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ANTIMICROBIAL PEPTIDE DEFENSES IN THE SALAMANDER, *AMBYSTOMA TIGRINUM*, AGAINST EMERGING AMPHIBIAN PATHOGENS

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ABSTRACT: Skin peptides were collected from living *Ambystoma tigrinum* larvae and adults and tested against two emerging pathogens, *Batrachochytrium dendrobatidis* and the *Ambystoma tigrinum* virus (ATV), as well as bacteria isolated from *A. tigrinum.* Natural mixtures of skin peptides were found to inhibit growth of *B. dendrobatidis, Staphylococcus aureus*, and *Klebsiella* sp., but activity against ATV was unpredictable. Skin peptides collected from salamanders held at three environmentally relevant temperatures differed in activity against *B. dendrobatidis.* Activity of the *A. tigrinum* skin peptides was found to be strongly influenced by pH.

Key words: *Ambystoma tigrinum*, *Ambystoma tigrinum* virus, amphibian decline, antimicrobial peptides, ATV, *Batrachochytrium dendrobatidis*, tiger salamander.

INTRODUCTION

Declines in amphibian populations have been observed worldwide since the early 1980s and have now become a global biologic concern (Blaustein and Wake, 1990; Daszak et al., 1999; Houlahan et al., 2000; Stuart et al., 2004; Beebee and Griffiths, 2005). Although factors such as habitat destruction, introduction of exotic species, UV radiation, and anthropogenic contaminants may be involved in declines, it is now apparent that disease is an important factor in many well-documented declines and extinctions. A recently discovered zoosporic fungus, *Batrachochytrium dendrobatidis*, has been implicated in declines and extinctions of frog populations in Central America and Australia (Berger et al., 1998; Lips, 1999; Lips et al., 2006). *Batrachochytrium dendrobatidis* has also been shown to infect adult tiger salamanders (*Ambystoma tigrinum*), although, in contrast to many frog species, *A. tigrinum* does not generally die from *B. dendrobatidis* infection (Davidson et al., 2003b). At the same time, another group of diseases caused by ranaviruses (Iridoviridae) have led to massive localized die-offs of frogs and salamanders in North America and Europe (Carey, 2000; Daszak et al., 1999, 2000; Collins and Storfer, 2003). *Ambystoma tigrinum* is highly susceptible to ranavirus infection (Jancovich et al., 1997). The apparently recent emergence of both *B. dendrobatidis* and ranaviruses suggests that the immune defenses of susceptible species may not be effective against these newly emergent pathogens (Carey et al., 1999; Carey, 2000; Rollins-Smith, 2001).

One important component of the immune defenses of amphibian skin is the capacity to secrete antimicrobial peptides (AMPs). The AMPs are small (10–50 amino acid residues), cationic, hydrophobic molecules produced in the granular skin glands of many amphibian species (reviewed by Andreu and Revas, 1999; Simmaco et al.,1999; Renaldi, 2002; Apponyi et al., 2004; Conlon et al., 2004). They are thought to bind to negatively charged residues in microbial membranes where peptide complexes form pores that lyse the cell or disrupt metabolic functions (Simmaco et al., 1999; Zasloff, 2002). The AMPs isolated from frogs and toads (anurans) have been found to act rapidly and effectively against *B. dendrobatidis* (Rollins-Smith et al., 2002a, b) and against representative ranaviruses including frog virus 3 and channel catfish virus (Chinchar et al., 2001, 2004). To date, few studies have examined whether salamanders (urodeles), members of the other major group of extant amphibians, also possess such peptides. Skin-derived protein fractions from the terrestrial salamander *Plethodon cinereus* were found to inhibit the bacterium *Staphylococcus aureus* but not *Escherichia coli* (Fredericks and Dankert 2000), while fatty acids extracted from this species inhibited *Bacillus cereus* but not 13 other species of bacteria and fungi (Rickrode et al., 1986). Our study is the first to examine the effect of skin secretions of salamanders on *B. dendrobatidis* or ranaviruses, the major pathogens implicated in recent amphibian declines.

