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Population trends associated with skin peptide defenses against chytridiomycosis in Australian frogs

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Abstract Many species of amphibians in the wet tropics of Australia have experienced population declines linked with the emergence of a skin-invasive chytrid fungus, *Batrachochytrium dendrobatidis*. An innate defense, antimicrobial peptides produced by granular glands in the skin, may protect some species from disease. Here we present evidence that supports this hypothesis. We tested ten synthesized peptides produced by Australian species, and natural peptide mixtures from five Queensland rainforest species. Natural mixtures and most peptides tested in isolation inhibited growth of *B. dendrobatidis* in vitro. The three most

active peptides (caerin 1.9, maculatin 1.1, and caerin 1.1) were found in the secretions of non-declining species (*Litoria chloris*, *L. caerulea*, and *L. genimaculata*). Although the possession of a potent isolated antimicrobial peptide does not guarantee protection from infection, non-declining species (*L. lesueuri* and *L. genimaculata*) inhabiting the rainforest of Queensland possess mixtures of peptides that may be more protective than those of the species occurring in the same habitat that have recently experienced population declines associated with chytridiomycosis (*L. namotis*, *L. rheocola*, and *Nyctimystes dayi*). This study demonstrates that in vitro effectiveness of skin peptides correlates with the degree of decline in the face of an emerging pathogen. Further research is needed to assess whether this non-specific immune defense may be useful in predicting disease susceptibility in other species.

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Introduction

In recent years much attention has been focused on the global problem of amphibian declines (Wake 1991; Alford and Richards 1999; Carey et al. 1999; Daszak et al. 1999; Houlahan et al. 2000; Kiesecker et al. 2001). The amphibian chytrid fungus, *Batrachochytrium dendrobatidis*, is an important pathogen of amphibians that has caused epizootics of chytridiomycosis, mass mortalities, and local extinctions of some amphibian populations in the western United States, Central America, Europe, Africa, and Australia (Berger et al. 1998; Longcore et al. 1999; Pessier et al. 1999; Lips 1999; Bosch et al. 2001; Bradley et al. 2002; Weldon and du Preez 2004). Genetic comparisons of 35 isolates of *B. dendrobatidis* from North America, Panama, Africa, and Australia demonstrate that all are very closely related and may

represent a newly emerging pathogen (Morehouse et al. 2003). This supports ecological, pathological, and biogeographical evidence that chytridiomycosis is an emerging infectious disease of amphibians (Berger et al. 1999; Daszak et al. 1999, 2000). Because this pathogen is thought to spread within a single host and also between hosts by infectious zoospores erupting from previously infected skin cells (Berger et al. 1998; Longcore et al. 1999; Pessier et al. 1999; Lips 1999), we have hypothesized that antimicrobial peptides secreted onto the skin surface may provide an effective immune defense for some species that express a potent antimicrobial peptide repertoire (Rollins-Smith et al. 2002b). Lack of lymphocytic infiltration in the chytrid-infected skin of frogs suggests that these animals have a poor cell-mediated naïve immune response against this pathogen (Berger et al. 1998; Pessier et al. 1999), and there is currently no evidence for a humoral defense. Thus, other innate immune defenses, such as antimicrobial peptides, may be of critical importance in maintaining protection from infection.

Antimicrobial peptides produced in the dermal granular glands of amphibians are small (12–46 amino acid residues), cationic, and hydrophobic. Most can adopt an α -helical conformation when bound to charged residues on target cell membranes. Resulting peptide complexes are thought to disturb the membrane, causing death of the target cell (reviewed in Nicolas and Mor 1995; Simmaco et al. 1998; Zasloff 2002; Conlon et al. 2004). Infectious zoospores of *B. dendrobatidis* may be vulnerable to the activity of this innate defense upon contact with the skin mucosal surface.

Our previous studies have demonstrated that a number of amphibian species produce antimicrobial peptides in the skin that can inhibit growth of *B. dendrobatidis* in vitro. The most active peptides completely inhibit growth of chytrid zoospores at concentrations below 20 μ M (brevinin-1TRa, brevinin-2Ob, esculentin-1A, esculentin-2L, palustrin-3A, ranalexin, ranatuerin-1). Other peptides inhibit at somewhat higher concen-

