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Skin defenses of North American salamanders against a deadly salamander fungus

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Abstract

The recently emerged fungal pathogen, *Batrachochytrium salamandrivorans* (*Bsal*) causes the lethal skin disease chytridiomycosis in susceptible salamander species and is predicted to emerge within the Americas with devastating consequences. Host responses to *Bsal* are variable but the factors underlying these differences are unknown. To investigate the role of skin-associated immune defenses against *Bsal* and the closely related, *B. dendrobatidis* (*Bd*), we sampled skin peptides from wild and captive North American salamanders (spotted salamanders [*Ambystoma maculatum*], n = 10; hellbenders [*Cryptobranchus alleganiensis*], n = 2; red-legged salamanders [*Plethodon shermani*], n = 18; and Ocoee salamanders [*Desmognathus ocoee*], n = 15) and conducted *in vitro* experimental assays to test whether salamander skin peptides inhibit chytrid growth. Interspecies differences in skin defenses against *Bsal* and *Bd*, and peptide mixtures were also assessed using a standardized measure and RP-HPLC, respectively. For *A. maculatum*, skin peptides inhibited *Bsal* and *Bd* growth, consistent with known *Bsal* resistance. *Cryptobranchus alleganiensis* skin peptides inhibited the growth of *Bsal* but not *Bd*. *Plethodon shermani* and *D. ocoee* skin peptides facilitated *Bsal* growth and had either no effect or inconsistent effects on *Bd* growth. With the exception of *A. maculatum*, most species had relatively weak skin defenses against both chytrid pathogens. Collectively, we demonstrate that salamander skin peptide defenses against chytrid pathogens are highly variable and not always equally effective against *Bsal* and *Bd*. By advancing knowledge about the factors underlying chytrid susceptibility, particularly *Bsal*, our findings will help inform conservation initiatives aimed at reducing disease impacts and biodiversity loss.

Introduction

In recent years, emerging fungal diseases have become significant drivers of biodiversity loss, raising serious concerns among the conservation community (Fisher *et al*., 2012; Scheele *et al*., 2019). Chytridiomycosis, an infectious skin disease of amphibians, is particularly concerning because chytridiomycosis has driven the decline of hundreds of amphibian species worldwide, deeming it one of the most devastating vertebrate diseases in recorded history (Scheele *et al*., 2019). Chytridiomycosis is caused by chytrid fungal pathogens, *Batrachochytrium dendrobatidis* (*Bd*) and *B. salamandrivorans* (*Bsal*) (Longcore *et al*., 1999; Martel *et al*., 2013). While *Bd* was initially discovered in the late 1900s and is now found globally, the recent emergence of *Bsal* has resulted in chytridiomycosis outbreaks and mass mortalities among captive and wild salamander populations in Europe (Longcore *et al*., 1999; Martel *et al*., 2013; Cunningham *et al*., 2015; Sabino-Pinto *et al*., 2015; Spitzen-van der Sluijs *et al*., 2016; Sabino-Pinto *et al*., 2018; Scheele *et al*., 2019). Although *Bsal* has not yet been detected outside of Europe or endemic regions of Asia, *Bsal* is predicted to emerge within the Americas by way of international trade (Grant *et al*., 2016; Klocke *et al*., 2017; Laking *et al*., 2017; Nguyen *et al*., 2017; Parrott *et al*., 2017). Given the widespread and destructive impacts *Bd* has had on amphibian biodiversity over the last several decades, *Bsal*'s potential to negatively impact hundreds of native salamander species is of utmost conservation concern (Gray *et al*., 2015; Grant *et al*., 2016; Scheele *et al*., 2019).

Despite *Bsal's* ability to infect a range of amphibian hosts, host responses to *Bsal* are highly variable (Martel *et al*., 2014; Carter *et al*., 2019). Until now, most of our understanding of the interspecific differences in host response to *Bsal* has come from a limited number of laboratory studies where individual amphibians were experimentally exposed to controlled doses of *Bsal* zoospores (infective life stage; range: 5×10^3 to 5×10^6 zoospores), and monitored for changes in infection load (the number of pathogenic organisms on a host; measured via genomic equivalents or GE), clinical signs of disease (e.g. ulcerative skin lesions, lethargy, skin sloughing, loss of righting reflex) and

mortality (Blooi *et al*., 2013; Martel *et al*., 2013; Martel *et al*., 2014; Blooi *et al*., 2016; Barnhart *et al*., 2019; Carter *et al*., 2019). Based on these initial studies, host responses to *Bsal* ranged from resistant (no infection or clinical signs of disease) to lethal (infection resulting in mortality) (Martel *et al*., 2014). Although *Bsal* can establish infection in at least one anuran species, chytridiomycosis and mortality has, thus far, only been documented for salamander hosts (Martel *et al*., 2014; Stegen *et al*., 2017).

Little is known about the factors underlying *Bsal* susceptibility but studies using an anuran-*Bd* model system find that susceptibility is dependent on infection load. Thus, interspecific differences in host responses to *Bsal* may also be explained, in part, by a species' ability to limit infections (Carey *et al*., 2006; Briggs *et al*., 2010; Wilber *et al*., 2017). For example, studies investigating *Bd* susceptibility in captive and free-living anurans demonstrated that the probability of survival was significantly increased when *Bd* infection loads were below an approximate species-specific threshold (Stockwell *et al*., 2010; Vredenburg *et al*., 2010; Wilber *et al*., 2016). A similar trend was observed for plethodontid salamanders experimentally exposed to *Bsal* where mortality was only observed at infection loads above a threshold of 15 000 GE suggesting that *Bsal* susceptibility may also be load-dependent (Carter *et al*., 2019). Furthermore, computational models examining the role of infection load in driving *Bd*-associated population extinctions showed that the probability of disease-induced extinction can be drastically decreased by reducing infection load (Stockwell *et al*., 2010). Therefore, salamander species capable of limiting *Bsal* infections would, in principle, also be less prone to population extinctions compared to species that accrue heavier infection loads.

As an amphibian's skin is the first line of defense against chytrid pathogens, skin-associated immune defenses may be important for limiting *Bsal* infections and predicting chytrid susceptibility (Rollins-Smith, 2009). Amphibian skin harbors an array of microbial symbionts (and metabolites) and host enzymes, and is populated by glands that release mucous and bioactive compounds onto the skin's surface, including peptides (Duellman *et al*., 1994; Austin, 2000; Zhao *et al*., 2006; Kueneman *et al*., 2014; Nikolaieva *et al*., 2018; Varga *et al*., 2019). While the specific skin secretion mixtures vary widely among amphibians and are influenced by environmental, genetic and physiological factors, many species secrete peptides with broad-spectrum antimicrobial activity (Simmaco *et al*., 1997; Tennessen *et al*., 2009; Conlon & Sonnevend, 2010; Krynak *et al*., 2016; Wang *et al*., 2016b). Thus, skin peptides are considered an important part of amphibian innate immunity (Woodhams *et al*., 2007b; Rollins-Smith, 2009). *In vitro* studies show that anuran skin peptides (isolates and mixtures) inhibit the growth of *Bd* (Rollins-Smith & Conlon, 2005a; Rollins-Smith, 2009). In addition, anuran species that released skin peptides with *in vitro* activity against *Bd* tended to have low infection loads and did not develop chytridiomycosis following *Bd* exposure (Woodhams *et al*., 2007a).

Compared to anurans, little is known about the role of skin peptides in protecting salamanders from chytrid pathogens, particularly *Bsal* (Nascimento *et al*., 2003; Woodhams *et al*., 2006b; Sheafor *et al*., 2008; König *et al*., 2015; Smith *et al*., 2018). Understanding the factors underlying *Bsal* disease dynamics, such as those influencing susceptibility are important for building computational models for simulating various disease scenarios and identifying amphibian populations likely to experience *Bsal*-mediated declines and extinctions. Further, such models are critical for informing proactive management strategies aimed at reducing disease impacts should *Bsal* emerge in the Americas (Woodhams *et al*., 2011; DiRenzo & Grant, 2019).

As a first step toward understanding the role of skin peptides in protecting salamander species against *Bsal* and *Bd*, we sampled skin peptides from wild and captive North American salamander species (spotted salamander, *Ambystoma maculatum;* hellbender, *Cryptobranchus alleganiensis;* red-legged salamander, *Plethodon shermani;* Ocoee salamander, *Desmognathus ocoee*) and conducted *in vitro* experimental assays to test the hypothesis that salamander skin peptides inhibit chytrid growth. We chose these species because they represent three different North American salamander families and exhibit a range of lifestyles: alternating between terrestrial and aquatic depending on breeding status (*Ambystomatidae: A. maculatum*), fully aquatic (*Cryptobranchidae*: *C. alleganiensis*), fully terrestrial (*Plethodontidae: P. shermani*) and semi-aquatic (*Plethodontidae: D. ocoee*) (Bishop, 2018). Each species also inhabits geographic regions likely to be impacted by *Bsal* should it reach North America (Yap *et al*., 2015; Richgels *et al*., 2016; Bishop, 2018; Pereira & Cusaac, 2019).

