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PREDICTED DISEASE SUSCEPTIBILITY IN A PANAMANIAN AMPHIBIAN ASSEMBLAGE BASED ON SKIN PEPTIDE DEFENSES

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ABSTRACT: Chytridiomycosis is an emerging infectious disease of amphibians caused by a chytrid fungus, *Batrachochytrium dendrobatidis*. This panzootic does not equally affect all amphibian species within an assemblage; some populations decline, others persist. Little is known about the factors that affect disease resistance. Differences in behavior, life history, biogeography, or immune function may impact survival. We found that an innate immune defense, antimicrobial skin peptides, varied significantly among species within a rainforest stream amphibian assemblage that has not been exposed to *B. dendrobatidis*. If exposed, all amphibian species at this central Panamanian site are at risk of population declines. In vitro pathogen growth inhibition by peptides from Panamanian species compared with species with known resistance (*Rana pipiens* and *Xenopus laevis*) or susceptibility (*Bufo boreas*) suggests that of the nine species examined, two species (*Centrolene prosoblepon* and *Phyllomedusa lemur*) may demonstrate strong resistance, and the other species will have a higher risk of disease-associated population declines. We found little variation among geographically distinct *B. dendrobatidis* isolates in sensitivity to an amphibian skin peptide mixture. This supports the hypothesis that *B. dendrobatidis* is a generalist pathogen and that species possessing an innate immunologic defense at the time of disease emergence are more likely to survive.

Key words: Antimicrobial peptide, *Batrachochytrium dendrobatidis*, chytridiomycosis, MALDI mass spectrometry, population decline.

INTRODUCTION

Emerging infectious diseases of wildlife present a growing threat to the conservation of biodiversity (Daszak et al., 2000; Cleaveland et al., 2001). Chytridiomycosis is an emerging infectious disease of amphibians caused by *Batrachochytrium dendrobatidis* that affects some species more than others within an assemblage (Berger et al., 1998; Lips et al., 2003b; Retallick et al., 2004; Woodhams and Alford, 2005). For example, chytridiomycosis-associated population declines in Queensland, Australia, may have contributed to the extirpation of some species from high elevations, whereas other species persisted in the same habitat (Richards et al., 1993; Berger et al., 1998). Pathogens such as *Ribeiroia ondatrae* and iridoviruses also affect amphibian species differently (Green et al., 2002; Johnson et al., 2002).

The global trend in amphibian population declines (Stuart et al., 2004) is demonstrated regionally in Central America. Although it is not the only agent of declines in this region, the southeastward spread of chytridiomycosis is depleting amphibian populations (Daszak et al., 2003; Lips et al., 2003a). Monitored populations have declined throughout Costa Rica (Pounds et al., 1997; Lips, 1998) and western Panama, and chytridiomycosis was associated with these events (Berger et al., 1998; Lips, 1999; Lips et al., 2003a).

Little is known about the factors that affect disease resistance or susceptibility in amphibians. Although there is an effort to predict the probability of population decline of species within the rainforest amphibian assemblage in Panama based on ecologic characteristics (Lips et al., 2003b), no studies have yet investigated

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the immune defenses of these species. Defense of the skin is critical for protection from many amphibian pathogens, including *Aeromonas hydrophila*, parasitic water molds (reviewed in Pessier, 2002; Rollins-Smith et al., 2002a), and *Ribeiroia ondatrae* (Johnson et al., 2001, 2002). *Batrachochytrium dendrobatidis* colonizes keratinized epithelium (Berger et al., 1998; Longcore et al., 1999; Pessier et al., 1999). Defense of amphibian skin involves adaptive and innate immune defenses (Carey et al., 1999). The skin of all vertebrates can develop adaptive immune responses. That is, antigen detected by antigen-presenting cells in the skin can activate both cell-mediated (T cell) and humoral (B cell) defenses. Lack of lymphocytic infiltration in the chytrid-infected skin of frogs suggests that these animals have a poor cell-mediated immune response against *B. dendrobatidis* (Berger et al., 1998; Pessier et al., 1999), and there is currently no evidence for a humoral defense. Innate defenses such as epithelial barriers, phagocytic cells, and antimicrobial peptides may be vital.

Recent evidence suggests that antimicrobial peptides may be important defenses against *B. dendrobatidis* infection. In vitro studies showed that many isolated antimicrobial peptides of frogs had potent activity against the fungal pathogen (Rollins-Smith and Conlon, 2005). Natural mixtures of skin peptides are also active (Rollins-Smith et al., 2002b; Woodhams et al., 2006), and this may be more relevant to the natural immune defense of amphibians given that some peptides may act in synergy to inhibit pathogen growth (Westerhoff et al., 1995; Rollins-Smith et al., 2002a). Anuran species show significant variation in the number, structure, and antimicrobial activity of skin peptides (Vanhoye et al., 2003; Apponyi et al., 2004; Conlon et al., 2004).

Susceptibility to disease may be partly controlled by immune defenses that vary among individuals and species. Our aim was to develop an index of predicted resistance to chytridiomycosis. In forming this index, we included measures of quantity of skin peptides produced and their potency against *B. dendrobatidis* in vitro. This index may be applied to predict the relative disease susceptibility of species within an assemblage. There are few other predictive tools to indicate the risk of disease emergence.