trations (Rollins-Smith et al. 2002a, b, c), but the release of peptides onto the skin under normal physiological conditions or in response to infection has not yet been quantified. Although these studies suggested that antimicrobial skin peptides can protect against invasive skin pathogens in vitro, there was little information available from species that are known to be susceptible or resistant to the amphibian chytrid fungus. The present studies examined the potency against *B. dendrobatidis* of purified skin peptides as well as natural mixtures of peptides from species that have experienced declines and from non-declining species of Australian frogs. Some species that produce peptides with potent in vitro activity against *B. dendrobatidis* may still be susceptible to infection. For example, some adult *Litoria chloris*, *L. caerulea*, and *L. genimaculata* were found with chytridiomycosis (Speare and Berger 2000). We found that in general, non-declining species had more effective peptide defenses than those species that have recently experienced declines associated with chytridiomycosis. Several other factors may contribute to variation among species in disease susceptibility or population decline including behavior, habitat specialization, life-history traits, stream association, fecundity, and range (Williams and Hero 1998; Lips et al. 2003; Retallick et al. 2004; Woodhams et al. 2003; Woodhams and Alford 2005). This study presents the first evidence that correlates antimicrobial skin peptide effectiveness in vitro and amphibian population declines associated with chytridiomycosis.

Materials and methods

Synthetic peptides

Table 1 lists the pure synthetic peptides examined in this study. These peptides were a generous gift from Dr. John Bowie. Each peptide was initially isolated from induced skin secretions of one or more of 13 Australian

Table 1 Ten synthetic peptides previously isolated from Australian frog skin secretions were tested in this study

Peptide	Species of Origin	Sequence	Reference
Aurein 2.1	<i>Litoria aurea</i> , <i>L. raniformis</i>	GLLDIVKKVVGAFGSL-NH ₂	Rozek et al. (2000)
Caerin 1.1	<i>L. caerulea</i> , <i>L. splendida</i> , <i>L. gilleni</i> , <i>L. ewingi</i>	GLLSVLGSAKHVLP VVPVIAEHL-NH ₂	Steinborner et al. (1997a, 1998); Wong et al. (1997); Wabnitz et al. (1998); Bowie et al. (1999)
Caerin 1.9	<i>L. chloris</i>	GLFGVLGSAKHVLP VVPVIAEKL-NH ₂	Steinborner et al. (1998)
Caerin 4.1	<i>L. caerulea</i>	GLWQKIKSAAGLDL S GIVEGIKS-NH ₂	Bowie et al. (1999)
Citropin 1.1 mod 17	<i>L. citropa</i>	GLFAVIKKVAAVIKLL-NH ₂	Wegener et al. (1999)
Dahlein 5.6	<i>L. dahlia</i>	GLLASLGKVFVGGYLAEKLPK-OH	Wegener et al. (1999)
Frenatin 3	<i>L. infrafrenata</i>	GLMSVLGHAVGNVVGFGFKPKS-OH	Raftery et al. (1996)
Maculatin 1.1	<i>L. genimaculata</i>	GLFGVLAKVAAHVPPAIAEHF-NH ₂	Wegener et al. (1999, 2001)
Tryptophyllin 1.2	<i>L. rubella</i>	FPWL-NH ₂	Steinborner et al. (1996)
Uperin 3.6	<i>Uperoleia mjobergii</i>	GVIDAAKKVVNVLKNLF-NH ₂	Chia et al. (1999)

Some peptides are found in more than one species, and may occur in other species that have not yet been studied

frog species as previously described (Raftery et al. 1996; Steinborner et al. 1996, 1997a, 1998; Wong et al. 1997; Rozek et al. 1998, 2000; Wabnitz et al. 1998; Bowie et al. 1999; Chia et al. 1999, 2000; Wegener et al. 1999, 2001). All peptides were synthesized (by Chiron Mimotopes, Clayton, VIC, Australia) using L-amino acids and standard Fmoc chemistry as previously described (Maeji et al. 1995). Citropin 1.1 mod 17 was synthesized with four amino acid substitutions as compared to the natural molecule (residue 4, A for D; residue 11, A for S; residues 14 and 15, K for G).