Ambystoma tigrinum virus (ATV), a ranavirus related to frog virus 3, causes mortality in many species of salamanders (*A. tigrinum, Ambystoma gracile, Ambystoma mexicanum*) and newts (*Notophtalamus viridescens*) (Jancovich et al., 1997, 2001; Davidson et al., 2003a). The ATV is responsible for localized die-offs of *A. tigrinum* across western North America (Jancovich et al., 2005). However, *A. tigrinum* larvae infected with ATV over a range of environmental temperatures (10 C, 18 C, and 26 C) differ markedly in their mortality and time to death (Rojas et al., 2005). At 26 C most larvae survive ATV exposure, but some retain the virus for up to 60 days. However, at 18 C mortality is rapid and total, while at 10 C mortality is total but requires far longer to be expressed. In cell culture, ATV replicates at all three temperatures, but viral replication is faster at warmer temperatures (Rojas et al., 2005).

One goal of this study was to determine whether secreted skin peptides from *A. tigrinum* could inhibit growth of *B. dendrobatidis* or inhibit infectivity of ATV *in vitro.* A further goal was to determine whether the observed pattern of virus-induced mortality in response to environmental temperatures could be explained by differential production or activity of antimicrobial peptides. Ambient temperature has been shown to influence AMP synthesis in anurans. For example, low environmental temperatures inhibited AMP production in the freeze-tolerant frog *Rana sylvatica* (Mattute et al., 2000). We assessed the efficacy of AMPs from salamanders maintained over a range of ambient temperatures against ATV, *B. dendrobatidis*, and potential bacterial pathogens.

MATERIALS AND METHODS

Animals

Laboratory-reared tiger salamander larvae (*A. tigrinum nebulosum*) were housed individually in small plastic Zip Lock® containers with 300 ml of aged tap water. Larvae were fed live black worms (*Lumbriculus variegatus*) twice weekly, and the water was changed weekly. For temperature acclimation experiments, 15 3-mo-old salamander larvae (mean body mass=2.48 g) were exposed to each experimental temperature (10 C, 18 C, and 26 C; total=45 larvae) for 2 wk prior to peptide collection. For large-scale collections, skin peptides were also collected from larval and small metamorphosed animals that were housed together in aquaria at room temperature (≈22 C). These animals were fed live black worms and crickets (*Acheta domesticus*).

Peptide collection

Skin peptides were collected using a modification of the techniques described by Rollins-Smith et al. (2002b). For temperature acclimation experiments, groups of five *A. tigrinum* larvae acclimated to the same ambient temperature were submerged in 50 ml of collection buffer (50 mM sodium chloride, 25 mM sodium acetate, pH=7.0) for 15 min to accumulate skin peptides from resting salamanders. The buffer was removed and acidified with 500 µl trifluoroacetic acid (TFA) and passed over a C-18 Sep-Pak cartridge (Waters Corporation, Milford, Massachusetts, USA). Immediately following removal of the initial buffer, salamanders were submerged in 50 ml of collection buffer containing 500 µl of 20 mM norepinephrine hydrochloride (Sigma Chemical Co., St. Louis, Missouri, USA; final concentration of norepinephrine=200 µM). After 15 min, this buffer was removed, acidified, and filtered as previously described. For assays requiring large quantities of peptides, samples were collected from groups of 3–5 salamanders maintained at room temperature using collection buffer containing 200 μM norepinephrine. All peptides were eluted from Sep-Paks with a solution of 70% acetonitrile, 29.9% water, and 0.1% TFA (v/v/v). Eluted peptides were concentrated to dryness using a Speed-Vac concentrator (Savant Instruments Inc., Holbrook, New York, USA), reconstituted with ultrapure water and filter-sterilized. Protein concentration of reconstituted peptides was determined using a Micro BCA assay (Pierce, Rockford, Illinois, USA) following the manufacturer's instructions except that bradykinin (Sigma Chemical Co.) was used to establish a standard curve (Rollins-Smith et al., 2002b). After analysis of protein content, the resting and norepinephrine stimulated samples were pooled for use in *B. dendrobatidis* growth inhibition assays.