When available, we used information on experimentally observed host responses to chytrid pathogens to guide our predictions. Thus, for *A. maculatum*, which appear to be *Bsal*-resistant, we predicted that skin peptides would inhibit the growth of *Bsal* (Martel *et al*., 2014; Barnhart *et al*., 2019). Because individuals of many species of *Plethodon* salamanders are often resistant to all but very high doses of *Bd*, we predicted that *P. shermani* skin peptides would inhibit the growth of *Bd* (Chinnadurai *et al*., 2009; Vazquez *et al*., 2009; Fonner *et al*., 2017). Less is known about responses of *C. alleganiensis* and *D. ocoee* to chytrid pathogens, precluding specific predictions. Finally, given the broad-spectrum nature of known antimicrobial skin peptides, we also predicted that *in vitro* responses would be similar for *Bsal* and *Bd* (Wang *et al*., 2016b).

To standardize and compare interspecies differences in skin defenses against *Bsal* and *Bd*, we attempted to use *in vitro* results to calculate minimum inhibitory concentrations (MIC) and predictive indices of resistance (PIR). Previous studies found that low MIC values and high PIR values are generally associated with species with strong skin defenses and increased resistance against *Bd* (Woodhams *et al*., 2006a; Woodhams *et al*., 2006b). Thus, we predicted that these values would correspond with known resistance of *A. maculatum* and *P. shermani*. In addition, skin peptides

were analyzed using reversed phase high performance liquid chromatography (RP-HPLC) to complement our *in vitro* assays by exploring species differences in peptide mixtures.

Collectively, this study aimed to narrow the gap in knowledge about the factors underlying salamander susceptibility to chytrid pathogens, particularly *Bsal*. We investigated the variation in pathogen-peptide responses to salamander skin peptides, as well as, the implications of skin peptides for protecting species against chytrid pathogens. Thus, our results will help inform conservation initiatives focused on reducing disease impacts and biodiversity loss.

Materials and methods

Animal use

Skin secretions were collected from adult salamanders of the following species: spotted salamander *Ambystoma maculatum*, hellbender *Cryptobranchus alleganiensis*, red-legged salamander *Plethodon shermani* and Ocoee salamander *Desmognathus ocoee*. Most of these species have a propensity to release milky skin secretions indicative of high peptide content (Largen & Woodley, 2008; Gall & Mathis, 2010; von Byern *et al*., 2017b). Animal collection was permitted by the Pennsylvania Fish and Boat Commission and the North Carolina Wildlife Resources Commission. All animal use was approved by Duquesne University's Institutional Animal Care and Use Committee (IACUC No. 1708-07).

Skin secretions were collected from *A. maculatum* and *C. alleganiensis* in the field. Male and female *A. maculatum* (n = 10; mean mass SD. of 7 individuals: 16.4 2.4 g) were hand captured from Allegheny County, Pennsylvania in March 2017. Male *C. alleganiensis* (n = 2; mean mass SD: 566.0 42.4 g) were hand captured from Crawford County, Pennsylvania in June 2017. Because *A. maculatum* and *C. alleganiensis* are relatively large in body size, repeated sampling of skin secretions was not necessary to collect enough skin peptides for *in vitro* assays. Following capture, *A. maculatum* and *C. alleganiensis* were immediately sampled for skin secretions, rinsed and released.

Skin secretions were collected from *P. shermani* and *D. ocoee* in the laboratory. Male and female *P. shermani* (n = 18; mean mass SD: 3.1 0.8 g) and *D. ocoee* (n = 17, mean mass SD: 2.2 0.5 g) were hand captured from Macon County, North Carolina in August 2017. Because *P. shermani* and *D. ocoee* are small-bodied and release small amounts of skin peptide (personal observation), repeated sampling events were necessary to collect enough material for *in vitro* assays. Thus, individuals were maintained in captivity for approximately one year for this study. Captive salamanders were individually housed within 16 × 16 × 5 cm plastic home-boxes lined with moistened paper towels, maintained on a 14:10 light: dark cycle at 16°C, and fed wax worms bi-weekly.

Collection and preparation of skin peptides

Salamander skin secretions were collected using methods adapted from Woodhams *et al*. (2006b) and Pereira *et al*. (2018) . Salamanders were individually submerged in collection buffer (CB, 50 mmol 1^{-1} sodium chloride, 25 mmol 1^{-1} sodium acetate, pH 7) within a plastic bag or bin. The CB volume used for each species was estimated based on body size. Specifically, CB volumes of 10 mL were used for small-bodied (*P. shermani* and *D. ocoee*), 100 mL for medium-bodied (*A. maculatum*) and 350 mL for large-bodied (*C. alleganiensis*) species. Salamanders were firmly massaged by the collector's hands for 10 minutes to stimulate release of skin secretions into CB, removed from CB, rinsed with nanopure water and returned to the field or home-box. Each skin secretion sample was immediately acidified with trifluoroacetic acid (TFA, final concentration 1%) and stored at –80°C.

Acidified skin secretion samples were processed individually to extract and concentrate peptide mixtures. Samples were centrifuged up to 6500 revolutions per minute for 30 minutes and passed through a syringe filter (0.2 μm PES syringe filter, GE Healthcare Life Sciences, Pittsburgh, PA, USA) to remove sticky components of the skin secretions (e.g. carbohydrates and large proteins; von Byern *et al*. (2017a)) that can interfere with subsequent processing steps. Samples were then passed through a C-18 Sep-Pak cartridge (cat. no. WAT 051910, Waters Corp., Milford, MA, USA) previously primed with 100% methanol followed by 0.1% TFA to enrich for peptides (Conlon, 2007). Sep-Pak cartridges were washed in 0.1% TFA and retained compounds were eluted with 70% acetonitrile and 0.1% TFA. Wash, sample passage and elution steps were repeated twice to maximize the amount of peptide obtained from each skin secretion sample. Eluted compounds were further concentrated by drying in a Speed-Vac concentrator (Savant Instruments, Marietta, OH, USA) and resuspended in nanopure water. Peptide content of each resuspended sample was estimated using a Micro BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA) following the manufacturer's instructions except that bradykinin (Sigma Chemical Co., St. Louis, MO, USA) was used as the standard (Rollins-Smith *et al*., 2002b).

Skin peptide samples were individually adjusted with sterile nanopure water to a final concentration of 1000 or 2000 ug mL^{-1}. Peptide samples were pooled within each species to ensure that we would have enough material at the desired concentration to complete *in vitro* assays. For *A. maculatum* and *C. alleganiensis*, skin peptides from only a few animals collected within a single skin secretion sampling event were individually adjusted to final concentrations of 1000 to 2000 μg mL^{-1} and pooled. Two sets of pooled peptides were prepared to complete *in vitro* assays for *A. maculatum*, while only one set of pooled peptides was required to complete *in vitro* assays for *C. alleganiensis* (Table 2). For *P. shermani* and *D. ocoee*, skin peptides from numerous animals were individually adjusted to a final concentration of 1000 μg mL^{-1} and pooled (Table 2). Skin peptides from *P. shermani* were collected within a single skin secretion sampling event and used to prepare one set of pooled peptides (Table 2). For *D. ocoee*, several individuals were sampled twice separated by a 5-month interval to generate enough

material for *in vitro* assays. Amphibian skin peptides are typically replenished within several weeks of being depleted, therefore, we did not expect duplicate sampling of individuals to alter peptide abundance or composition (Rollins-Smith *et al*., 2005b). Skin peptides collected from each sampling event were used to prepare two sets of pooled peptides for *D. ocoee* (Table 2).

Pooled skin peptides were filter-sterilized (0.2 μm Supor® Membrane syringe filter, PALL Life Sciences, New York, NY, USA), measured for pH, and serially diluted to six concentrations (range: 31.3 to 1000 μg mL^{-1}). Serial dilutions were used in *in vitro* assays with the goal of determining the minimal inhibitory concentration (MIC) for each salamander species. The pH of each set of pooled peptides (for all species) was 6.