Collection of skin peptides from rainforest stream frogs

We sampled skin secretions from adult amphibians of five species (listed below) at several natural stream sites in the wet tropics of Queensland, Australia (Woodhams and Alford 2005). For these species there are good long-term population trend data available (Richards et al. 1993; McDonald and Alford 1999, Northern Queensland Threatened Frog Recovery Team 2001). In this system, population declines were associated with chytridiomycosis (Berger et al. 1998, 1999). The IUCN red list conservation status of each species is shown in parentheses (for international, national, and regional conservation status of each species see AmphibiaWeb <http://amphibiaweb.org/>). The species sampled were: *Nyctimystes dayi* (endangered), *Litoria rheocola* (endangered), *L. nannotis* (endangered), *L. genimaculata* (lower risk, near threatened), and *Litoria lesueuri* (lower risk, least concern). Adults were exclusively males except for *L. nannotis*. Their skin secretions were collected using a norepinephrine immersion technique adapted from one described by Rollins-Smith et al. (2002c). This method was designed to induce skin secretions from a variety of small tropical amphibians and tadpoles and avoid the need for prolonged handling and injection of norepinephrine into very small frogs. Briefly, frogs were captured by hand in new plastic bags, weighed and measured. For each gram of body weight, 10 ml collecting buffer (50 mM sodium chloride, 25 mM sodium acetate, pH 7.0) (Nutkins and Williams 1989) were added to the container, and norepinephrine (bitartrate salt Sigma, St. Louis, MO, USA) was added to a final concentration of 100 μ M. Animals remained largely submerged in the buffer for 15 min while skin secretions accumulated. Animals were then removed from the container and released, and the buffer containing peptides was acidified with 1.0 ml 100% HCl (Sigma, St. Louis, MO, USA) per 100 ml of collecting buffer to inactivate endoproteases that are also secreted (Resnick et al. 1991; Steinborner et al. 1997b). The skin peptides were partially purified as described previously with some minor modifications (Rollins-Smith et al. 2002c). Using a large syringe, the collecting buffer with peptides was passed over C-18 Sep-Pak cartridges (Waters Corporation, Milford, MA, USA). The Sep-Paks were stored in vials with a small amount of 0.1% HCl

and shipped to Vanderbilt University in Nashville, Tennessee. Peptides bound to the Sep-Paks were eluted with 70% acetonitrile, 29.9% water, 0.1% trifluoroacetic acid (TFA) (v/v/v) and concentrated to dryness by centrifugation under vacuum. Total concentration of skin peptides recovered after Sep-Pak purification was determined by Micro BCA Assay (Pierce, Rockford, IL, USA) following manufacturer's instructions, except that bradykinin (RPPGFSPFR) (Sigma Chemical, St. Louis, MO) was used to establish a standard curve (Rollins-Smith et al. 2002c).

Culture and maintenance of *Batrachochytrium dendrobatidis*

Two isolates of *B. dendrobatidis* were used in these studies. The first, designated #197 (or type isolate JEL197), was isolated from a diseased blue dart poison frog (*Dendrobates auratus*) by Longcore et al. (1999). The second, designated VM1, was isolated by V. Miera and E. Davidson from a diseased Western chorus frog (*Pseudacris triseriata*). Both were grown on TGH agar or in H broth at 22–23°C as previously described (Rollins-Smith et al. 2002a, b, c). Broth cultures were passaged twice weekly to assure that cells were in an active phase of growth. Zoospores were harvested as previously described (Rollins-Smith et al. 2002a). After separation of mature cells, the zoospore population was > 99% pure.

Batrachochytrium dendrobatidis growth inhibition assay

Pure synthetic peptides or natural 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, c). For the determination of minimum inhibitory concentration and percent growth inhibition at a standard peptide concentration, approximately 5×10^4 mature cells or 5×10^5 zoospores in 50 μ l of broth were plated in replicates of five into 96-well flat-bottomed microtiter plates (Costar, Corning, NY, USA) to which serial dilutions of the peptides were added. The optical density at 492 nm (OD_{492}) was obtained daily with a Titertek ELISA plate reader for each experimental well and for control wells containing broth but no peptides (positive control with unhindered fungal growth) or 0.4% paraformaldehyde (negative control with killed fungus) (Rollins-Smith et al. 2002a, b, c). To calculate the growth inhibition of cultures at the peak of growth, plates containing adult cells were maintained for 4 days, and plates with zoospores for 7 days. However, data from earlier time points were used if older plates became contaminated. Minimal inhibitory concentration (MIC) is defined as the lowest concentration of added peptide at which no significant growth was observed.

Determination of MIC equivalents in skin secretions

To compare the relative effectiveness of skin peptides, we developed a measure of the mean MIC equivalents per gram of body weight (gbw) and mean MIC equivalents per cm² of surface area for each species. An MIC equivalent is the total amount of peptides (μg) recovered from each frog after elution from the C-18 Sep-Paks (concentrated to dryness and dissolved in 1.0 ml of water) per 1 g of body weight (or per 1 cm² surface area of frog) divided by the experimentally determined MIC (μg/ml) for each species. For example, if a total of 50 μg of peptide were recovered from a 10 g frog (5 μg/gbw) and the average MIC for that species was 100 μg/ml, then that sample of skin secretions would contain 0.05 MIC equivalents/gbw. Surface area of frogs in cm² was calculated using a standard equation from McClanahan and Baldwin (1969): Surface area = 9.90 (weight in grams)^{0.56}

Statistical comparisons

To test for differences among species in MIC (μg/ml), MIC equivalents per gram body weight, and MIC

equivalents per cm² surface area, one-way ANOVAs were performed after the distributions of data were found to meet the assumptions for parametric tests. Pre-planned ANOVA contrasts were used to test for differences between the two non-declining species and the three species that have experienced recent population declines. Student's *t* tests were used to compare significance of growth inhibition. All statistics were performed using SPSS version 10 (SPSS Inc., 1999).