C-18 Sep-Paks were utilized in sample collection in order to ensure that peptides were the only antimicrobial chemicals present in the final sample. In addition, because an aqueous buffer was used, there is little likelihood that antimicrobial contaminants such as free fatty acids (FFAs) would be present in samples. Nevertheless, to ensure that samples did not contain antimicrobial fatty acids, colorimetric analyses were performed (Itaya and Ui, 1965; Itaya, 1977), and no detectable levels of fatty acids were observed (<0.01 μ equivalents FFA/ml) . Moreover, the aqueous portion of the samples that contained peptides retained antimicrobial function when used in *B. dendrobatidis* growth inhibition assays.

Batrachochytrium assays

Batrachochytrium dendrobatidis (isolate 277) was isolated from *A. tigrinum* (Davidson et al., 2003a) and maintained in culture as described by Rollins-Smith et al. (2002a). Between 5×10^4 and 5×10^5 zoospores in a volume of 50 μl of TGhL broth were plated in four replicates in a 96-well microtiter plate to which 50 μl of peptide dilutions were added. Positive control wells (maximal growth) contained 50 μl of broth with zoospores and 50 μl of either sterile HPLC-grade water or 10 mM pH 3.6 acetate buffer without peptides. Growth at 10 days was measured as increased optical density at 492 nm with a Titertek Multiskan plate reader (Titertek Instruments Inc., Huntsville, Alabama, USA). The mean optical density of each treatment at Day 0 was used as a no-growth baseline control. Minimal inhibitory concentration (MIC) is defined as the lowest concentration of crude peptides at which no growth was detectable. To obtain MIC values, a four-parameter sigmoidal regression curve was calculated for growth inhibition assays using SigmaPlot software version 8.0 (Systat Software Inc., Richmond, California, USA). The MIC was then calculated as the point where the 95% confidence limit above the lower asymptote (y0) crossed the regression line (Fig. 1). The relative amounts of active peptides produced by each acclimation treatment were compared using MIC equivalent measures per gram of body weight and per cm^2 of surface area. MIC equivalents are defined as the total amount of peptides recovered (μg) per gram weight or per cm^2 surface area divided by the experimentally determined MIC (μg/ml) for each treatment (Woodhams et al., 2005). Surface area of the salamanders in cm^2 was calculated using the following allometric equation: surface area$=8.42 \times (\text{weight in grams})^{0.694}$ (Whitford and Hutchinson, 1967).

Virus infectivity assays

The ATV was propagated in *Epithelioma papulosum cyprini* (EPC) cells (Fijan et al., 1983) in Minimal Eagle's Medium (MEM; Sigma Chemical Co.) containing 10% fetal bovine serum (FBS) until full cell lysis was observed. The preparation containing approximately 1×10^7 plaque forming units (PFUs)/ml, was frozen and thawed, divided into 200 μl aliquots, and refrozen. Assay of peptide antiviral activity followed procedures described by Chinchar et al. (2004). Briefly, 10 μl of ATV preparation was added to 10 μl of each undiluted filter-sterilized peptide preparation and incubated for 1 hr at 26 C. Controls included ATV incubated with 10 μl sterile HPLC-grade water or with 10 mM pH 3.6 acetate buffer. In early experiments, some samples of concentrated skin peptides mixed with ATV preparations in MEM tissue culture medium produced a change in color, suggesting that the peptide mixtures were acidic. Using a glass electrode, we observed that the peptide preparations ranged from about pH 2 to pH 5, with most preparations at pH 3.5–4.0. To determine the molarity, we titrated 200 μl samples of crude peptide preparations using 2, 4, 6, 8, or 10 μl aliquots of 50 mM Tris buffer pH 7.3. The molarity of two separate preparations approximated 10 mM. For this reason we included 10 mM sodium acetate buffer, pH 3.6 as a control in all further assays to determine whether activity of peptide preparations or reduction of virus activity might be due in part to pH effects. Preparations titrated from initial pH 4.0 to pH 5.2,