Some species may have strong peptide defenses and unique skin peptide profiles or "fingerprints." Matrix-assisted laser desorption/ionization mass spectrometry (MALDI MS) is a rapid method for obtaining a profile or "fingerprint" of peptides secreted from amphibian skin (Conlon et al., 2004). In addition to differentiation of species, skin peptide profiles can reveal variation within species and indicate evolutionary relationships (Apponyi et al., 2004). Individual variation in the peptide profile may indicate novel protective peptides that correspond to antimicrobial potency. Identifying these protective peptides in other species may also improve disease-risk prediction.

MATERIALS AND METHODS

Collection of skin peptides

Skin peptides were sampled in January 2004 from adult amphibians of nine species in the rainforest of Omar Torrijos H. National Park, El Copé, Coclé, Panama (8°40′N, 80°37′W) at an elevation of approximately 850 m. Amphibian populations at this site have been monitored since 1997 (Lips et al., 2003b). The species sampled included anurans *Atelopus varius* ($n=8$), *Centrolene ilex* ($n=5$), *Centrolene prosoblepon* ($n=7$), *Colostethus panamensis* ($n=12$), *Eleutherodactylus punctariolus* ($n=5$), *Phyllomedusa lemur* ($n=9$), and *Rana warszewitschii* ($n=1$); and urodeles *Bolitoglossa colonnea* ($n=2$) and *Bolitoglossa schizodactyla* ($n=10$). Monitoring for the presence of *B. dendrobatidis* is ongoing and consists of swabbing the ventral surface or drink patch, thighs, and toe webbing 10 times each with sterile toothpicks (two per individual) and preserving them in 70% ethanol. *Batrachochytrium dendrobatidis* was not detected on over 800 individual amphibians sampled before August 2004 using polymerase chain reaction (PCR) analysis (Pisces Molec-

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ular, Boulder, Colorado, USA; Lips, unpubl. data). As the amphibians sampled were not infected, the assemblage was thought to be naïve to the pathogen.

For comparison with Panamanian amphibians, we also sampled skin peptides from species with known susceptibility or resistance (Table 1): *Rana pipiens* (BioCorporation, Alexandria, Minnesota, USA; $n=5$ adults), *Xenopus laevis* (Xenopus I, Dexter, Michigan, USA; $n=5$ adults), and *Bufo boreas* (Colorado Division of Wildlife, John W. Mumma Native Aquatic Species Restoration Facility, Alamosa, Colorado, USA; $n=10$ newly metamorphosed). Survival of natural populations of *R. pipiens* and *X. laevis* infected with *B. dendrobatidis* (Green et al., 2002; Weldon et al., 2004; Ouellet et al. 2005) and resistance to experimental exposure (Rollins-Smith and Conlon, 2005; Carey, unpubl. data) demonstrate that these species are quite resistant to disease development. We found that some of the *R. pipiens* and *X. laevis* sampled in our laboratory colony carry a sublethal infection of *B. dendrobatidis* by PCR analysis of skin swabs (A. Hyatt, CSIRO, Australian Animal Health Laboratory, Geelong, Victoria, Australia). In contrast, *B. boreas* was highly susceptible to *B. dendrobatidis* in natural populations (Muths et al., 2003), and in exposure experiments (Blaustein et al., 2005). Although mortality rate depended on dose and time of exposure, 100% mortality was reached within 26 days after exposure of toads to 1,000 zoospores (Carey, unpubl. data).

Skin secretions containing a mixture of peptides were collected from amphibians after administration of norepinephrine by subcutaneous injection as described by Rollins-Smith et al. (2002b). This method was designed to provide a standardized stimulus to induce skin secretions from a variety of small tropical amphibians and to avoid the more painful transcutaneous electrostimulation procedure, which requires calibration for each species (Tyler et al., 1992). Amphibians were captured by hand inside new plastic bags and weighed within 0.1 g. With minimal handling, frogs were injected with 10 nmol (0.01 ml) per gram body weight of norepinephrine (bitartrate salt, Sigma, St. Louis, Missouri, USA). At this moderate level of stimulation, the granular glands release significant quantities of material, but peptide stores are not fully depleted and are naturally replenished (Rollins-Smith et al., 2005). Hoffman and Dent (1977) found that the granular glands of some urodeles discharged in response to acetylcholine rather than α-adrenergic agents. Sufficient peptides were collected from salamanders (*Bolitoglossa*) without injection.

All animals were placed into 50 ml of collecting buffer (50 mM sodium chloride, 25 mM sodium acetate, pH 7.0) (Nutkins and Williams, 1989), and they remained largely submerged for 10 min while skin secretions accumulated. Animals were then removed from the containers and released, and the buffer containing peptides was acidified to a final volume of 1% HCl (Sigma) to inactivate endogenous peptidases that might also have been secreted (Resnick et al., 1991; Steinborner et al., 1997). The acidified collection buffer with skin secretions was immediately passed over C-18 Sep-Pak cartridges (Waters Corporation, Milford, Massachusetts, USA). The Sep-Paks were stored in vials with 2–5 ml of 0.1% HCl. Peptides bound to the Sep-Paks were eluted with 70% acetonitrile, 29.9% water, and 0.1% trifluoroacetic acid (v/v/v) and concentrated to dryness by centrifugation under vacuum. Although the Sep-Pak procedure removes salts and other contaminants, deionized water may be superior to collection buffer for mass spectrometry analyses. The total concentration of skin peptides recovered after Sep-Pak purification was determined by Micro BCA Assay (Pierce, Rockford, Illinois, USA) following manufacturer's instructions, except that bradykinin (RPPGFSPFR; Sigma) was used to establish a standard curve (Rollins-Smith et al., 2002b).