Results

Inhibition of *B. dendrobatidis* growth by pure synthetic skin peptides

Although each of the peptides shown in Table 1 (except tryptophyllin 1.2 and dahlein 5.6) had previously been shown to inhibit bacterial growth (Raftery et al. 1996; Steinborner et al. 1997a, 1998; Rozek et al. 1998; Bowie et al. 1999; Chia et al. 1999, 2000; Wegener et al. 1999), nothing was known about their ability to inhibit growth of *B. dendrobatidis*. The three most potent of the ten peptides tested were caerin 1.9, caerin 1.1, and maculatin 1.1. Caerin 1.9 inhibited growth of mature chytrids and

Fig. 1 Growth inhibition of mature *Batrachochytrium dendrobatidis* cells by eight isolated peptides: **a** Maculatin 1.1, **b** Caerin 1.1, **c** Caerin 1.9, **d** Dahlein 5.6, **e** Frenatin 3, **f** Citropin 1.1, **g** Uperin 3.6, **h** Aurein 2.1. Growth measured on day 4 (peak of growth) as optical density at 492 nm (OD₄₉₂) (mean ± SE, n = 5). *Open circles* represent the negative control, 0.4% paraformaldehyde-killed cells (PF), and *filled circles* represent the positive control, live cells with no added peptides. *Asterisks* (*) indicate significantly less than positive control for growth by Student's *t* test (*P* ≤ 0.05). Minimal inhibitory concentration (MIC) is defined as the lowest concentration of added peptide at which no significant fungal cell growth was observed

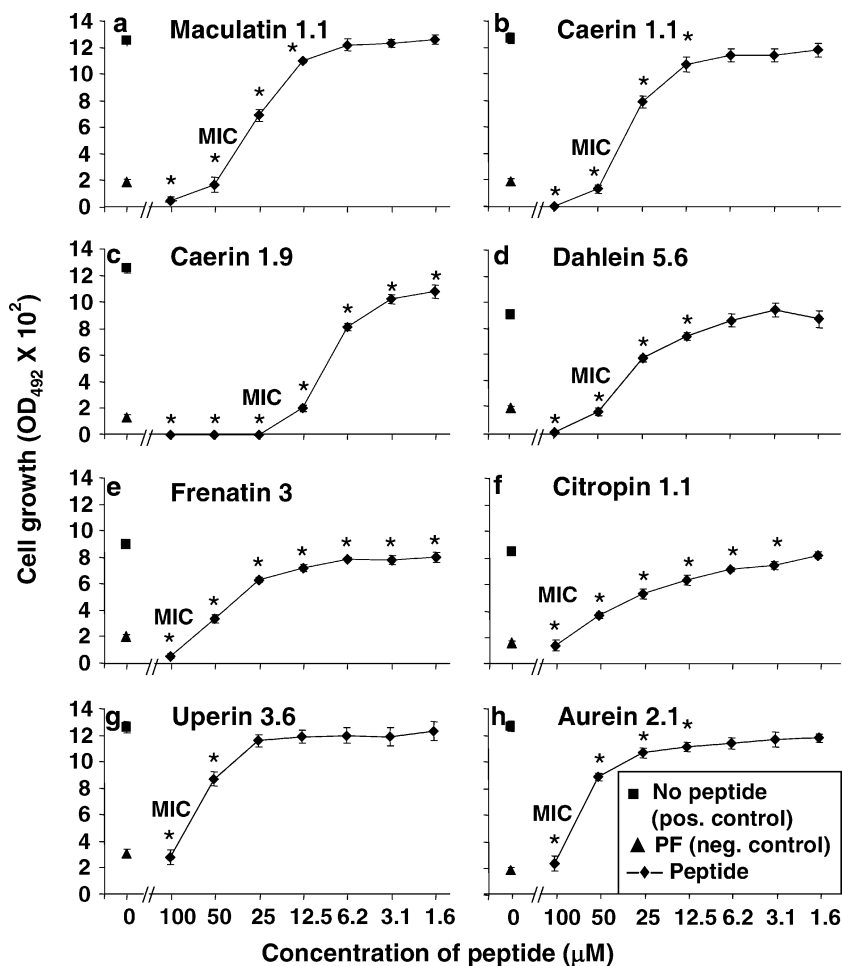


Table 2 Minimal inhibitory concentration (MIC) values for synthetic peptides tested against two *Batrachochytrium dendrobatidis* isolates