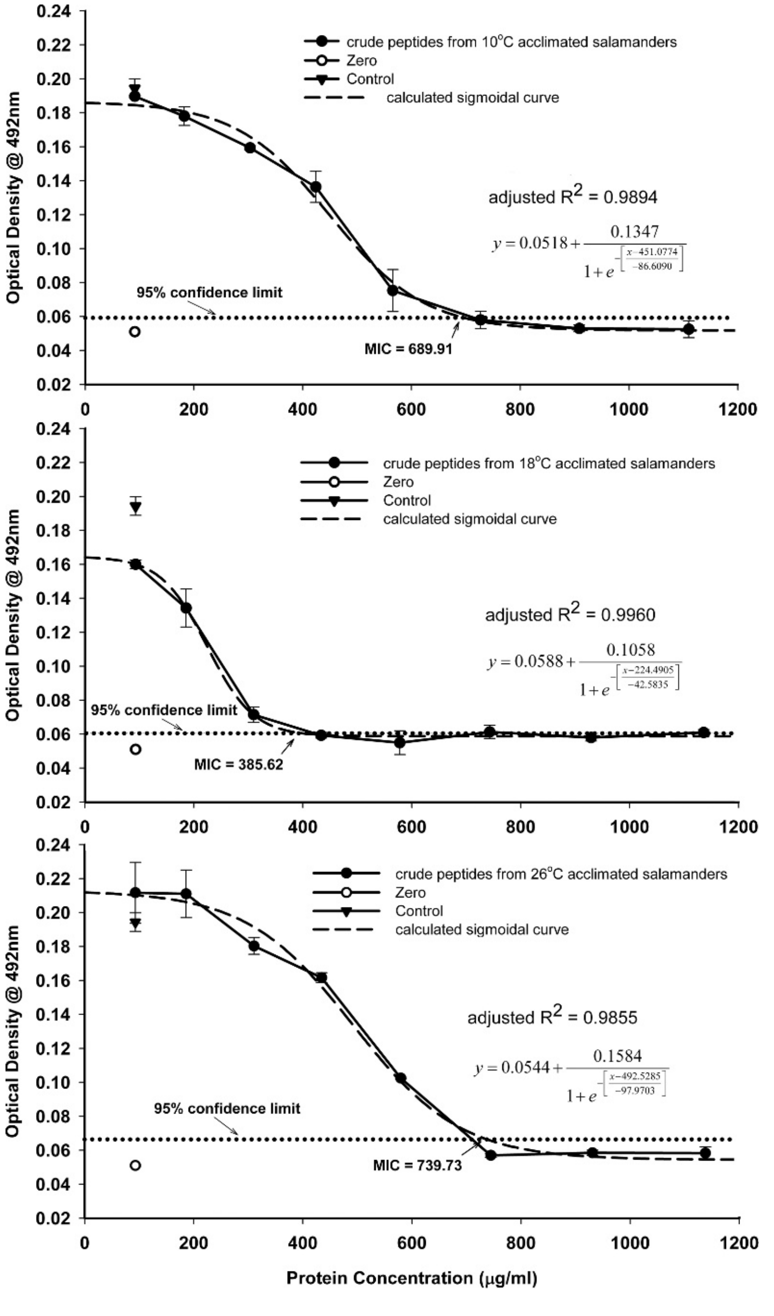

FIGURE 1. Growth inhibition of *Batrachochytrium dendrobatidis* zoospores by skin peptides from larval *Ambystoma tigrinum* acclimated to ambient temperatures of 10 C, 18 C, and 26 C. Data points are means of four replicates±SE. Four parameter sigmoidal regression curves were calculated for each assay, and the minimum inhibitory concentration (MIC) was calculated as the point where the 95% confidence limit crosses the regression line.

5.8, 6.4, and 6.7 were assayed against *B. dendrobatidis*. Peptide-virus preparations were then diluted in MEM with 2% FBS and a standard plaque assay using EPC cells was performed. Plaques in replicate wells were counted and averaged after approximately 2 wk incubation at 18 C (Jancovich et al., 1997). Activity of the peptide preparations is expressed as percentage reduction in PFU's compared with positive controls (ATV incubated with sterile water alone).