Culture and maintenance of *B. dendrobatidis*

Seven isolates of *B. dendrobatidis* (Table 2) were maintained by standard methods (Rollins-Smith et al. 2002a, b). A peptide mixture from *R. pipiens* was tested against each isolate at concentrations of 25, 50, 100, 150, 200, 250, 500, and 1,000 μg/ml through 7 days of growth (see below for method). Isolate JEL 310 (originating from *Smilisca phaeota* from Fortuna, Panama, isolated by P. Murphy) was used for assays of peptide activity for all species unless noted otherwise.

Predictive index of resistance and statistics

To compare the relative immunologic resistance of each species based on in vitro growth inhibition assays, we developed an index of predicted resistance to *B. dendrobatidis*. The predicted resistance was calculated by multiplying the mean percent inhibition of pathogen growth at a standard peptide concentration of 50 μg/ml by the quantity of peptides produced per gram body weight (gbw) for each species. To test for differences among species in quantity of peptides collect-

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TABLE 1. Comparison of skin peptide defenses against *Batrachochytrium dendrobatidis* among Panamanian species and reference species.

Species	Total peptides recovered (μg/g body weight)			% growth inhibition of *B. dendrobatidis* at 50 μg/ml		
	Mean	SE	*n*	Mean	SE	*n*
Xenopus laevis[a]	1,895.4	182.1	5	32.5	5.2	5
Centrolene prosoblepon	766.5	451.0	7	36.9	15.6	7
Phyllomedusa lemur	2,248.5	351.2	9	6.6	3.1	9
Rana pipiens[a]	816.7	74.5	5	20.9	1.4	5
Rana warszewitschii	450.7		1	21.4		1
Bolitoglossa schizodactyla[b]	183.4	74.2	10	18.8	3.0	10
Colostethus panamensis	529.2	86.5	12	4.5	1.9	11
Bufo boreas[a]	41.3	7.5	10	59.2	7.1	10
Centrolene ilex	611.8	267.9	5	1.3	1.1	4
Atelopus varius	137.1	61.7	10	5.0	1.7	8
Eleutherodactylus punctariolus	77.5	33.6	5	1.1	0.9	5
Bolitoglossa colonnea[b]	973.0	275.1	2	0.0	0.0	2

[a] Sampled in the laboratory.

[b] Sufficient peptides were collected from salamanders without norepinephrine injection.

ed per gbw (μg/gbw), growth inhibition of *B. dendrobatidis* (%), and predicted resistance Kruskal-Wallis tests were performed with SPSS v. 12 (SPSS Inc., Chicago, Illinois, USA). Species were classified as having the potential for either strong predicted resistance or weak predicted resistance with Hierarchical Cluster Analysis (a species classified as potentially strongly resistant had at least one individual within that cluster), selecting Squared Euclidean Distances to measure between points and Ward linkage to measure the distance between two clusters. The variables total peptides/gbw and percent growth inhibition were included in the analysis.

MALDI mass spectrometry of peptides

Mixtures of amphibian skin peptides were analyzed by MALDI MS. A PerSeptive Biosystems Voyager Elite mass spectrometer (Applied Biosystems, Foster City, California, USA) was operated in reflector, delayed extraction, and positive ion mode. A stainless

TABLE 2. Comparison of growth inhibition among geographically distinct *Batrachochytrium dendrobatidis* isolates caused by a reference mixture of *Rana pipiens* skin peptides.

Isolate	Host	Location	Mean *B. dendrobatidis* growth inhibition at 50 μg/ml[a] (%±SE) [*n*]	MIC[b] (μg/ml)
JEL 197 (type isolate)	*Dendrobates azureus*	National Zoological Park, Washington, D.C., USA	58.1±3.5 [3]	250
LJR 119	*Rana muscosa*	Sierra Nevada, California, USA	38.4±5.5 [3]	1,000
JEL 254	*Rana pipiens*	Orono, Maine, USA	50.3 [1]	500
JEL 275	*Bufo boreas*	Clear Creek Co., Colorado, USA	43.0±14.9 [2]	500
JEL 308	*Eleutherodactylus caryophyllaceus*	Fortuna, Panama	40.2 [1]	500
JEL 310	*Smilisca phaeota*	Fortuna, Panama	51.9±5.5 [3]	500
VM 1	*Pseudacris triseriata*	Arizona, USA	43.9 [1]	250

[a] A laboratory *Rana pipiens* was subcutaneously injected with 80 nmol/g body weight NE-HCl to induce a maximal peptide secretion for use as a reference mixture. Peptides were stored at −20 C until use.