Peptide	MIC Against Isolate #197 Mature Cells (μM)	MIC Against Isolate#VM1 Mature Cells (μM)	MIC Against Isolate #197 Zoospores (μM)	MIC Against Isolate #VM1 Zoospores (μM)
Caerin 1.9	25	25	50	25
Maculatin 1.1	50 [2]	50 [2]	25	50
Caerin 1.1	50 [2]	50 [2]	50	25
Dahlein 5.6	100 [2]	100 [2]	200 [4]	200 [3]
Frenatin 3	100	100	100	100
Uperin 3.6	100	100	100	100
Citropin 1.1 Mod 17	100–200 [2]	100–200 [2]	100–200 [3]	100–200 [4]
Aurein 2.1	200 [3]	200 [2]	200 [2]	200 [2]
Caerin 4.1	> > 200 [2]	> > 200 [3]	> > 200 [3]	> > 200 [2]
Tryptophyllin 1.2	> > 200 [2]	> > 200 [2]	> > 200 [2]	> > 200 [2]

Brackets indicate the number of replicate assays. If no brackets are shown, the peptide was tested once. The symbol (> >) indicates that a MIC was not detected within the range tested from 200 μM and below, thus, caerin 4.1 and tryptophyllin 1.2 were inactive in these assays

zoospores at concentrations above 6 μM (MIC 25–50 μM), and both caerin 1.1 and maculatin 1.1 inhibited growth at concentrations above 12.5 μM (MIC 50 μM) (Fig. 1, Table 2). Dahlein 5.6, frenatin 3, and citropin 1.1 were the next most potent, inhibiting growth at concentrations above 12.5–25 μM (MIC 100 μM) (Fig. 1, Table 2). Uperin 3.6 and aurein 2.1 showed moderate inhibition at concentrations above 50 μM (MIC 100 μM) (Fig. 1 and Table 2). Caerin 4.1 and tryptophyllin 1.2 were inactive at concentrations as high as 200 μM (Table 2).

Growth inhibition of a second isolate of *B. dendrobatidis*

Little is known about the genetic variability of the pathogenic amphibian chytrid (Morehouse et al. 2003). It is possible that isolates from different species and different geographic locations could differ in their sensitivity to immune defense mechanisms. Therefore, we tested the peptide sensitivity of a second isolate (VM1) from a diseased Arizona frog (*P. triseriata*). In comparison experiments, nearly identical activity was demonstrated against both chytrid isolates. Comparisons of the sensitivity of type isolate 197 and isolate VM1 are shown (Fig. 2, Table 2).

Growth inhibition of *B. dendrobatidis* by natural mixtures of skin peptides

The data presented above (Fig. 1, Tables 1 and 2) provide information about the potency of isolated peptides. However, most amphibian species express multiple peptides (Vanhoye et al. 2003), and a more complete understanding of the effectiveness of the antimicrobial peptide repertoire of each species can be reached by examining the activity of natural mixtures of skin peptides. We examined the potency against *B. dendrobatidis* of natural mixtures of peptides from five species of stream-associated frogs from Queensland rainforests

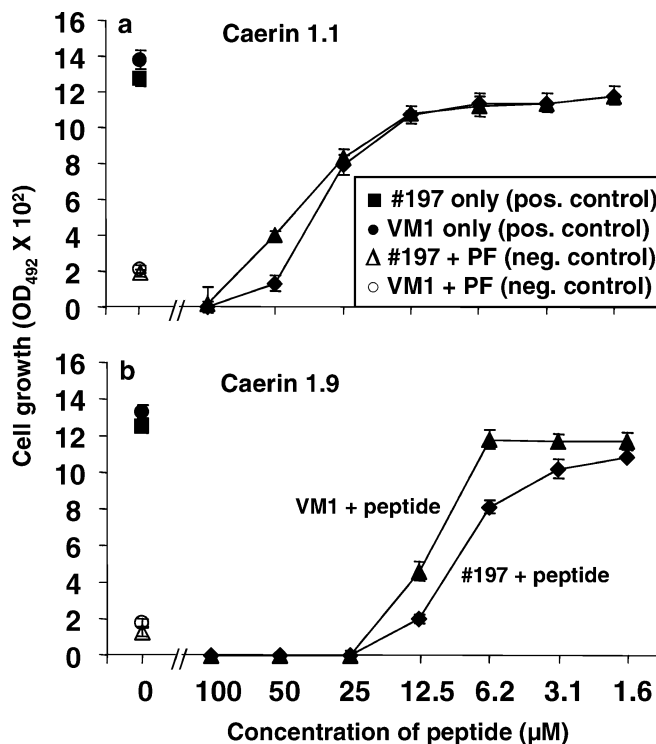


Fig. 2 Growth inhibition of *B. dendrobatidis* mature cells of isolates #197 (type isolate) and #VM1 by **a** caerin 1.1 and **b** caerin 1.9. Growth measured on day 4 (peak of growth) as optical density at 492 nm (OD_{492}). Open symbols represent the negative control, 0.4% paraformaldehyde-killed cells (PF), and filled symbols represent the positive control, live cells with no added peptides