Bacteria assays

Escherichia coli (ATCC no. 23716), *S. aureus* (ATCC no. 25923), and *Aeromonas hydrophila* (ATCC no. 4308B) were cultured for 24 hr in 10 ml Luria broth at 25–26 C; *B. cereus* was cultured in 10 ml nutrient broth at 25–26 C. Each bacterial culture was diluted to ca. 10^4 bacteria/ml in 0.9% NaCl, and 10 μl of this dilution was mixed with 10 μl undiluted natural peptide mixtures. Controls included sterile HPLC-grade water or 10 mM pH 3.6 acetate buffer. The mixture was incubated at 25–26 C for 1 hr, then added to 1 ml saline and further dilutions were made and plated.

Bacteria were also isolated from an adult *A. tigrinum* that had been reared in the laboratory. This salamander was wiped with a sterile cotton swab that was then inoculated onto NYSM agar (nutrient agar, yeast extract, salts medium; Yousten et al., 1984), trypticase soy agar, MacConkey's agar, and thioglycollate broth (McCampbell, 2001). Cultures were incubated for 48 hr at 26 C. Nine representative colonies that each appeared to be different bacterial species were chosen for assay. Bacteria were identified based on cell shape and size, colony color, sporulation, motility, Gram stain, catalase test, pattern of growth on plate, and changes during life cycle. Enterotube II© tests (Beckton Dickinson, Cockeysville, Maryland, USA) were also employed. The activity of undiluted skin peptide preparations was determined against these isolates using the technique used for known species (above). When our results demonstrated that the pH 3.6 buffer control alone inhibited most bacterial species, we repeated the experiments including 20 μl of nutrient broth in the incubation mixture to reduce the effect of pH alone.

To test the influence of the media used in assays against ATV, *B. dendrobatidis*, and bacteria on pH of the AMP preparations, we added equal quantities of 10 mM pH 3.6 acetate buffer to each of the media. The pH was increased by MEM (ATV cell culture medium) to 6.65, by TGhL (*B. dendrobatidis* medium) to 5.82, and by nutrient broth (added to bacterial assays) to 5.17, whereas dilution with 0.9% NaCl resulted in pH of 3.90.

Statistical tests

Effectiveness of skin peptide mixtures as average MIC equivalents were compared by a one-tailed Student's *t*-test. A P value≤0.05 was generally considered to be significant.

RESULTS

Protein content

Skin secretions from salamanders collected using acetate buffer alone had significantly lower protein content than did samples extracted using acetate buffer containing 200 μM norepinephrine (one-tailed Student's *t*-test). Even though peptide extraction using norepinephrine took place immediately following extraction with plain acetate buffer, norepinephrine induced samples were on average 28% higher.

Antimicrobial activity against *Batrachochytrium dendrobatidis*

Growth of *B. dendrobatidis* was inhibited by peptides collected from larval *A. tigrinum.* Highly active preparations led to immediate loss of mobility of zoospores, observable within 1 hr, and no *B. dendrobatidis* growth was observed in these preparations. In addition, there was a distinct relationship between the temperature at which salamander larvae were held and the activity of the peptides collected from these animals against *B. dendrobatidis.* The peptides released by animals acclimated to 18 C were more potent (i.e., MICs were reduced), and MIC equivalents were higher than those from salamanders acclimated to either 10 C or 26 C (one-tailed Student's *t*-test) (Fig. 1 and Table 1). Preparations that fully inhibited *B. dendrobatidis* growth at an initial pH of 4.0 lost partial activity at pH 6.4 and nearly all activity at pH 6.7 when titrated with pH 7.3 Tris buffer.

Inhibition of ATV infectivity

Skin peptides from larval *A. tigrinum* were unpredictable in their activity against ATV among preparations. Peptides collected from larvae held at 10, 18, or 26 C reduced viral PFU's between 13% and 66%, but activity was not related to the

TABLE 1. Summary of skin peptides and antimicrobial activity against *Batrachochytrium dendrobatidis* from larval *Ambystoma tigrinum* acclimated to a range of ambient temperatures. Minimum inhibitory concentration (MIC) equivalents are calculated per gram body weight (gbw) and per cm^2 of skin surface area. Values shown are means±SD.