Culturing of chytrid fungi

Chytrid fungi, *B. salamandrivorans* (*Bsal* isolate AMFP) and *B. dendrobatidis* (*Bd* isolate JEL-197) were cultured and used to generate zoospores for *in vitro* assays from April 2018 to May 2019. *Bsal* cultures were maintained in TGhL broth (16 g tryptone, 4 g collagen hydrolysate, 2 g lactose, 1 L deionized water) and passaged twice per week to maintain active growth (Robinson *et al*., 2020). *Bd* cultures were maintained in tryptone broth (16 g tryptone, 1 L deionized water) and passaged weekly (Rollins-Smith *et al*., 2002a). We had little success reviving cryopreserved *Bsal* or *Bd* stock cultures, requiring liquid cultures to be continuously maintained and passaged. Consequently, *Bsal* and *Bd* passage numbers increased throughout the experimental period. One milliliter of each liquid culture (B*sal*: passage numbers 38 to 126; *Bd*: passage numbers 97 to 134) was individually added to TGhL agar plates containing ampicillin sodium salt (Sigma-Aldrich, St. Louis, MO, USA) and streptomycin sulfate (MP Biomedicals, LLC, Santa, Ana, CA, USA) and incubated for 5 days at 15°C (*Bsal*) and 23°C (*Bd*). Zoospores were harvested by flooding agar plates with 2 to 3 mL of broth, incubating plates for at least 30 minutes and passing the collected broth through sterile nylon mesh (N301, BioDesign Inc., Carmel, NY, USA) to remove remaining zoosporangia (reproductive life stage). A 50 μL aliquot of collected zoospores was stained with trypan blue and enumerated using a hemocytometer. The remaining collected zoospore volume was adjusted with broth to a final concentration of 1×10^6 zoospores mL^{-1} and used for *in vitro* assays (Robinson *et al*., 2020).

In vitro assays

In vitro assays were set up individually within a single 96-well microtiter plate for each salamander species-fungus combination and completed during April 2018 to May 2019. We set up multiple plates per salamander species-fungus combination but the number of plates for each combination was limited by the amount of pooled peptides available per salamander species. Within each plate, 50 μL of serial diluted skin peptides was combined with 50 μL zoospores (3 replicate wells per dilution). Positive control wells (6 replicate wells) consisted of 50 μL sterile nanopure water (no skin peptides) and 50 μL zoospores. Negative control wells (6 replicate wells) consisted of 50 μL sterile nanopure water and 50 μL heat-killed zoospores. Additional negative controls for microbial contamination consisted of 50 μL broth and 50 μL sterile nanopure water (6 replicate wells) or 50 uL broth and 50 μL serial diluted skin peptides (2 replicate wells per dilution). Plates were incubated at the optimal growth temperatures for *Bsal* (15°C) and *Bd* (23°C) for 8 days to allow fungi to complete several growth cycles (Longcore *et al*., 1999; Martel *et al*., 2013). Optical density at 490 nm (OD_{490}) was read daily with an accuSkan GO plate reader (Fisher Scientific, Hampton, NH, USA) and used to quantify fungal growth.

Analyzing skin peptide effects on fungal growth

Fungal growth was quantified for each set of replicate wells (positive and negative controls, chytrid + skin peptides) within an individual *in vitro* assay (i.e. one plate) by subtracting the OD_{490} on day 0 (day of setup) from OD_{490} on day 6 or 7 (during active growth phase) to generate the change in OD (ΔOD_{490}). A positive value for ΔOD_{490} was interpreted as increased fungal growth and a negative or zero value for ΔOD_{490} was interpreted as no fungal growth. It is worth noting that as fungal growth increases, developing chytrid thalli tend to clump together. The movement of these thalli clumps within the wells of a plate can alter OD measurements increasing variation in ΔOD_{490} values.

To further describe the ability of skin peptides from each salamander species to inhibit chytrid growth, we used ΔOD_{490} values for well sets containing chytrid and water (positive control) and chytrid and 500 μg mL^{-1} skin peptides to calculate *% change* (see formula below). This allowed us to quantify fungal growth in the presence of skin peptides relative to fungal growth in the absence of skin peptides (i.e. maximum growth). Although little is known about the peptide concentrations naturally available on the skin surface of salamanders, we chose to focus on the 500 μg mL^{-1} skin peptide concentration because this was the highest within-well concentration included in *in vitro* assays for all salamander species-fungus combinations. Moreover we expected that if a species produced skin peptides capable of inhibiting chytrid growth, inhibitory effects would be most pronounced at the 500 μg mL^{-1} skin peptide concentration. We also calculated *% change* for negative controls (broth + water, heat-killed chytrid) to demonstrate a lack of fungal growth in these well sets. A negative value for *% change* was interpreted as inhibitory and a positive value was interpreted as facilitatory.

$$\%\,change = \left[\frac{\left(\Delta OD_{490}\text{ of }500\,\mu g\text{ mL}^{-1}\text{ skin peptides}\right) - \left(\Delta OD_{490}\text{ of positive control}\right)}{\Delta OD_{490}\text{ of positive control}}\right]$$

Statistical methods

Statistical analyses of peptide collection and *in vitro* assay data were completed in SigmaPlot version 11.0. Peptide collection data were log transformed to meet assumptions of parametric statistics and analyzed with a one-way ANOVA to test for differences among salamander species in the amount of skin peptides collected (μg) and the amount of skin peptides collected per gram body mass (μg per gbm). For *in vitro* assays, pairwise comparison tests were conducted to compare fungal growth in well sets containing chytrid and 500 ug mL^{-1} skin peptides (ΔOD_{490} of 500 ug mL^{-1} skin peptides, n = 3 replicate wells) with well sets containing chytrid and water (ΔOD_{490} of positive control, n = 6 replicate wells). We used t-tests when assumptions of parametric statistics were satisfied and Mann-Whitney U tests when assumptions were not satisfied.

Standardized measures for interspecies comparisons

To standardize and compare *in vitro* pathogen-peptide responses across species, it is common to determine minimal inhibitory concentrations (MIC) and predictive indices of resistance (PIR) (Woodhams *et al*., 2006a; Woodhams *et al*., 2006b). MIC is defined as the lowest concentration of skin peptides in which no fungal growth is observed. Lower MIC values (below 280 μg mL^{-1}) generally indicate stronger skin defenses (Woodhams *et al*., 2006a). Because serially diluted skin peptides never completely inhibited the growth of *Bsal* or *Bd* (see results), we were unable to calculate MIC values. Alternatively, we calculated predicted indices of resistance (PIR) (Woodhams *et al*., 2006b).

Unlike MIC, PIR assesses fungal growth inhibition at a single, standard peptide concentration (typically 50 μg mL^{-1}) (Woodhams *et al*., 2006b). Higher PIR values (20 to 75) generally indicate stronger skin defenses (Woodhams *et al*., 2006b). To calculate PIR, we adapted methods from Woodhams *et al*. (2006b). First, we determined the percent *Bsal* or *Bd* inhibition at 62.5 μg mL^{-1} skin peptides (serial dilution closest to 50 μg mL^{-1}) for each individual *in vitro* assay. We then multiplied the mean percent inhibition (of multiple plates per species-fungus combination) by the mean total peptides collected per gram body mass (μg per gbm; Table 1).

Reversed-phase high performance liquid chromatography (RP-HPLC)

Pooled skin peptide samples were sent to the University of Pittsburgh's Peptide and Peptoid Synthesis Core Facility for analysis by RP-HPLC. Peptides in 100 μL of each sample and a 'no skin peptide' control (phosphate buffered saline, not previously processed by C-18 Sep-Pak) were separated and analyzed using a Waters Alliance chromatography system. Briefly, a C-12 reverse-phase column (Jupiter® 4 μm Proteo 90 Å, LC Column, 150 × 1 mm, Phenomenex, Torrance, CA) was equilibrated in 0.1% TFA (aqueous) followed by injection and separation of each sample using a linear mobile phase gradient from 0 to 70% acetonitrile/0.1% TFA at a flow rate of 0.1 mL min^{-1} for 60 minutes. Peak detection using a Waters 2487 dual wavelength UV-absorbance detector set to 220 nm was followed by individual peptide integration using an Empower™ 3 software package (Waters Division, Milford, MA, USA). Each peak on the resulting chromatogram was interpreted as an individual peptide.

Table 1 Amount of skin peptide collected from salamander skin secretions (mean SD)

Species	n	Total peptide collected (μg)		Total peptide collected per gram body mass (μg per gbm)	
Ambystoma maculatum	7*	1663	975	100	56
Cryptobranchus alleganiensis	2	36 769	14 711	64	21
Plethodon shermani	18	361	234	122	80
Desmognathus ocoee	17	163	110	74	48

* Body mass data were unavailable for three individuals of *A. maculatum*, therefore mean data were generated from only 7 of the 10 individuals used in this study.