[b] MIC = minimal inhibitory concentration, the lowest concentration at which no growth was detectable.

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steel 100 well plate was spotted with 0.5 µl sample and subsequently 0.5 µl matrix solution and allowed to air-dry. Samples of peptide mixtures were tested at 100 or 1,000 µg/ml if enough material was available. The matrix solution consisted of 10 mg/ml α-cyano-4-hydroxycinnamic acid (Sigma), 60% volume of acetonitrile, and 40% volume of 1% trifluoroacetic acid. For external calibration the following peptide standards were applied: bradykinin fragment 1–7 (monoisotopic mass 757.4) and bovine insulin (average mass 5,734.5) (Sigma). Each sample was analyzed by averaging signals from 256 consecutive laser shots. The MS data was acquired in the m/z range 500 to 7,000, truncated at m/z 4,500 and baseline-corrected with Data Explorer v4.4 (Applied Biosystems). The peak values shown represent the monoisotopic mass, $[M+H]^+$. A few signals may show secondary peaks 22 mass units greater than the primary peak and probably represent a peptide plus sodium adduct $[M+Na]^+$. Spectra may also show peaks at 568.1 and 650.0 because of matrix or background.

Growth inhibition of *B. dendrobatidis*

Peptide mixtures diluted to a known concentration were assayed for their ability to inhibit growth of *B. dendrobatidis* as previously described (Rollins-Smith et al., 2002a, b). Insufficient material was available from most of the small amphibians sampled for the determination of minimal inhibitory concentration. Instead, the percent growth inhibition at a standard peptide concentration of 50 µg/ml was measured. This low concentration may more accurately reflect conditions experienced in nature, because high concentrations must be induced from amphibian skin artificially (Rollins-Smith et al., 2005). Approximately 5×10^4 zoospores in 50 µl of broth were plated in replicates of five in 96-well flat-bottomed microtiter plates (Costar, Corning, New York, USA) to which we added serial dilutions of the peptides. The pH of this growth medium was 6.5–7.0. The optical density at 490 nm was obtained daily for 7 days with an MRX Microplate Reader with Revelation software (Dynex Technologies, Inc., Chantilly, Virginia, USA) for each experimental well and for control wells containing broth but no peptides (positive control) or broth with heat-killed *B. dendrobatidis* (negative control).

Active zoospores were counted on a standard hemocytometer slide to examine the effects of peptide mixtures on the activity of zoospores through time. For example, zoospores from isolate 254 (from *R. pipiens*) were diluted in water (adjusted to pH 2.5 to match the peptide solution) and in a solution containing 50 µg/ml peptide (pH 2.5). On opposite sides of the hemocytometer were placed the peptide-zoospore mixture and the water-zoospore mixture. The active zoospores were counted from each side alternately and the time recorded at each count.

RESULTS

Characterizing amphibian skin peptide defenses by MALDI MS

Skin peptide profiles obtained by MALDI MS demonstrated marked differences among species (Figs. 1, 2). Some *Xenopus laevis* mass signals matched previously described antimicrobial peptides from that species including PGLa (m/z 1,968) and magainin-1 and 2 (m/z 2,409, 2,466) (Fig. 1A; Rollins-Smith and Conlon, 2005). Similarly, some *Rana pipiens* mass signals matched previously described antimicrobial peptides of the brevinin 1 family (m/z 2,563, 2,577, and 2,593) and ranatuerin-2P (m/z 3,000) (Fig. 1B; Goraya et al., 2000). Thus, natural mixtures of peptides from some species analyzed here contained conventional antimicrobial peptides. Most Panamanian species demonstrated complex mixtures of 5–25 peptides. Some of these peptides will be sequenced in ongoing studies. Only minor variations in peptide profiles were evident within species. Clusters of peptides with masses similar to antimicrobials described in other species (i.e., patterns similar to the brevinin 1 family in *Rana pipiens*, Fig. 1B) are displayed in *C. prosoblepon* and two *Bolitoglassa* salamanders, but not in the two bufonids (Fig. 2).

Peptide recovery and potency

Among the 12 amphibian species sampled there were significant differences in the total quantity of peptides recovered (µg/g; Kruskal-Wallis test; $\chi^2_{11}=62.12$; $P<0.001$) and antifungal activity (% inhibition at 50 µg/ml; Kruskal-Wallis test; $\chi^2_{11}=51.45$; $P<0.001$; Table 1). *B. boreas*, *A. varius*, and *E. punctariolus* produced