(Figs. 3,4). Although the peptide mixtures from each of the five species inhibited growth of chytrid zoospores, species varied widely in the concentration of peptides necessary to completely inhibit growth (MIC) (ANOVA, $F_{4,10} = 19.912$, $P < 0.001$; Fig. 3) and in the amount of effective peptides on the surface of the skin (MIC equivalents per gbw and per cm^2 ; (ANOVA, $F_{4,43} = 3.307$, $P = 0.027$, and $F_{4,43} = 3.043$, $P = 0.027$, respectively; Fig. 4). The most potent natural peptide mixture occurred in the secretions of *L. lesueuri*. These

Fig. 3 Growth inhibition of *B. dendrobatidis* zoospores by natural mixtures of peptides from **a** *Litoria lesueuri*, **b** *L. genimaculata*, **c** *L. nannotis*, **d** *L. rheocola*, and **e** *Nyctimystes dayi*. Cell growth (mean \pm SE, $n=5$) measured on day 7 (peak of growth) as optical density at 492 nm (OD_{492}) for all panels except **a** measured on day 1. *Open circles* represent the negative control, 0.4% paraformaldehyde-killed cells (PF), and *filled circles* represent the positive control, live cells with no added peptides. *Asterisks* (*) indicate significantly less than positive control for growth by Student's *t* test, $P \leq 0.05$. Minimal Inhibitory Concentration (MIC) is defined as the lowest concentration of added peptide at which no significant growth was observed

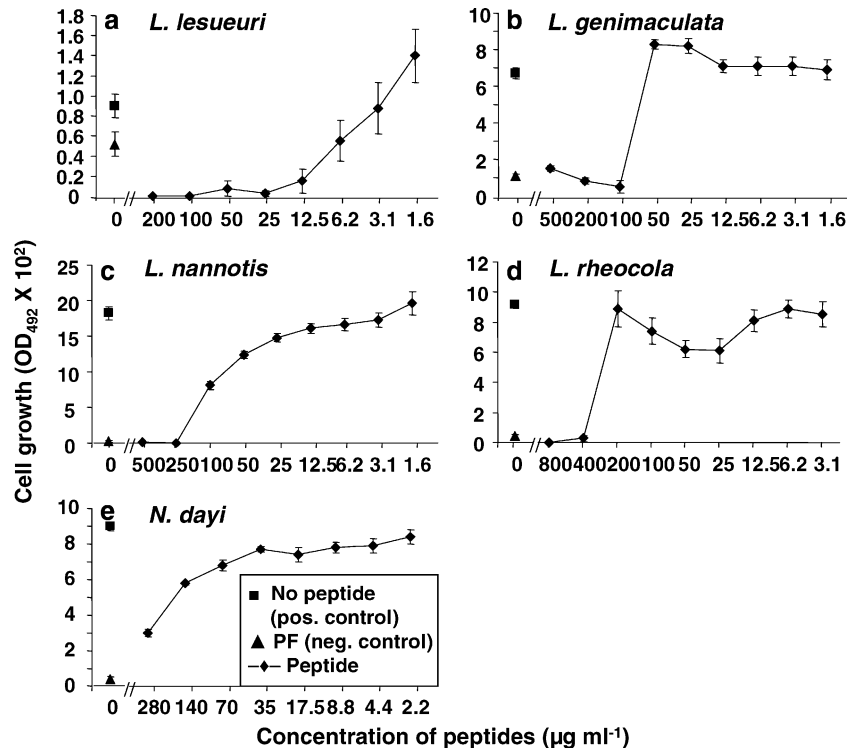
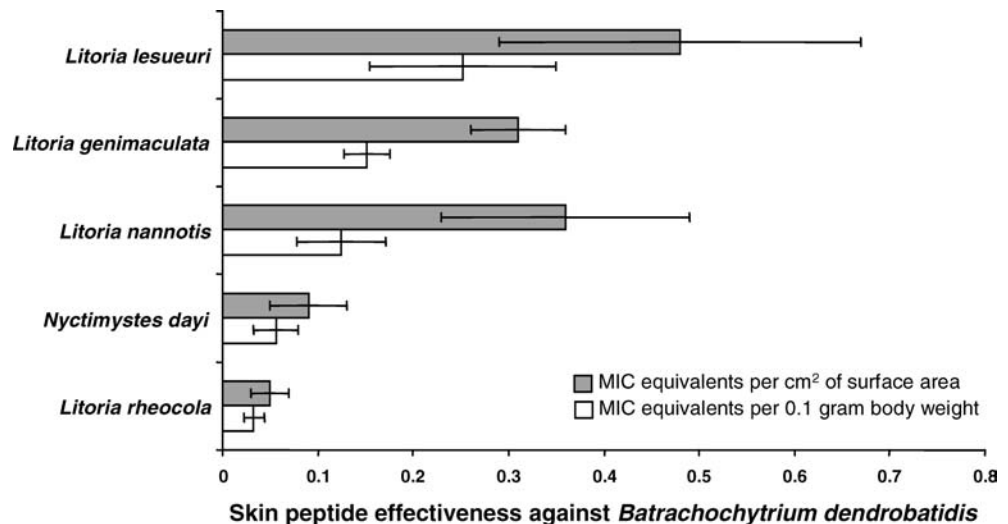