Results

Skin peptide collection

Among the four salamander species, we found significant differences in the amount of skin peptides obtained (μg; $F_{3,43} = 43.14$, $P < 0.001$; Table 1). The greatest amounts of skin peptide were collected from *A. maculatum* and *C. alleganiensis* (Table 1). In contrast, we obtained very little skin peptide from *P. shermani* and *D. ocoee* (Table 1). When the amount of skin peptides obtained from each salamander species was corrected for body mass (μg per gbm), we found no significant interspecies differences ($F_{3,43} = 1.48$, $P = 0.235$; Table 1).

Effects of skin peptides on fungal growth and predictive indices of resistance (PIR)

We tested the ability of skin peptides to inhibit the growth of *Bsal* and *Bd* by conducting a total of 19 *in vitro* assays comprising four salamander species and at least two plates per salamander species-fungus combination (Table 2). In each plate, fungal growth of positive controls (chytrid + water) was evident between Days 2 and 4 and remained evident throughout the 8-day incubation period (see Figure 1 for representative examples). No fungal growth was observed for any of the negative controls (heat-killed chytrid + water,

Table 2 Collection site, preparation and use of salamander skin peptides for *in vitro* assays and RP-HPLC analysis

Species	Location	Peptide preparation	Individuals pooled	Chytrid tested	Plate ID Nos.	RP-HPLC analysis
Ambystoma maculatum	Field	Set 1	5	*Bsal*	1, 3	Yes
		Set 2	5	*Bsal*	2	Yes
				Bd	12, 13	
Cryptobranchus alleganiensis	Field	Set 1	2	*Bsal*	4, 5	Yes
				Bd	14, 15	
Plethodon shermani	Captive	Set 1	18	*Bsal*	6, 7, 8, 9	Yes
				Bd	16, 17	
Desmognathus ocoee	Captive	Set 1	13	*Bsal*	10, 11	Yes
		Set 2	15	–	–	Yes
		Set 1 and 2, combined	–	*Bd*	18, 19	No

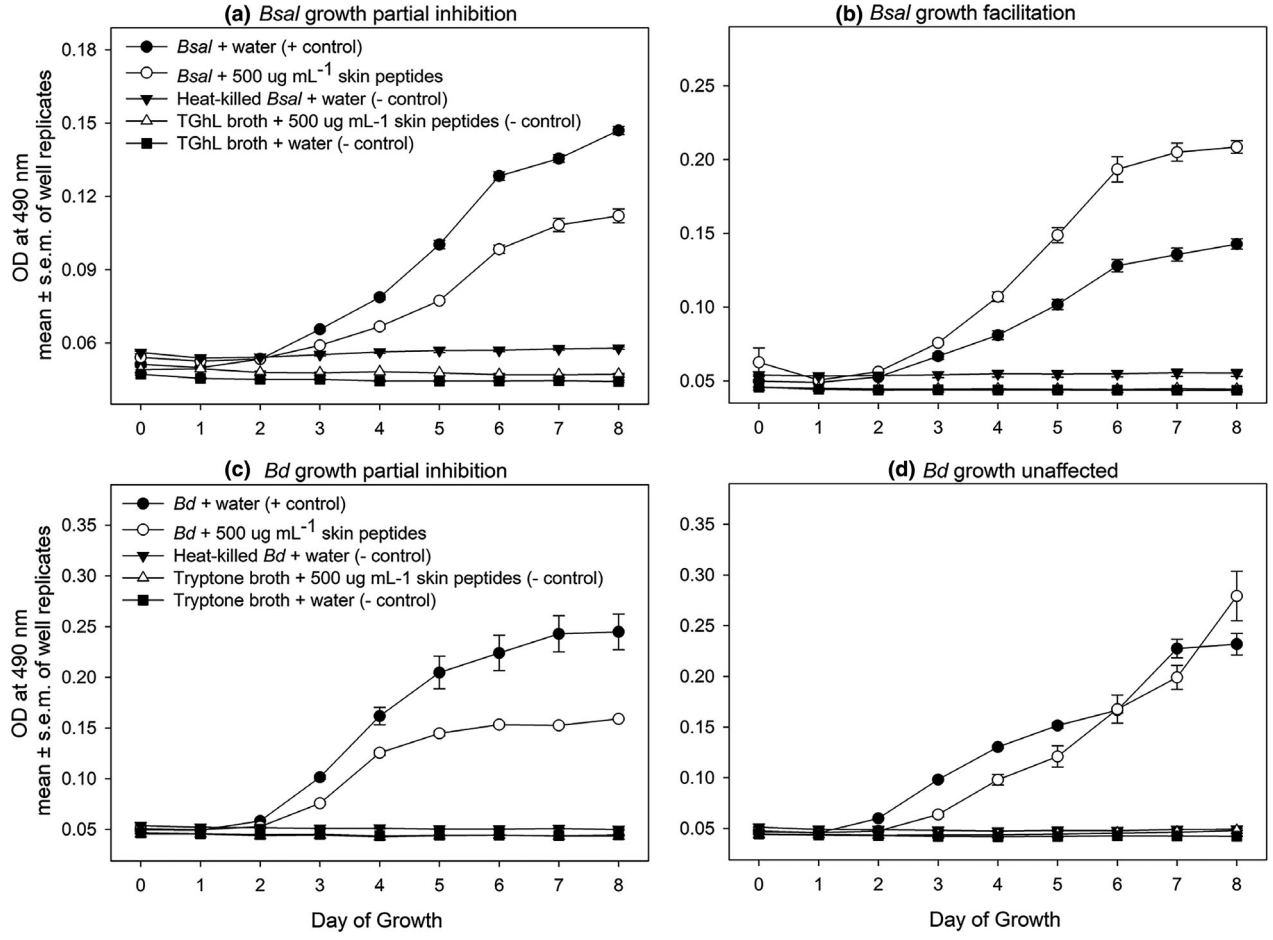

Figure 1 Representative examples of growth curves of *Batrachochytrium salamandrivorans* (*Bsal*, top panels) and *B. dendrobatidis* (*Bd*, bottom panels) zoospores (10^6 zoospores mL^{-1} starting density) when combined with 500 μg mL^{-1} salamander skin peptides *in vitro*. Each panel corresponds to a single representative *in vitro* assay (i.e. one plate). For each assay, fungal growth was measured via optical density (OD) for 8 days and averaged for each set of well replicates. Each plate included a set of positive controls (chytrid + water, n = 6 replicate wells), negative controls (heat-killed chytrid + water and broth + water, n = 6 replicate wells; broth + 500 μg mL^{-1} skin peptides, n = 2 replicate wells), and chytrid + 500 μg mL^{-1} skin peptides (n = 3 replicate wells). (a) *Bsal* growth partially inhibited by *Ambystoma maculatum* skin peptides; (b) *Bsal* growth facilitated by *Desmognathus ocoee* skin peptides; (c) *Bd* growth partially inhibited by *A. maculatum* skin peptides; (d) *Bd* growth not affected by *Cryptobranchus alleganiensis* skin peptides.

Table 3 Effects of salamander skin peptides on the growth of *Batrachochytrium salamandrivorans* (*Bsal*) and *B. dendrobatidis* (*Bd*)

	Bsal					*Bd*				
Species	Plate ID	Effect (avg. of single plate)	Test statistic	*P*-value	Overall effect (avg. of plate replicates)	Plate ID	Effect (avg. of single plate)	Test statistic	*P*-value	Overall effect (avg. of plate replicates)
Ambystoma maculatum	1	79% inhibitory	t(6) = 15.83	<0.001*	60% inhibitory	12	46% inhibitory	t(7) = 6.82	<0.001*	46% inhibitory
	2	64% inhibitory	t(7) = 15.10	<0.001*		13	47% inhibitory	U = 0.00	0.024*	
	3	36% inhibitory	t(7) = 7.70	<0.001*			-	-	-	
Cryptobranchus alleganiensis	4	47% inhibitory	t(6) = 7.14	<0.001*	46% inhibitory	14	15% inhibitory	t(7) = 0.46	0.245	No effect
	5	44% inhibitory	t(7) = 5.00	0.002*		15	16 % inhibitory	t(7) = 1.66	0.142	
Plethodon shermani	6	40% facilitatory	t(7) = −7.19	<0.001*	28% facilitatory	16	2% inhibitory	t(7) = 0.46	0.660	No effect
	7	6% inhibitory	U = 9.00	1.00		17	11% facilitatory	U = 2.00	0.095	
	8	19% facilitatory	t(7) = 6.85	<0.001*			–	–	–	
	9	24% facilitatory	U = 0.00	0.024*			–	–	–	
Desmognathus ocoee	10	43% facilitatory	U = 1.00	0.048*	55% facilitatory	18	11% inhibitory	t(7) = 1.50	0.177	Inconclusive
	11	66% facilitatory	U = 1.00	0.048*		19	48% inhibitory	t(7) = 11.85	<0.001*	

The 'Effect' column shows the average (avg.) *% change* in fungal growth in the presence of 500 μg mL^{-1} skin peptides (n = 3 replicate wells) relative to growth in the absence of skin peptides (n = 6 replicate wells) within a single plate. When this change in growth was statistically significant (indicated by *, pairwise comparison tests), effects observed for single plates were averaged and are shown in the 'Overall effect' column for each species. Nonsignificant effects were reported as 'No effect'.

broth + water or skin peptides; see Figure 1 for representative examples).