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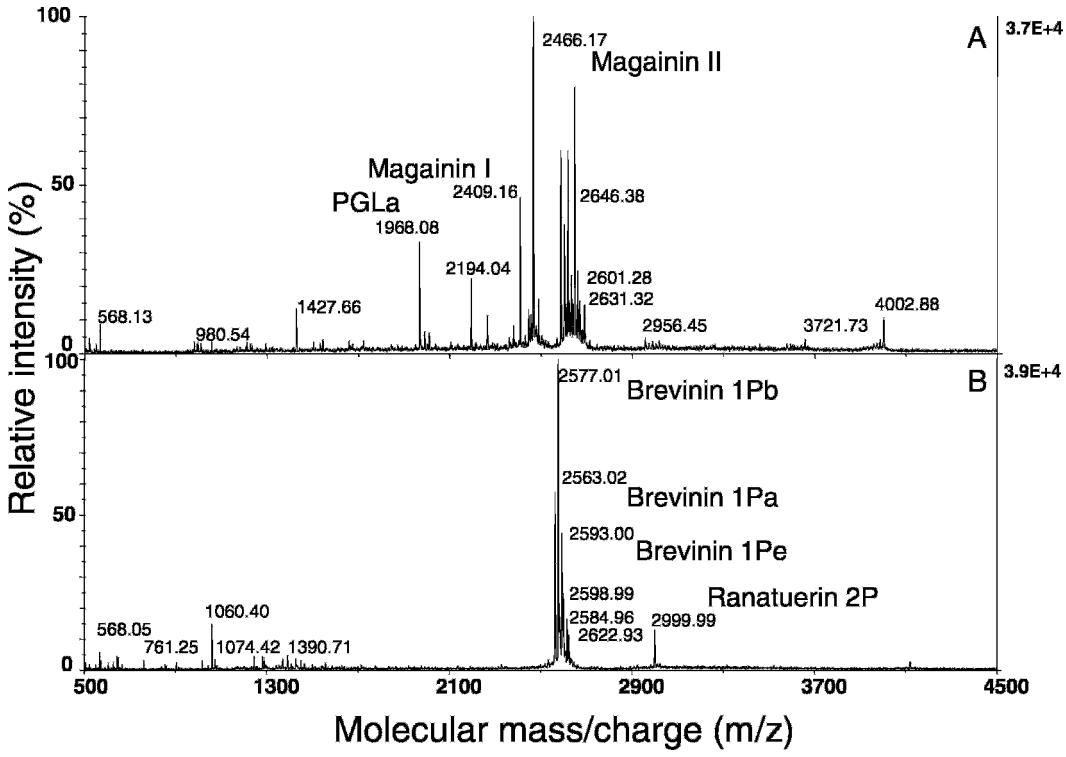


FIGURE 1. MALDI mass spectrometry profiles of skin peptides from *Xenopus laevis* (A) and *Rana pipiens* (B). Major antimicrobial peptides that were previously described are labeled (reviewed in Rollins-Smith and Conlon, 2005).

the smallest quantity of skin peptides (41–137 μg/gbw; Table 1). *Phyllomedusa lemur* and *X. laevis* produced the most copious peptide secretions (approximately 2,000 μg/gbw; Table 1). However, the most active peptide mixtures against *B. dendrobatidis* (percent inhibition at 50 μg/ml peptides; Table 1) were produced by *B. boreas* (59.2%), *C. prosoblepon* (36.9%), and *X. laevis* (32.5%). Thus, amphibians producing more copious skin secretions did not necessarily produce more effective peptides against *B. dendrobatidis*. For example, *B. boreas* peptides were highly potent at inhibiting *B. dendrobatidis* growth (Table 1) in a dose-dependent manner (Fig. 3). However, because so little total quantity of peptides is released from the skin, the predicted immunologic resistance to *B. dendrobatidis* was low (Fig. 4). The secretions of *B. boreas* were primarily composed of small peptides in the m/z range of 700–760 (Fig. 2E). At concentrations of 50 μg/ml, *B. boreas* peptides did not inhibit *B. dendrobatidis* (isolate JEL 275) zoospore activity for up to 40 min, and activity was comparable to that of a water control at a matching pH of 2.5. In contrast, 50 μg/ml *R. pipiens* peptides (pH 2.5) inhibited *B. dendrobatidis* (isolate JEL 254) zoospore activity completely on contact.

Predicted resistance to chytridiomycosis

The quantity and quality of skin peptide mixtures from each of 12 species were combined to predict immunologic resistance to *B. dendrobatidis* (Fig. 4). Predicted resistance was significantly different among the species (Kruskal-Wallis test; $\chi^2_{11}=39.89$; $P<0.001$). A hierarchic cluster analysis divided the species into two classes. Those species with strong predicted resistance included *X. laevis*, *C.*

