (Rollins-Smith et al., 2002b; Conlon et al., 2007; Woodhams et al., 2007). It is important to consider that host defenses may change temporally, differ among conspecifics (Holden et al., 2015) or be altered by itraconazole treatment. Itraconazole inhibits certain cytochrome P450 enzymes (Schuster, 1993), which are sometimes expressed by prokaryotes. Little information is available on which bacterial genera express them; but investigations of some Pseudomonas, Bacillus, Acinetobacter, and Sphingomonas species have failed to detect the expression of these enzymes (McLean, Leys & Munro, 2015). We did not investigate the role of the adaptive immune system in these frogs, which could also provide protection from chytridiomycosis. Our use of a different Bd strain for the AMP and bacterial assays may also influence results, however, the focus of this study is to compare defenses between two species, not the defenses themselves.

Using anti-Bd bacteria in bioaugmentation therapy is promising, but will require large-scale sampling of naturally occurring skin bacteria (Bletz et al., 2013). Here, we have discovered at least four novel anti-Bd strains, but the only bioaugmentation therapy candidate for G. nebulanastes is Pseudomonas sp. 3, whose anti-Bd capabilities require further validation. Since the females of G. excavitor and G. nebulanastes keep eggs in their dorsal brood pouch until the hatching of froglets (Duellman, Catenazzi & Blackburn, 2011), vertical transmission of cutaneous bacteria between a mother and offspring is likely, as seen for other amphibians with parental care (Banning et al., 2008).

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References


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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

Figure S1. Comparison of temperatures experienced by frogs during experimental housing at Wayqecha Field Station, 2850 m a.s.l. (thick lines, 28 June–25 August 2012; daily average from temperatures taken at 10-min intervals) and environmental temperatures in natural microhabitats of Gastrotheca excubitor and G. nebulanastes (thin lines, daily averages from temperatures taken at hourly intervals during 2014 and 2015).

Figure S2. A visual example of how Bd inhibition was quantified for Bacteria-Bd growth inhibition trials.

Figure S3. (a) Percentage survival in Bd exposed and control Gastrotheca excubitor. (b) Variation in log-transformed zoospore equivalents (Bd infection intensity) in Bd exposed individuals. (c) Variation in log-transformed zoospore equivalents (Bd infection intensity) in control (non Bd-exposed) individuals.

Figure S4. Sample based peptide accumulation curves for peptides sampled from (a) Gastrotheca excubitor and (b) G. nebulanastes.

Figure S5. Representative mass spectra for G. nebulanastes.

Figure S6. Representative mass spectra for G. excubitor.

Table S1. Sample sizes (per species), purpose, and collection year for frogs used in this study.

Table S2. Genbank accession numbers for the strains sampled in this study.

Table S3. Amount of peptides in peptide mixtures recovered from the skin of Gastrotheca excubitor and G. nebulanastes.

Appendix S1. Methods for disease resistance experiments.


Appendix S4. Results for antimicrobial peptide (AMP) Bd-growth inhibition assays.