Statistical comparisons were used to compare fungal growth at a skin peptide concentration of 500 μg mL^{-1} with growth in the absence of skin peptides (Table 3). At 500 μg mL^{-1}, skin peptides had one of three effects on fungal growth: partial inhibition, facilitation, or no effect (Table 3). Examples of partial inhibition or facilitation of *Bsal* growth are shown in Figure 1a and b, respectively. Examples of partial inhibition or no effect on *Bd* growth are shown in Figure 1c and d, respectively. Below, we describe fungal growth responses more generally. We also report the PIR value, which assesses changes in fungal growth at a skin peptide concentration of 62.5 μg mL^{-1}.

Skin peptides from *A. maculatum* inhibited the growth of *Bsal* and *Bd* (Table 3, Figure 2). For *Bsal*, growth inhibition was first evident at 62.5 μg mL^{-1} (2 out of 3 plates; mean of 2 plates: 19% inhibition) and increased with increasing concentration (125 μg mL^{-1}, mean of 3 plates: 28% inhibition) and plateaued at 500 μg mL^{-1} (mean of 3 plates: 60% inhibition). For *Bd*, growth inhibition was evident at 125 μg mL^{-1} (1 out of 2 plates; 32% inhibition) and 250 μg mL^{-1} (mean of 2 plates: 30% inhibition) and plateaued at 500 ug mL^{-1} (mean of 2 plates: 46% inhibition). At peptide concentrations below 125 μg mL^{-1}, *Bd* growth appeared to be greater and more variable relative to the positive control. Because similar 'spikes' in chytrid growth were also observed at low peptide concentrations (generally below 125 μg mL^{-1}) for other species-fungus combinations (see Figures 3–5), we believe that these 'spikes' reflect variation in OD measurements caused by clumping thalli and are methodological in nature. Lastly, for *Bsal* and *Bd*, growth inhibition at 500 and 1000 ug mL^{-1} were similar. The PIR value for *A. maculatum* was 19 for *Bsal* but 0 for *Bd*.

Skin peptides from *C. alleganiensis* inhibited the growth of *Bsal* but had little effect on the growth of *Bd* (Table 3, Figure 3). For *Bsal*, growth inhibition was first evident at 250 μg mL^{-1} (mean of 2 plates: 23% inhibition) and plateaued at 500 μg mL^{-1} (mean of 2 plates: 46% inhibition). *Bsal* growth inhibition at 500 and 1000 ug mL^{-1} were similar. For *Bd*, little to no inhibition was observed regardless of concentration, with maximum growth inhibition observed at 500 μg mL^{-1} (mean of 2 plates: 16%; Figure 3), which was not statistically different from growth in the absence of

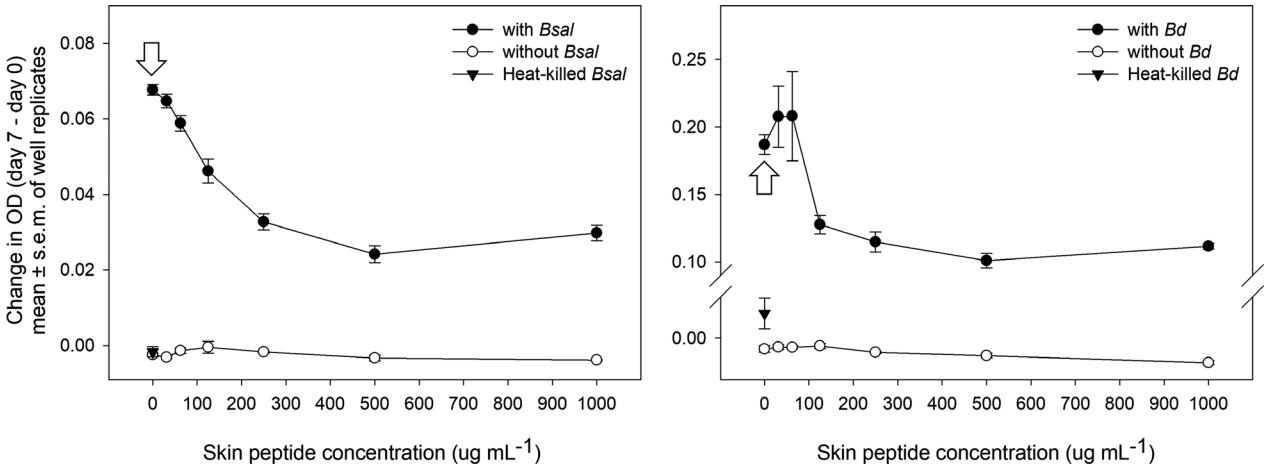

Figure 2 Changes in *in vitro* growth of *Batrachochytrium salamandrivorans* (*Bsal*, left panel) and *B. dendrobatidis* (*Bd*, right panel) zoospores (10^6 zoospores mL^{-1} starting density) when combined with *Ambystoma maculatum* skin peptides. Each panel corresponds to a single representative *in vitro* assay (i.e. one plate). For each assay, changes in fungal growth were measured via changes in optical density (OD) over a 7-day period and averaged for each set of replicate wells. Closed circles represent wells that contained live chytrid zoospores with varying amounts of skin peptide (chytrid + water, n = 6 replicate wells; chytrid + serial diluted skin peptides, n = 3 replicate wells per dilution). The closed triangle represents heat-killed chytrid + water (n = 6 replicate wells). Open circles represent wells without chytrid zoospores (broth + water, n = 6 replicate wells; broth + serial diluted skin peptides, n = 2 replicate wells per dilution). White arrows indicate positive controls (live chytrid + water, n = 6 replicate wells) representing maximum fungal growth in the absence of skin peptides. The change in fungal growth at the 500 μg mL^{-1} skin peptide concentration was statistically compared to the positive control using pairwise comparison tests.

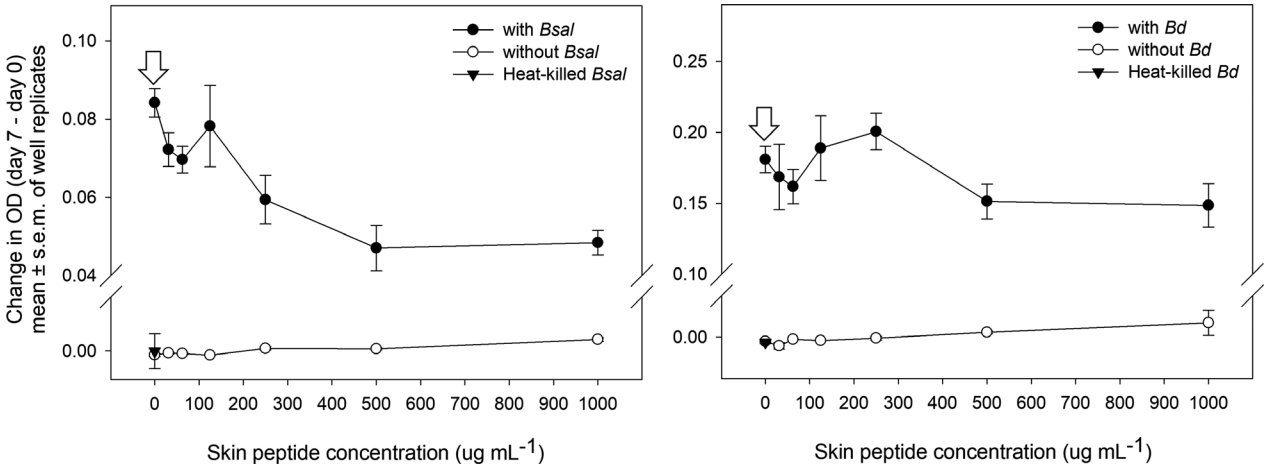

Figure 3 Changes in *in vitro* growth of *Batrachochytrium salamandrivorans* (*Bsal*, left panel) and *B. dendrobatidis* (*Bd*, right panel) zoospores (10^6 zoospores mL^{-1} starting density) when combined with *Cryptobranchus alleganiensis* skin peptides. Each panel corresponds to a single representative *in vitro* assay (i.e. one plate). See Figure 2 caption for details.

peptides (Table 3). The PIR value for *C. alleganiensis* was 0 for *Bsal* and *Bd*.