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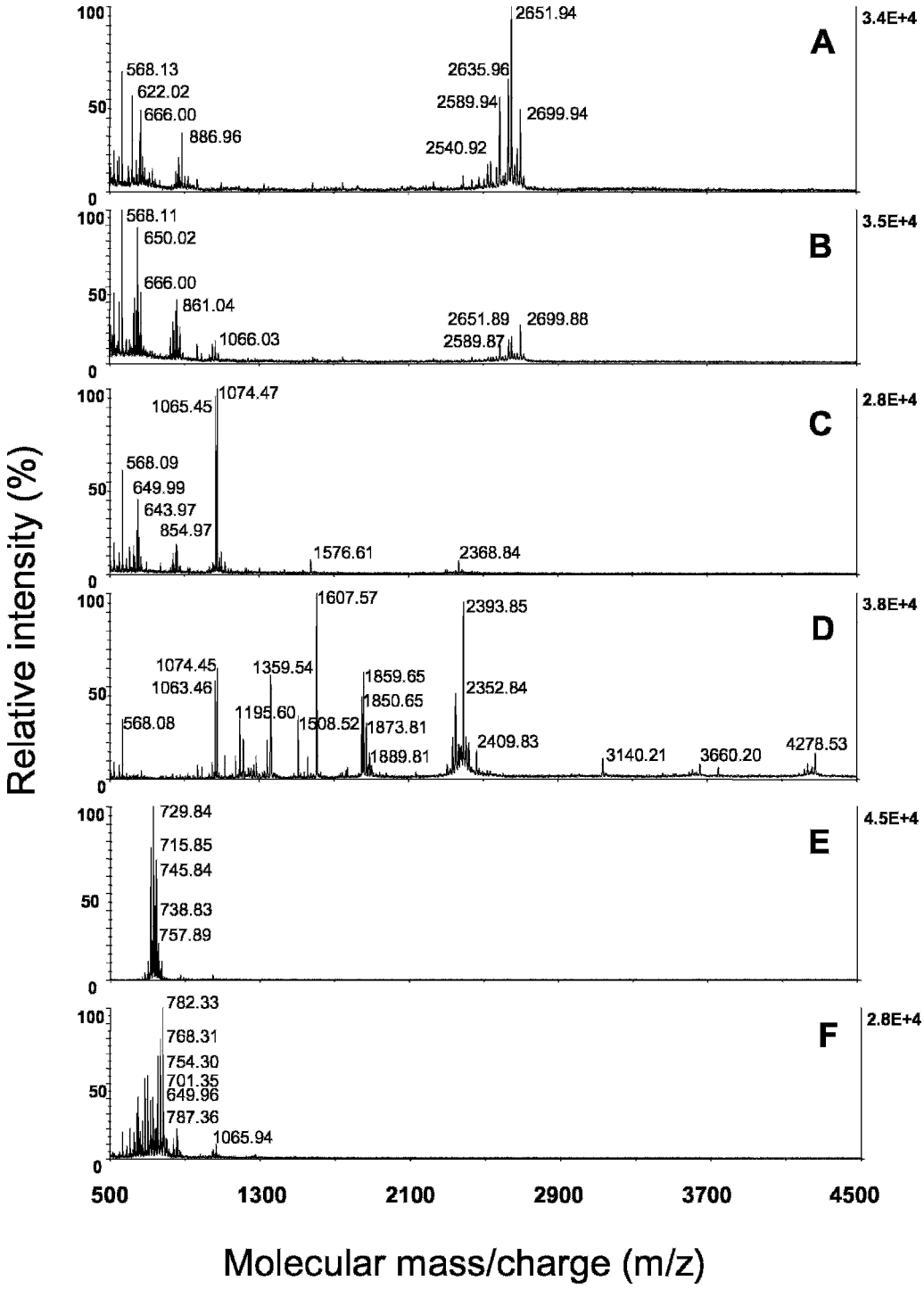


FIGURE 2. MALDI mass spectrometry profiles of skin peptides. (A) *Centrolene prosoblepon* (84.7% *B. dendrobatidis* growth inhibition), (B) *Centrolene prosoblepon* (13.6% *B. dendrobatidis* growth inhibition), (C) *Bolitoglossa schizodactyla*, (D) *Bolitoglossa colonnea*, (E) *Bufo boreas*, (F) *Atelopus varius*.

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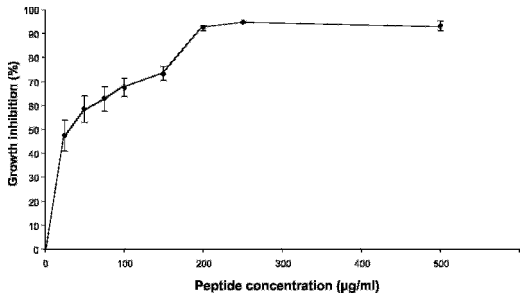


FIGURE 3. *Batrachochytrium dendrobatidis* (isolate JEL 274) zoospore growth inhibition (±SE) measured on day 7 with various concentrations of *Bufo boreas* skin peptide mixtures collected after injection of frogs ($n=10$) with 1–2 nmol/gbw norepinephrine-bitartrate.

prosoblepon, and *P. lemur*. Individuals of *X. laevis* and *R. pipiens* with known sublethal infections had relatively strong to moderate predicted resistance. Conversely, *B. boreas* had relatively weak predicted resistance and is a species known to be susceptible to infection and lethal disease (Fig. 4; Muths et al., 2003). The susceptibility of most Panamanian species is not known. On the basis of our index, we predict that *C. prosoblepon* and *P. lemur* will have a more robust skin peptide defense against chytridiomycosis than other species. However, variation in growth inhibition was high for *C. prosoblepon* (Table 1), and peptide defenses may be variable among individuals. A comparison of two MALDI MS profiles from *C. prosoblepon* individuals with 13.6% and 84.7% *B. dendrobatidis* growth inhibition shows relatively higher peaks of some peptides, particularly in the cluster around 2,651.9 Da, for the individual with greater antifungal activity (Fig. 2A, B). These peptides have not yet been described.