Temperature at which larvae were acclimated (C)	*n*	Total peptides per mass (μg/gbw)	Total peptides per surface area (μg/cm^2)	MIC against *B. dendrobatidis* zoospores (μg/ml)	MIC equivalents per gbw	MIC equivalents per cm^2
10	15	400.34±73.7	80.36±17.1	690	0.580±0.10	0.116±0.023
18	15	330.96±31.1	61.13±4.93	385	0.860±0.10[a]	0.159±0.016[b]
26	14	325.16±81.84	61.15±14.95	740	0.439±0.11	0.083±0.020

[a] Significantly greater than that of peptides collected from salamanders acclimated to 10 C by one tailed-Student's *t*-test ($P \leq 0.05$) or acclimated to 26 C by one-tailed Student's *t*-test ($P \leq 0.01$).

[b] Significantly greater than that of peptides collected from salamanders acclimated to 10 C by one tailed-Student's *t*-test ($P \leq 0.1$) or acclimated to 26 C by one-tailed Student's *t*-test ($P \leq 0.005$).

temperature at which the salamanders were housed. The activity of these preparations did not differ significantly whether based on total protein content, surface area of the animals, or total body weight of the animals. Activity of skin peptides from metamorphosed salamanders against ATV was also unpredictable. Some peptide preparations from metamorphs reduced PFUs up to 75%, but when the activity was expressed in terms of surface area, body weight or protein content of the preparation, the values were not significantly different from the peptides collected from larvae. None of the peptide mixtures alone had any observable effect on cultured EPC cells.

Antimicrobial activity against bacteria

Nine species of bacteria isolated from the skin of a single laboratory-reared *A. tigrinum* metamorph were tentatively identified as *Arthrobacter* sp., *Staphylococcus cohnii, Proteus mirabilis, Aureobacterium* sp., *Carnobacterium* sp., *Providencia rettgeri, Vibrio* sp., *Klebsiella* sp., and one unidentified species in the family Myxobacterium. Growth of all of these species except *Vibrio* sp. and Myxobacterium, as well as laboratory cultures of *E. coli, S. aureus, A. hydrophila*, and *B. cereus*, were inhibited 26–100% by the skin peptide preparations alone. Growth of all bacterial species except Myxobacterium was also inhibited between 18% and 99% by 10 mM pH 3.6 acetate buffer. However, when an equal volume of nutrient broth was added to the incubation mixture containing peptides, raising the pH to ca. 5.2, only two species were inhibited. *Staphylococcus aureus* was inhibited by 90% (±0.7%) and *Klebsiella* sp. by 83% (±13%; average of two assays). None of the tested species was inhibited by pH 3.6 acetate buffer when nutrient broth was added to the incubation mixture (Table 2).

DISCUSSION

This study is one of the first to demonstrate antimicrobial activity of skin peptides from salamanders against pathogens associated with amphibian declines. Skin peptides from *A. tigrinum* were effective inhibitors of the growth of the pathogenic skin fungus, *B. dendrobatidis*, and several bacterial species isolated from *A. tigrinum.* Salamander peptides also inhibited infectivity of ATV virus, but less predictably. Our results closely agree with observations of diseases of *A. tigrinum* in the field. This species does not generally die of *B. dendrobatidis* even when heavily infected (Davidson et al., 2003b), but is readily killed by low concentrations of ATV (Jancovich et al., 1997; Brunner et al., 2004, 2005).

We also examined differences in peptides produced by amphibians at environ-

TABLE 2. Activity of *Ambystoma tigrinum* crude antimicrobial peptide extracts against bacteria isolated from the skin of *A. tigrinum* or against laboratory strains of bacteria, with or without addition of equal volumes of nutrient broth. Average of two experiments.

Bacterial species	Larval peptide alone, % inhibition	Larval peptide plus nutrient broth, % inhibition	pH 3.6 buffer alone, % inhibition	pH 3.6 buffer plus nutrient broth, % inhibition
Arthrobacter sp.	95	0	62	0
Aureobacterium sp.	30	0	29	0
Carnobacterium sp.	70	0	99	0
Klebsiella sp.	80	83	74	0
Proteus mirabilis	80	0	66	0
Providencia rettgeri	99	0	18	0
Staphylococcus cohnii	90	0	66	0
Vibrio sp.	0	0	40	0
Myxobacteria[a]	0	0	0	0
Escherichia coli[b]	90	0	26	0
Staphylococcus aureus[b]	90	90	30	0
Aeromonas hydrophila[b]	100	0	96	0
Bacillus cereus[b]	98	0	82	0

[a] Genus and species not determined.