Skin peptides from *P. shermani* did not inhibit the growth of *Bsal* or *Bd* (Table 3, Figure 4). In fact, *Bsal* growth was facilitated in the presence of skin peptides. Facilitation was apparent at concentrations ranging from 31.25 to 250 ug mL^{-1}. When statistically comparing growth at 500 μg mL^{-1} with growth in the absence of peptides, significant *Bsal* growth facilitation was observed in 3 of 4 plates (mean of 3 plates: 28% facilitation). *Bd* growth was not affected by skin peptides. The PIR value for *P. shermani* was 0 for *Bsal* and *Bd*.

Skin peptides from *D. ocoee* did not inhibit the growth of *Bsal* and had inconsistent effects on the growth of *Bd* (Table 3, Figure 5). For *Bsal*¸ growth was facilitated in the presence of skin peptides. *Bsal* facilitation was first evident

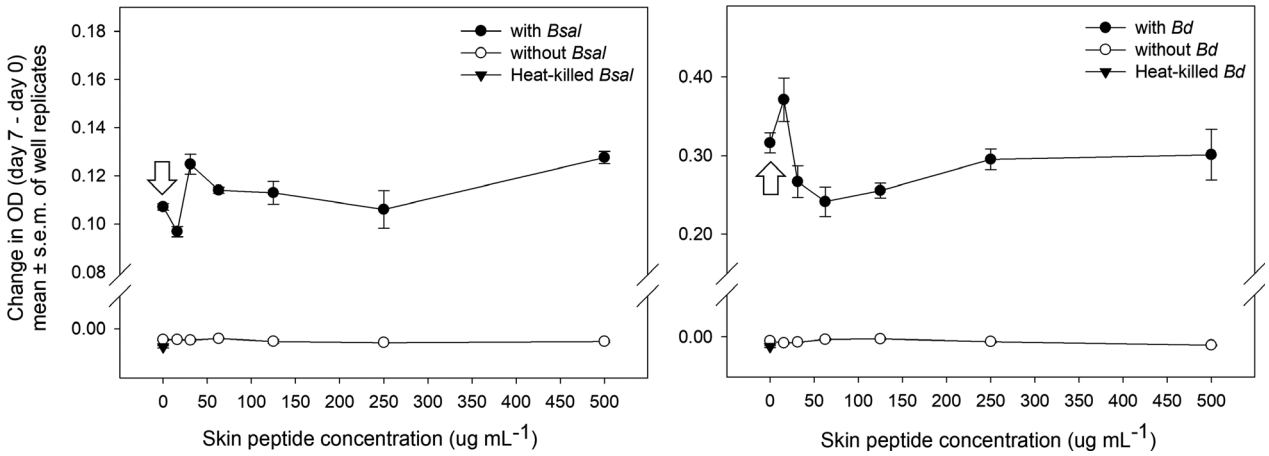

Figure 4 Changes in *in vitro* growth of *Batrachochytrium salamandrivorans* (*Bsal*, left panel) and *B. dendrobatidis* (*Bd*, right panel) zoospores (10^6 zoospores mL^{-1} starting density) when combined with *Plethodon shermani* skin peptides. Each panel corresponds to a single representative *in vitro* assay (i.e. one plate). See Figure 2 caption for details.

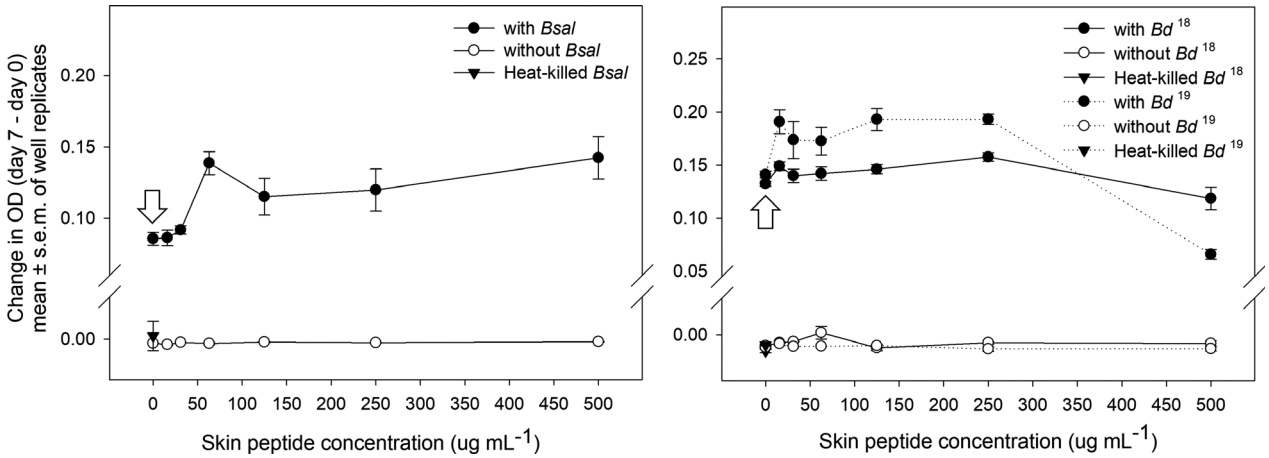

Figure 5 Changes in *in vitro* growth of *Batrachochytrium salamandrivorans* (*Bsal*, left panel) and *B. dendrobatidis* (*Bd*, right panel) zoospores (10^6 zoospores mL^{-1} starting density) when combined with *Desmognathus ocoee* skin peptides. The left panel corresponds to a single representative *in vitro* assay (i.e. one plate) and the right panel depicts 2 assays (plate ID 18 and 19). See Figure 2 caption for details.

at 32.1 (plate ID 10) and 62.5 μg mL^{-1} (plate ID 11) and maintained up to 500 μg mL^{-1} (mean of 2 plates: 55% facilitation). For *Bd*, slight growth inhibition was observed at 500 μg mL^{-1} in the first plate (plate ID 18; 11% inhibition) but was not statistically significant from growth in the absence of skin peptides suggesting that skin peptides from *D. ocoee* did not affect *Bd* growth. However, in the second plate, skin peptides from *D. ocoee* appeared to facilitate *Bd* growth up to 31% at concentrations ranging from 15.6 to 250 μg mL^{-1} but inhibit growth at 500 μg mL^{-1} (plate ID 19; 48% inhibition). The PIR value for *D. ocoee* was 0 for *Bsal* and *Bd*.

RP-HPLC analysis of skin peptides

As expected, no notable peaks were observed for the phosphate buffered saline (PBS, no skin secretion) control (Figure 6). Each of the two sets of pooled skin peptides from *A. maculatum* had a roughly similar peptide profile with up to 80 different peptide peaks. Two peaks comprised approximately 50% of the total skin peptides of each of the two sets (Figure 6). The single sets of skin peptides from *C. alleganiensis* and *P. shermani* identified 44 and 35 peptide peaks, respectively, with none comprising more than 16% of the total skin peptides within each set (Figure 6).

Peptide profiles for the two sets of pooled skin peptides from *D. ocoee* differed, despite being collected from many of the same individuals (Figure 6). For example, set 1 included two peptide peaks comprising approximately 54% of the total skin peptides, whereas set 2 included peptide peaks with a similar separation profile as set 1 but comprised less than 15% of total skin peptides representing a 72% decrease. Nonetheless, the number of peptide peaks (range: 28 to 38) was similar between the two sets (Figure 6).