Antimicrobial peptide sensitivity of *B. dendrobatidis* isolates

A reference peptide mixture was obtained from a laboratory *R. pipiens* and tested against seven geographically different *B. dendrobatidis* isolates. The growth-inhibitory activity of peptide mixtures was not significantly different among chytrid isolates (analysis of variance; $F_{6,7}=1.006$; $P=0.489$; Table 2). The mean inhibition (%±SE) was 47.5±3.0 at a peptide concentration of 50 µg/ml. The minimal inhibitory concentration of the mixture was usually between 250 and 500 µg/ml (Table 2).

DISCUSSION

Predicting immunologic resistance to chytridiomycosis

The ability to predict disease susceptibility is an important step in the conservation of amphibians at risk of disease-associated population declines. Ecologic correlates of disease may provide valuable indicators of susceptibility (Lips et al., 2003b). These, in combination with indicators of host immunologic resistance, create a valuable tool for disease ecology and wildlife management. For example, species with potentially weak predicted defenses may be targeted for captive husbandry. However, the link between in vitro peptide effectiveness and protection in the wild is tentative (Woodhams et al., 2006).

Differences among species were found in both predicted resistance to chytridiomycosis and profiles of skin peptide mixtures examined by mass spectrometry. The index of predicted resistance to chytridiomycosis indicates weak levels of resistance for most species of Panamanian amphibians studied, similar to the predicted resistance of *B. boreas*, a species known to be susceptible.

We found that MALDI MS was a rapid method to obtain unique skin-peptide profiles, and can be used for phylogenetic comparisons. The bufonids (true toads) *B. boreas* and *A. varius* had similar skin peptide profiles characterized by a set of small peptides not typical of the larger antimicrobial peptides found in other species (Zasloff, 2002; Apponyi et al., 2004; Rollins-Smith and Conlon, 2005). Conlon et al. (2004) did not detect

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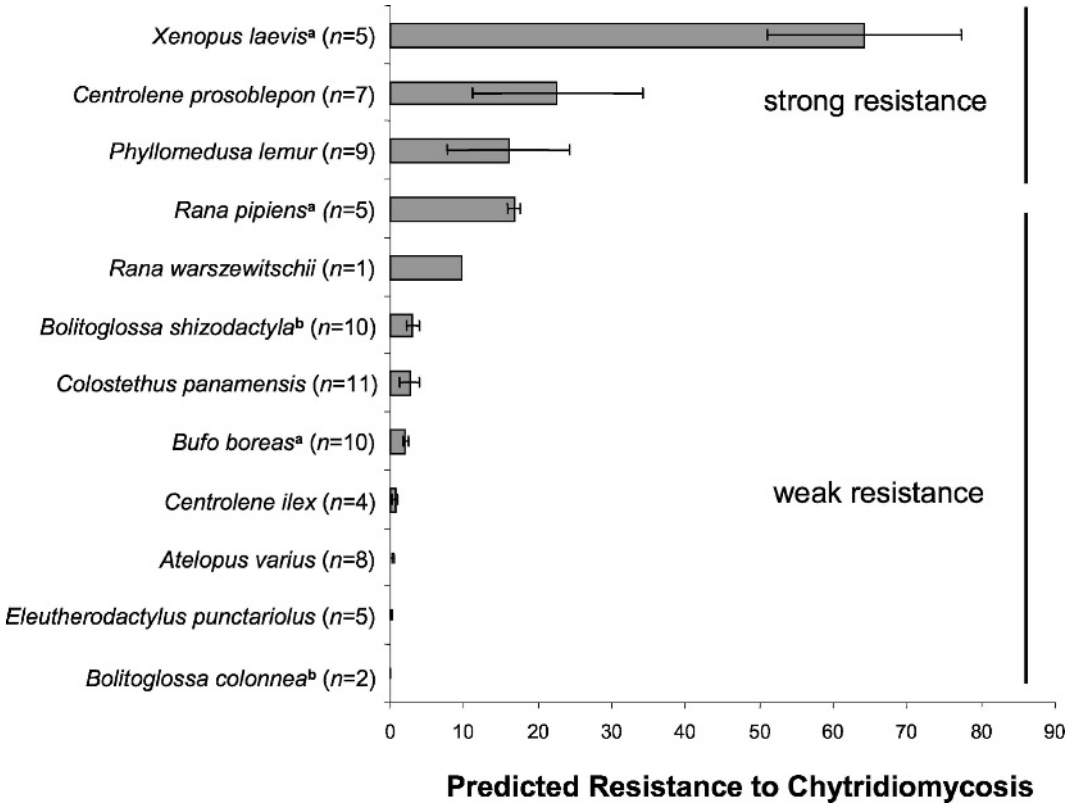


FIGURE 4. Predicted immunologic resistance (±SE) of amphibians to *Batrachochytrium dendrobatidis* calculated by taking the mean percent inhibition of in vitro pathogen growth (at a standard peptide concentration of 50 μg/ml) and multiplying by the quantity of peptide produced per g body weight for each species. (a) Sampled in the laboratory; (b) peptides were collected from salamanders without norepinephrine injection.

antimicrobial peptides in *Bufo* skin. Similarly, *A. varius* may lack conventional antimicrobial peptides. The small peptides found in these species may have alternative functions. We found that the peptide mixtures of *B. boreas* were active against *B. dendrobatidis* but did not completely kill the fungus. Although the *B. boreas* peptide mixtures inhibited fungal growth in vitro, the small quantities of peptides produced by the toads may not provide significant protection in vivo.