Fig. 4 Skin peptide effectiveness against *B. dendrobatidis* as indicated by MIC equivalents (\pm SE) per cm² of surface area and per 0.1 g body weight of five species of stream-dwelling Queensland frogs. *Litoria lesueuri* ($n=5$) and *L. genimaculata* ($n=24$) (non-declining species) were significantly different than *L. nannotis* ($n=7$), *Nyctimystes dayi* ($n=6$), and *L. rheocola* ($n=4$) (ANOVA contrasts, $P < 0.05$). MIC is defined as the lowest concentration of added peptide at which no significant fungal cell growth was observed



mixtures inhibited zoospore growth at concentrations above 25 µg/ml with an MIC of 25–50 µg/ml (Fig. 3). *L. genimaculata* peptides were the next most potent, inhibiting at concentrations above 100 µg/ml with an MIC of 100–280 µg/ml (Fig. 3). Peptides from *L. nannotis* were somewhat less potent. They inhibited zoospore growth when present at concentrations above 25 µg/ml with an MIC of 200–250 µg/ml (Fig. 3). Peptides from *N. dayi* and *L. rheocola* were the least potent, inhibiting only at high concentrations with MICs estimated between 400–425 (Fig. 3). The MICs of the endangered species were significantly greater than those of the common species (ANOVA two by three contrasts, $P < 0.05$). While MIC is a useful measure of the activity

of the peptide mixtures, it provides little information about the relative amount of active peptides that are present on the skin surface. A comparison of MIC equivalents demonstrated that the endangered species, *N. dayi* and *L. rheocola*, have the least available active peptides per g or per cm² of skin surface (Fig. 4).

Discussion

Although some species may be well-buffered against global change, others may have constrained immune defenses or be otherwise restricted physiologically, biogeographically, or behaviorally, increasing the risk of

extinction (Loehle 2003; Williams et al. 2003; Norris and Harper 2004; Thomas et al. 2004). Innate immune defenses may vary among species and perhaps populations. Some amphibian species are more susceptible to disease-associated population declines than others (Berger et al. 1998; Lips et al. 2003; Retallick et al. 2004). This study demonstrates that an innate defense, antimicrobial skin peptides, may contribute to protecting some amphibians from an emerging pathogen.

Effectiveness of isolated peptides in vitro

Our studies demonstrate that several species of Australian amphibians have at least one antimicrobial skin peptide that can strongly inhibit the growth of *B. dendrobatidis* in vitro. They include *L. chloris* (possesses caerin 1.9), *L. genimaculata* (possesses maculatin 1.1), *L. caerulea*, *L. splendida*, *L. gilleni*, and *L. ewingi* (all possess caerin 1.1) and *L. dahlia* (possesses dahlein 5.6). Four of these species, *L. chloris*, *L. genimaculata*, *L. caerulea*, and *L. ewingi* are known to be susceptible to infection by *B. dendrobatidis* (Speare and Berger 2000). Thus, the presence of a single peptide that is potent in vitro is not sufficient to protect all individuals of a species against chytrid infection. Like other immune responses, antimicrobial peptide expression may be suppressed by corticosteroids released in response to stress (Norris 1996; Miele et al. 1998; Rollins-Smith et al. unpublished data). The effects of pollutants such as pesticides on peptide expression are not known.

Comparison of geographically separated chytrid isolates

These studies allowed us to test whether isolates of *B. dendrobatidis* from widely different geographic locations differed in their susceptibility to antimicrobial skin peptides. Isolate VM1 came from a diseased chorus frog (*P. triseriata*) collected in the state of Arizona. Isolate JEL197 is the type for the species originally isolated from a diseased dart poison frog (*D. auratus*) housed at the Washington National Zoo (Longcore et al. 1999). Both were equally susceptible to all of the 10 purified peptides examined in this study (Fig. 2, Table 2). This suggests that these two *B. dendrobatidis* isolates have not evolved differential abilities to escape destruction by amphibian skin peptides, and supports the hypothesis that this pathogen has recently emerged (Daszak et al. 1999).