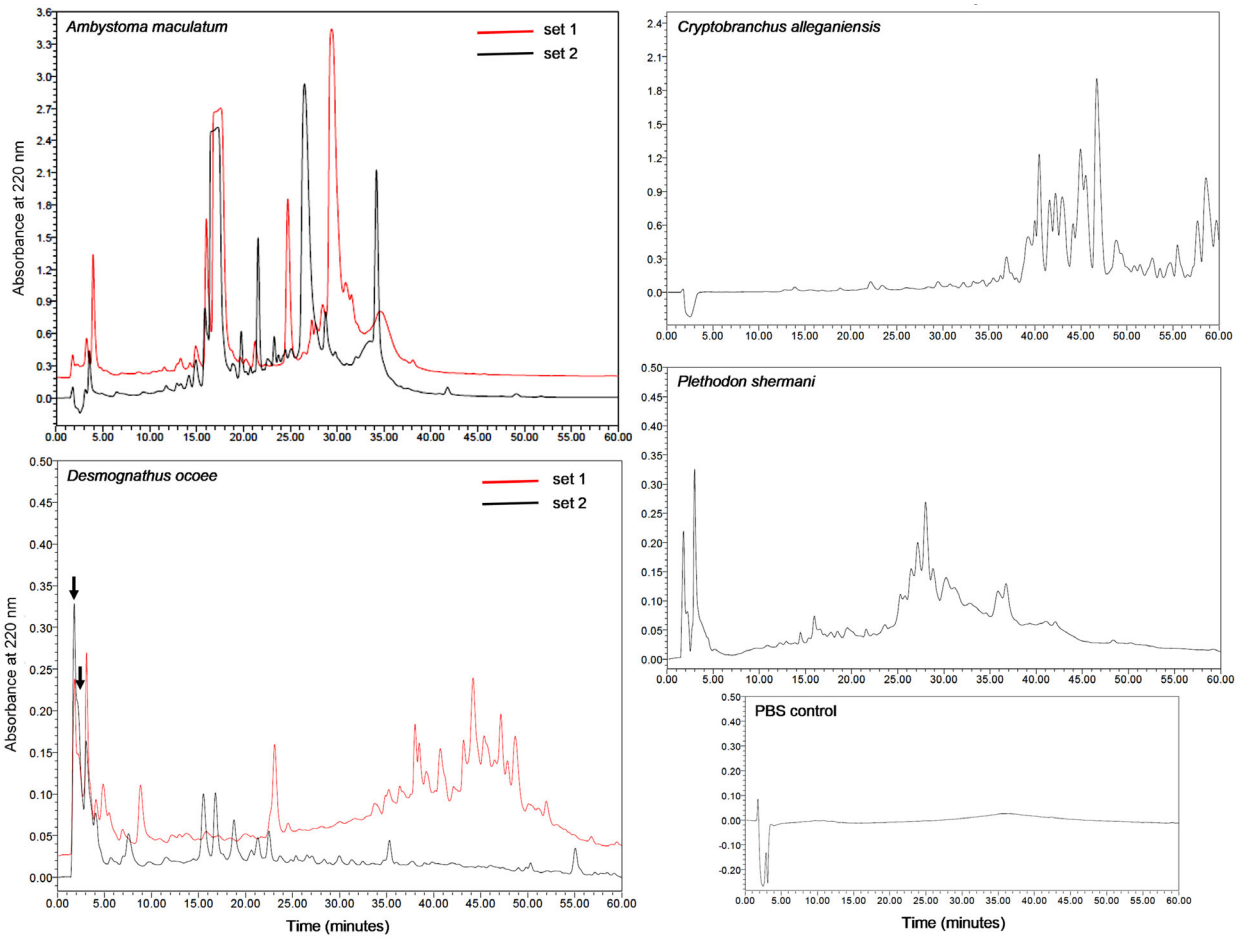

Figure 6 Separation of peptides collected from salamander skin secretions and phosphate buffered saline (PBS, no skin peptide control) by RP-HPLC. A single set of skin peptides was analyzed for *C. alleganiensis* and *P. shermani* (sets represented by black line only), whereas two sets were analyzed for *A. maculatum* and *D. ocoee* (each set represented by a black or red line). For *D. ocoee*, the black arrows indicate two peptide peaks comprising approximately 54% of total peptides in set 1 but 15% in set 2.

Discussion

The present study used *in vitro* experimental assays to investigate the role of skin-associated immune defenses, particularly skin peptides, in protecting North American salamander species against chytrid fungal pathogens linked to amphibian biodiversity loss worldwide (Skerratt *et al*., 2007; Martel *et al*., 2013). This is one of the first studies to test whether amphibian skin peptides inhibit the growth of the recently emerged chytrid pathogen, *Batrachochytrium salamandrivorans* (*Bsal*) and whether inhibitory effects are similar against both *Bsal* and the closely related chytrid pathogen, *B. dendrobatidis* (*Bd*). Among the four salamander species, skin peptides either partially inhibited, facilitated or had no effect on chytrid growth. In addition, *Bsal* and *Bd* responses to skin peptides were not always similar. Lastly, we found interspecies differences in skin defenses against both chytrid pathogens and peptide mixtures using a standardized measure (i.e. predictive index of resistance) and RP-HPLC, respectively.

Consistent inhibition of chytrid growth in spotted salamanders

As we predicted, skin peptides from spotted salamanders (*Ambystoma maculatum*) inhibited the growth of *Bsal*, consistent with the observation that *A. maculatum* is *Bsal* resistant (Martel *et al*., 2014; Barnhart *et al*., 2019). Similar results were found for *Bd*. Considering that skin peptides from two other salamander species (tiger salamanders, *A. tigrinum;* and three-toed amphiuma, *Amphiuma tridactylum*) fully inhibited *Bsal* and/or *Bd* growth at concentrations between 75 and 750 μg mL^{-1}, it was unexpected that *A. maculatum* skin peptides did not also completely inhibit the growth of *Bsal* or *Bd* at even the highest concentration of 1000 μg mL^{-1} (Sheafor *et al*., 2008; Pereira *et al*., 2018). It was also unexpected that predictive index of resistance (PIR) values were not similar for both chytrid pathogens. Woodhams *et al*. (2006b) predicted that species with PIR values between 20 and 75 had stronger skin defenses and were

more resistant to *Bd* compared to species with lower PIR values. Therefore, while the *Bsal* PIR of 19 is consistent with moderate skin defenses of other chytrid resistant amphibians and with experimental studies showing that *A. maculatum* is *Bsal* resistant, the *Bd* PIR of 0 echoes weak skin defenses of more susceptible species (Woodhams *et al*., 2006b). Nevertheless, our *in vitro* results suggest that concentrations as low as 62.5 and 125 μg mL^{-1} can inhibit the growth of *Bsal* and *Bd*, respectively (Woodhams *et al*., 2006b). Although we do not know how much peptide is naturally available on the skin of *A. maculatum*, collectively, our findings suggest that skin peptides are an important component of the defensive arsenal for *A. maculatum* for both chytrid pathogens.

Inconsistent inhibition of chytrid growth in hellbenders

In contrast to results for *A. maculatum*, skin peptides did not have similar effects on *Bsal* and *Bd* growth in the other salamander species. This was evident in hellbenders (*Cryptobranchus alleganiensis*), where skin peptides inhibited the growth of *Bsal* but not *Bd*. These results are contrary to our prediction that skin peptides would have similar effects on the growth of both chytrid pathogens. Only three previous studies have compared *Bsal* and *Bd* responses to amphibian skin peptides (Pereira *et al*., 2018; Smith *et al*., 2018; Woodhams *et al*., 2020). Skin peptides from fire salamanders (*Salamandra salamandra*) and Sierra Nevada yellow-legged frogs (*Lithobates* [= *Rana*] *sierrae*) inhibited the growth of both chytrid pathogens similarly (Smith *et al*., 2018; Woodhams *et al*., 2020). Likewise, skin peptides from the aquatic salamander, three-toed amphiuma (*Amphiuma tridactylum*) had potent inhibitory activities against both *Bsal* and *Bd* (Pereira *et al*., 2018). It is important to note that the zoospore isolates (i.e. strains) used in Pereira *et al*. (2018) were the same as what was used in the current study, indicating the study differences were not due to differences in chytrid strains. Furthermore, methods used for processing skin peptides in (Pereira *et al*., 2018) and Woodhams *et al*. (2020) were also similar to those used in the current study. Therefore, our study provides the first evidence that amphibian skin peptide defenses are not always equally effective against *Bsal* and *Bd*.

There are several possibilities for why skin peptides had different effects on the growth of *Bsal* and *Bd*. First, results could reflect intrinsic differences between chytrid pathogens. Peptides that inhibit microbial growth, including those collected from amphibian skin are commonly termed antimicrobial peptides (AMPs) (Nascimento *et al*., 2003). AMPs are found throughout all living kingdoms and kill a wide range of bacterial, fungal, and viral pathogens (Wang *et al*., 2016a). The broad-spectrum killing ability of AMPs is generally attributed to non-specific membranolytic mechanisms (Reddy *et al*., 2004). However, some AMPs such as buforin II (isolated from the stomach of the Asian toad [*Bufo bufo garagrizans*]) use non-lytic mechanisms to interrupt specific intracellular processes making peptides more efficient at killing some microbes than others (Park *et al*., 1998; Scocchi *et al*., 2016). We speculate that the specific skin peptides of *C. alleganiensis* may utilize a mode of action that targets one chytrid more than the other potentially explaining differences in chytrid-peptide responses *in vitro*. In contrast, skin peptides of *A. maculatum* may employ a more generalized mode of action that has similar efficacy against both *Bsal* and *Bd*.