[b] Laboratory strains.

mentally relevant temperatures and related these differences in peptide activity to sensitivity of the host to a series of pathogens. Ambient temperature has a strong effect on susceptibility of *A. tigrinum* to ATV (Rojas et al., 2005), but the influence of temperature on susceptibility to *B. dendrobatidis* has not been investigated. The ambient temperature at which tiger salamanders were acclimated had a notable influence on the effectiveness of skin peptides against *B. dendrobatidis.* Although acclimation temperature did not significantly affect the total amount of peptides produced per gram body weight or per cm^2 of skin surface area, the animals housed at their preferred ambient temperature of 18 C synthesized crude peptide mixtures that demonstrated greater antifungal activity as denoted by their low MIC (Fig. 1). Animals acclimated to ambient temperature 8 C higher or lower showed MIC values that were over two times greater than what was observed at 18 C. Similarly, MIC equivalents show that salamanders acclimated to 18 C generate more available active peptides per gram or per unit of skin surface than do animals maintained at 10 C or 26 C (Table 1).

The peptide content of skin secretions released by *A. tigrinum* following induction with 200 μM norepinephrine appears to be far greater than was observed in studies examining peptides released by anurans induced in the same way. For example, Woodhams et al. (2005), using collection procedures similar to those in this study, examined five species of Australian frogs and found total peptide levels ranging from 10.5 to 29.6 μg/gram body weight. This is in contrast to the 325–400 μg/gram body weight collected from tiger salamanders (Table 1). It should be noted that peptide concentrations in both studies were determined by micro-BCA assay using bradykinin as a standard. This assay measures the number of peptide bonds and the presence of cysteines, cystine, tryptophan, and tyrosine (Weichelman et al., 1988). Because this is one of the few studies to examine urodele skin peptides, we are not sure if copious skin peptide production is common to all salamanders or if it is specific to *A. tigrinum.* Even though tiger salamanders produce large quantities of peptides, their effectiveness against *B. dendrobatidis* is less than the smaller amounts of peptides made by many anurans, suggesting that many nonactive peptides were also collected from salamanders by this technique. For instance, the crude peptides from most frogs generally have MICs in the range of 25–200 μg/ml against *B. dendrobatidis* zoospores (Rollins-Smith et al., 2002a; Woodhams et al., 2005). Only frogs that are highly susceptible to chytrid infection have demonstrated MICs as high as those in *A. tigrinum* (310–740 μg/ml). The function of the nonactive peptides is unknown at this time: they may be remnants of degraded proteins, antimicrobial peptide precursors, antimicrobial peptides that are active against microbes other than *B. dendrobatidis*, or structural components of urodele skin mucus not related to antimicrobial defense.

Although *A. tigrinum* is generally not killed by *B. dendrobatidis*, adult animals do become heavily infected after exposure to zoospores. Tiger salamander peptides may not be active enough or present in sufficient quantities on the skin to inhibit zoospore attachment. However, the peptides may be involved in clearing of infection or prevention of mortality. Heavily infected animals can eventually reduce or eliminate their infections (Davidson et al., 2003b). We do not yet clearly understand how *B. dendrobatidis* kills infected hosts or why some species such as *A. tigrinum* become heavily infected but do not generally suffer mortality from this pathogen. Nevertheless, our findings indicate that animals that are exposed to suboptimal environmental temperatures may be at greater risk of infection due to a decrease in antimicrobial peptide effectiveness.