Comparison of skin peptide in vitro activity and conservation status of host

Both individual purified peptides and natural mixtures induced growth inhibition of *B. dendrobatidis* in vitro, and may therefore contribute to the immune defense against chytridiomycosis in nature. We would expect

potency to be underestimated if antimicrobial peptides are quickly degraded on the skin (Steinborner et al. 1997b). By limiting the time the frogs spent in collection buffer to 15 min, and immediately acidifying the collection buffer and passing this over C-18 Sep-Pak cartridges, the skin peptide collection technique limited the activity of endoproteases to degrade the antimicrobial peptides. This was confirmed by MALDI-MS analysis of a limited number of samples (D. Woodhams, unpublished observation). For MIC determination, at least three replicate growth inhibition assays for each species incubated for 7 days; day 1 was used as an endpoint for *L. lesueuri* samples that became contaminated later in the experiment (representative assays are shown in Fig. 3). All five of the Australian frog species we examined produced skin peptide mixtures that can inhibit the growth of the amphibian chytrid zoospores in vitro. Although little variation was observed among individuals examined here, preliminary studies of geographically distinct populations of Northern Leopard Frogs (*Rana pipiens*) suggests that peptide defenses vary at the population level (D. Woodhams and L. Rollins-Smith unpublished data). Additional populations of Australian species should be examined in future studies.

The term “antimicrobial peptide” usually designates peptides of a specific structure and mode of operation. It is unknown whether all of the species of Australian wet tropics frogs studied here produced traditional antimicrobial peptides. A previous examination of skin secretions from *L. lesueuri* found no specific antibacterial peptides (Doyle et al. 2002). However, a recent genetic study of the *L. lesueuri* species group distinguished three species (Donnellan and Mahony 2004); animals studied here were within the range of *L. wilcoxii*. It is possible that small peptides or synergistic effects of peptides within natural mixtures were responsible for the observed anti-*B. dendrobatidis* activity. We prefer the term “skin peptide defenses” when referring to natural mixtures of peptides that may or may not contain antimicrobial peptides. Variation existed among species in the quantity and potency of skin peptides. MIC equivalents per body weight or per surface area provide combined measures of skin peptide effectiveness in vitro. By these measures there was a clear pattern suggesting that species that have experienced recent population declines (*L. nannotis*, *L. rheocola*, and *N. dayi*) have peptides of lower in vitro effectiveness than non-declining species (*L. lesueuri* and *L. genimaculata*). This correlation should be tested on more species and systems to determine whether this non-specific immune defense may be useful in predicting disease susceptibility in threatened amphibian assemblages.

Many of the species examined here have been exposed to *B. dendrobatidis* in the wild. Of the five stream-associated species from Queensland rainforests, all can be infected and infection prevalence varies among species but is usually less than 10% in adults (Woodhams and Alford 2005). Several species have disappeared from this habitat before their peptide defenses could be

examined (McDonald and Alford 1999; Northern Queensland Threatened Frog Recovery Team 2001). Persisting species may be those with strong peptide defenses.

Although *L. nannotis* populations declined in association with chytridiomycosis, its peptide defenses are intermediate among the species examined (Fig. 4). This species sometimes released copious frothy secretions in response to handling. However, only individuals that did not respond in this way prior to treatment with norepinephrine were used in the analysis. It is important to recognize that other factors, such as developmental stage, behavior, and environmental conditions may also influence host defenses and susceptibility to chytrid infection, and these parameters should be tested. Further studies comparing the skin peptide defenses of frogs with their susceptibility to experimental infection with *B. dendrobatidis* will be instructive. Differences in immune function are one likely factor explaining why *B. dendrobatidis* affects some species more than others within an assemblage. Other factors are also involved, such as differences in behavior (temperature tolerance and basking behavior; Woodhams et al. 2003), life-history traits, community dynamics (Woodhams and Alford 2005), niche specialization, stream breeding, fecundity, and biogeography (Laurance et al. 1996; Williams and Hero 1998; Lips et al. 2003; Retallick et al. 2004). Although the population dynamics of amphibian assemblages are affected by numerous factors, innate immune defenses may be particularly important in systems that include virulent disease.

In summary, ten isolated peptides and natural peptide mixtures from five species were examined for *B. dendrobatidis* growth inhibition. Isolated amphibian skin peptides potently inhibited growth of the amphibian chytrid fungus *in vitro*. Common species tended to have more effective natural mixtures of skin peptides (in vitro) than endangered species. These results suggest that skin peptides are an important defense against chytridiomycosis *in vivo*. They also suggest that skin peptide defenses may influence host-pathogen interactions and disease-regulated population trends.

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