Phylogenetic constraints on innate immune defenses may help explain the taxon bias in declines found by the Global Amphibian Assessment (Stuart et al., 2004). For example, Bufonidae is one of four amphibian families that contain significantly more than the average number of rapidly declining species (Stuart et al., 2004). This taxon has a distinctively thick epidermis, often with a heavily keratinized and roughened structure (Elias and Shapiro, 1957), and skin peptide profiles of this family are notably less complex than in other taxa. These features may increase susceptibility to chytridiomycosis.

Only two Panamanian species, *C. prosoblepon* and *P. lemur*, were predicted to be more resistant than North American *R. pipiens*, which survives infection with *B. dendrobatidis* (Ouellet et al., 2005). MALDI MS indicated that potentially novel antimicrobial peptides are present in the skin-peptide mixtures of *C. prosoblepon*. The large variability in the growth inhibition activity of *C. prosoblepon* peptides may indicate that not all individuals

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are equally defended. Those individuals that are better defended may survive chytridiomycosis epizootics. We hypothesize that variation in skin peptide defense is reduced at sites of disease emergence where small populations of survivors persist. For example, at Fortuna, Panama, populations of many amphibian species declined in 1996 and chytridiomycosis is the suspected agent of declines (Lips, 1999), but small populations of *C. prosoblepon* persist. These surviving populations may have enhanced skin peptide defenses relative to populations that are naïve to this strong selection pressure.

Batrachochytrium dendrobatidis–resistant *R. pipiens* experienced population declines in the past, although the causes are not fully known (NatureServe, 2004). A preserved *R. pipiens* from Colorado, 1974, represents the earliest known infection of that species (Carey et al., 1999), and recent studies found infected frogs in apparently healthy wild populations (Green et al., 2002). Historic evidence suggests that *B. dendrobatidis* is enzootic in *R. pipiens* (Ouellet et al., 2005). The strength of *X. laevis* resistance is consistent with a coevolutionary history with the fungus. Studies of preserved museum specimens have found the earliest *B. dendrobatidis* infection in *X. laevis* from South Africa in 1938, and enzootic infections were found in nondeclining populations in South Africa (Weldon et al., 2004). Antimicrobial peptide defenses may evolve more readily than components of the adaptive immune system (Zasloff, 2002; Vanhoye et al., 2003). Rapid peptide variation may result from error-prone DNA polymerases (Vanhoye et al., 2003). As disease advances through Panama, there is the opportunity to study this strong selection pressure on the evolution of peptide immune defenses.

The disease resistance observed in *R. pipiens* and *X. laevis* stands in contrast to the sensitivity of *C. panamensis* and *Eleutherodactylus cruentus*. When frogs were brought into captivity after collection from El Copé, Panama, in September 2004, mass mortality resulted from naturally contracted chytridiomycosis, and wild populations later disappeared (Carey and Voyles, unpublished data). Many other Panamanian species are showing susceptibility to chytridiomycosis, the first signs of which appeared at El Copé in October 2004 (Lips et al., unpubl. data). Dead amphibians of species examined here were found positive for *B. dendrobatidis*. The extent of the declines of these populations is not yet known.

Comparison of *B. dendrobatidis* isolates

Piotrowski et al. (2004) compared the growth curves of *B. dendrobatidis* at various temperatures and found similar growth among isolates. We also found little variation among isolates in sensitivity to an amphibian skin peptide mixture. It is not known whether an isolate of *B. dendrobatidis* or any other organism can be artificially selected for resistance to an antimicrobial peptide. Although infection experiments comparing survival of amphibians after exposure to different *B. dendrobatidis* isolates are still needed, our results suggest a lack of adaptation to particular species or environments and therefore little genetic differentiation among isolates. Genetic comparisons of 35 *B. dendrobatidis* isolates from North America, Panama, Africa, and Australia demonstrate that all are closely related (Morehouse et al., 2003). This supports ecologic, pathologic, and biogeographic evidence that chytridiomycosis is an emerging infectious disease of amphibians (Daszak et al., 1999; Weldon et al., 2004). This also suggests that *B. dendrobatidis* is a generalist pathogen and that species possessing an innate immunologic defense at the time of emergence are more likely to survive.

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isolates, A. Hyatt and the AAHL for diagnosis of *B. dendrobatidis* infections, L. Manier and P. Chaurand for assistance with mass spectrometry, and L. Reinert for collecting *Xenopus* peptides. Approval for animal procedures was granted from Vanderbilt University and the University of Colorado. Permits were provided through the Smithsonian Tropical Research Institute from the Autoridad Nacional del Ambiente, Republic of Panama, and the US Fish and Wildlife Service. This research was supported by a subcontract to L. R.-S. from an NSF Integrated Research Challenges in Environmental Biology grants IBN-9977063 and DEB-0213851 (James Collins, P.I.) and NSF grant IBN-0131184 to L.R-S. D.C.W. was supported by the Immunobiology of Blood and Vascular Systems Training Program (NIH 5 T32 HL069765-02).

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