In addition, the chytrid pathogens may vary in their secretion of proteolytic enzymes. Proteolytic enzymes are secreted by many fungal pathogens and are important for modulating host immune defenses (Marcos *et al*., 2016). While the production of secretory enzymes in *Bsal* has not been studied, over 32 serine peptidase genes have been identified in *Bd* (Sun *et al*., 2011). Furthermore, the cleavage of temporin A (an anti-fungal amphibian skin AMP) by a *Bd* subtilisin-like protease suggests that proteolytic enzymes produced by *Bd* may be important for neutralizing amphibian skin peptide defenses (Rollins-Smith *et al*., 2003; Thekkiniath *et al*., 2013). Interestingly, Figure 1d shows that the growth of *Bd* was slightly inhibited by *C. alleganiensis* skin peptides until day 5 but then somehow recovered during days 6 through 8. We speculate that proteolytic enzymes secreted by mature *Bd* zoosporangia may have degraded *C. alleganiensis* skin peptides, thereby allowing *Bd* growth to continue.

Another explanation for different growth patterns of *Bsal* and *Bd* in the presence of skin peptides could relate to the disparity in incubation temperatures of *Bsal* (15°C) and *Bd* (23°C). While some AMPs maintain efficacy over a wide thermal range, an increase in as little as 10°C can reduce inhibitory activity in others by 50 percent (Chinchar *et al*., 2001; Kaur *et al*., 2004). Finally, our *in vitro* results could be influenced by methodological differences. For example, culture age (i.e. passage number) differed between *Bsal* and *Bd*, which may account for differing responses to skin peptides (Woodhams *et al*., 2006a; Langhammer *et al*., 2013).

No inhibition of chytrid growth in red-legged and Ocoee salamanders

Skin peptides from red-legged salamanders (*Plethodon shermani*) and Ocoee salamanders (*Desmognathus ocoee*) facilitated the growth of *Bsal* and had no effect or inconsistent effects on the growth of *Bd*. These results should be interpreted cautiously because skin peptides were collected from captive animals, and captivity can alter community structure of skin microbiota and abundance of antimicrobial skin peptides (Tennessen *et al*., 2009; Loudon *et al*., 2014; Kueneman *et al*., 2016; Woodhams *et al*., 2020). For example, Tennessen *et al*. (2009) found that captivity decreased the overall abundance of antimicrobial skin peptides in leopard frogs (*Lithobates* [= *Rana*] *pipiens*) but not the types of peptides secreted. In contrast, we found that free-living and captive salamanders released similar amounts of skin peptide proportional to body size suggesting that overall peptide production was not altered by captivity. Furthermore, RP-HPLC

data showed that the abundance of at least two peptides from *D. ocoee* decreased by 72% over a span of 5 months suggesting that their captivity may have altered the proportions of specific peptides produced.

We predicted that skin peptides from *P. shermani* would inhibit the growth of *Bd* based on apparent *Bd* resistance of other species of *Plethodon* salamanders (Chinnadurai *et al*., 2009; Vazquez *et al*., 2009). Contrary to our prediction, skin peptides from *P. shermani* had no effect on *Bd* growth. We do not think that the lack of inhibition is fully attributable to captivity because numerous studies demonstrate that skin peptides from captive anurans and salamanders inhibit the *in vitro* growth of chytrid pathogens and/or bacteria (Fredericks & Dankert, 2000; Gibble *et al*., 2008; Pereira *et al*., 2018).

One possible explanation for the lack of chytrid growth inhibition is that *P. shermani* and *D. ocoee* do not secrete AMPs. It is well established that not all amphibians secrete antimicrobial skin peptides (i.e. AMPs)(Conlon, 2011; Ladram & Nicolas, 2016). Moreover few AMPs have been characterized from the skin secretions of salamanders (Meng *et al*., 2013; Pei & Jiang, 2017). In plethodontid salamanders, skin secretions serve diverse biological functions (Dodd *et al*., 1974; Lutterschmidt *et al*., 1994; Woodley, 2010; Woodley, 2014). For example, previous studies suggest that *P. shermani* skin secretions are involved in scent marking, predator defense, and courtship (Largen & Woodley, 2008; Woodley, 2010; Woodley, 2015; von Byern *et al*., 2017c). Likewise, skin secretions for *D. ocoee* have also been related to courtship behaviors (Houck *et al*., 2008). Because RP-HPLC results confirm the presence of numerous peptides in samples collected from *P. shermani* and *D. ocoee*, the lack of inhibition was not attributable to a lack of peptide.

Species-specific effects on chytrid growth were consistent for each set of plates (e.g. inhibitory, facilitatory or no effect) except for *D. ocoee* – *Bd* plates. We do not understand the source of this variation because both plates were set up on the same day and used the same preparations of pooled skin peptides (combination of sets 1 and 2, Table 2) and *Bd* zoospores. However, this finding demonstrates the importance of replicating assays at least two times to ensure that results are repeatable.

Interspecies comparisons

We used the standardized measure, PIR to compare differences in skin defenses against chytrid pathogens across species. Based on previous studies, we predicted that the PIR would be above 10 (i.e. PIR value indicative of weak skin defenses) for those species showing resistance to chytrid pathogens (*A. maculatum* against *Bsal* and *P. shermani* against *Bd*) (Woodhams *et al*., 2006b). While the PIR for *A. maculatum* against *Bsal* was 19, PIR was 0 for all other species-fungus combinations. Our results suggest that with the exception of *A. maculatum* and *Bsal*, most of the species included herein have relatively weak skin defenses against chytrid pathogens. Although previous work predicted that species with PIR values less than 10 were at greater risk of experiencing *Bd*-mediated population declines, the implications of PIR in the current study are unclear (Woodhams *et al*., 2006b). Additional research is needed to fully assess host responses and risk of these species to *Bsal* and/or *Bd* – mediated population declines.

RP-HPLC results also revealed variation in the complexity of salamander skin peptides across species. Consistent with previous work, the salamander species studied herein produced complex peptide mixtures consisting of 28 to 80 different types (Woodhams *et al*., 2006b). Although each peptide profile (i.e. relative number and distribution of peptide peaks; Figure 6) was generated using skin peptides from multiple conspecifics from a single source population, the overall patterns of each peptide profile clearly illustrate that skin peptide mixtures greatly varied among the four salamander species, consistent with the differences in *in vitro* activities.

Previous studies show that amphibians within the same species tend to secrete similar peptide types. In fact, such peptide 'profiles' have been used as taxonomic markers for species identification (Conlon *et al*., 2004). Thus, we expected that peptide profiles of duplicate skin peptide preparations for *A. maculatum* and *D. ocoee* would overlap, indicating strong similarity between each of the two sets, but this was not always the case. Although *A. maculatum* sets shared a large degree of overlap, very little overlap was observed for the two sets of *D. ocoee* skin peptides. As described earlier, it is possible that captivity and repeated sampling explain these differences.

Caveats to interpretation of *in vitro* data

It is crucial to acknowledge an important limitation of our study. Skin peptides are only one feature of a complex skin repertoire that includes skin microbiota and host enzymes like lysozymes (Zhao *et al*., 2006; Nikolaieva *et al*., 2018; Rebollar *et al*., 2020; Rollins-Smith, 2020). Thus, *in vitro* results using skin peptides alone likely do not fully explain interspecies variation in susceptibility to chytrid pathogens. In addition, skin peptide production is dynamically modulated by the skin microbiota (Matutte *et al*., 2000; Woodhams *et al*., 2020). Hence, the skin peptide repertoire reflects differences in skin microbiota which, in turn, are influenced by factors like captivity or geographic location of individuals that were sampled in the field (Kueneman *et al*., 2014; Loudon *et al*., 2014; Krynak *et al*., 2016). Despite this limitation, assessing *in vitro* responses has been a useful tool in previous studies of the anuran-*Bd* system, and we feel that our results provide important insight towards better understanding the disease-dynamics of chytrid pathogens, particularly *Bsal* (Woodhams *et al*., 2006a; Woodhams *et al*., 2006b; Grogan *et al*., 2018; Barnhart *et al*., 2019).

Conclusion

We demonstrated that salamander skin peptides and inhibitory activities against chytrid pathogens are highly variable and not always equally effective against *Bsal* and *Bd*, despite

being closely related fungal lineages (Martel *et al*., 2013). While skin peptides may inhibit chytrid growth, thereby reducing infection load and overall susceptibility in some salamander species, skin peptides, particularly those lacking apparent antimicrobial activity, may serve numerous other biological functions in others. We demonstrated that PIR calculated for *A. maculatum* for *Bsal* corresponded to known *Bsal* resistance, but additional research is needed for species for which host responses to *Bd* and/or *Bsal* are unknown to fully evaluate the usefulness of such standardized measure in estimating the species-specific risks of chytrid pathogens in salamanders. By advancing knowledge about the factors underlying chytrid susceptibility, particularly *Bsal*, our findings will help inform conservation initiatives aimed at reducing disease impacts and further biodiversity loss .

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