The isolation of at least nine species of culturable bacteria on the skin of a single metamorphosed salamander held under clean laboratory conditions suggests that bacteria and antimicrobial peptides maintain a balance on the skin and within the mucus layer of the animals. Bacteria resident on the skin may be involved in stimulation of antimicrobial peptide production by salamanders, as observed with frogs (Miele et al., 1998; Boman, 2000; Mangoni et al., 2001), or may themselves produce antimicrobial substances (Jack et al., 1996). Similar bacterial species (*Proteus mirabilis, Staphylococcus* spp., *Providentia (Proteus) rettgeri, Klebsiella* spp.) were isolated from diseased anurans held in the National Aquarium in Baltimore (McCampbell, 2001), and *Klebsiella* and *Proteus vulgaris* were isolated from healthy frogs by Boman (2000), suggesting that these and related bacteria are common residents of amphibian skin. Harris et al. (2006) have demonstrated that bacteria in three genera found on the skin of two salamander species, *Plethodon cinereus* and *Hemidactylium scutatum*, inhibit the growth of *B. dendrobatidis.*

The low pH values of natural skin peptide mixtures isolated from *A. tigrinum* led us to ask whether the activity of these preparations against *B. dendrobatidis*, ATV, or bacteria could be related to pH. When an *A. tigrinum* skin peptide preparation with an initial pH of 4.0 was combined with a Tris buffer of pH 7.4, activity against *B. dendrobatidis* was reduced above pH 6.4 and nearly eliminated at pH 6.7, suggesting that these peptides are most active at low pH. *Batrachochytrium dendrobatidis* and ATV were not found to be inhibited by pH 3.6 buffer alone, but all bacterial species were inhibited by this buffer to varying degrees. Both *B. dendrobatidis* and ATV were exposed to the buffer and skin peptides in the presence of media that were capable of raising the pH, whereas bacteria were initially exposed in the presence of unbuffered saline. The partial inhibition of most bacteria by low pH buffer suggests that the pH of the preparations was responsible for the broad range of activity of the peptides against bacteria. When nutrient broth was added, inhibitory activity against most bacterial species was lost. However, two bacterial species, *S. aureus* and *Klebsiella* sp., continued to be inhibited by *A. tigrinum* skin peptides in the presence of nutrient broth (Table 2). Jack et al. (1996) demonstrated that a peptide isolated from the bacterium *Carnobacterium piscicola* retained antimicrobial activity at a low pH, but was inactivated at neutral or alkaline pH. Interestingly, a member of this bacterial genus was isolated from the skin of an *A. tigrinum* metamorph (Table 2). Our results suggest that the effect of pH should be taken into consideration in all assays of antimicrobial peptides. *Staphylococcus aureus* is also inhibited by peptides from plethodontid salamanders (Fredericks and Dankert, 2000) and several frog species (Zasloff, 1987; Goraya et al., 1998, 2000; Matutte et al., 2000; Conlon et al., 2004). *Klebsiella pneumoniae* was shown to be inhibited by Magainin 2, a peptide from *Xenopus laevis* (Zasloff, 1987). Failure of *A. tigrinum* peptides to inhibit *Aeromonas hydrophila*, a common secondary pathogen of amphibians (Taylor et al., 2001), is similar to what has been reported with frog peptides (Rollins-Smith et al., 2002b).

Crude mixtures of skin peptides produced at 10 C, 18 C, and 26 C were active against ATV, but their activity was unpredictable among preparations. Our initial hypothesis, that the markedly different responses of *A. tigrinum* to ATV observed at three environmentally relevant temperatures (Rojas et al., 2005) is due to differences in production of antimicrobial peptides, was therefore not supported. Rather, our results suggest that the observed differences in susceptibility and time to death at the three experimental temperatures may largely be due to differences in immune functions other than skin peptides. However antimicrobial peptides may still have a role in protecting the salamanders when they are exposed to low concentrations of virus in the field.

The range of activity of crude A. tigrinum peptide preparations observed against *B. dendrobatidis*, ATV, and bacteria suggest that *A. tigrinum* produces several antimicrobial peptides, which may act synergistically as found by Rollins-Smith et al. (2002b) for two peptides from *X. laevis*. These peptides may differ both qualitatively and quantitatively from one animal to another, as a result of life stage (larva vs. metamorph), diet, exposure to microorganisms, or stress.

In summary, skin peptides from *A. tigrinum* were effective inhibitors of growth or infectivity of fungal and bacterial pathogens of importance to amphibian health. Peptides from salamanders held at optimal temperatures were most effective. Further studies aim to isolate and characterize these antimicrobial peptides and determine their relatedness to other previously described amphibian antimicrobial peptides.

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