The Journal of Experimental Biology 216, 2908-2916

doi:10.1242/jeb.084145

## RESEARCH ARTICLE

# Skin peptides protect juvenile leopard frogs (*Rana pipiens*) against chytridiomycosis

James D. Pask[1], Tawnya L. Cary[2] and Louise A. Rollins-Smith[1,3,4,*]

[1]Department of Pathology, Microbiology and Immunology, Vanderbilt University School of Medicine, Nashville, TN 37232, USA, [2]Department of Zoology, University of Wisconsin-Madison, Madison, WI 53706, USA, [3]Department of Pediatrics, Vanderbilt University School of Medicine, Nashville, TN 37232, USA and [4]Department of Biological Sciences, Vanderbilt University, Nashville, TN 37235, USA

*Author for correspondence (louise.rollins-smith@vanderbilt.edu)

### SUMMARY

**One issue of great concern for the scientific community is the continuing loss of diverse amphibian species on a global scale. Amphibian populations around the world are experiencing serious losses due to the chytrid fungus, *Batrachochytrium dendrobatidis*. This pathogen colonizes the skin, leading to the disruption of ionic balance and eventual cardiac arrest. In many species, antimicrobial peptides secreted into the mucus are thought to contribute to protection against colonization by skin pathogens. Although it is generally thought that antimicrobial peptides are an important component of innate immune defenses against *B. dendrobatidis*, much of the current evidence relies on correlations between effective antimicrobial peptide defenses and species survival. There have been few studies to directly demonstrate that antimicrobial peptides play a role. Using the northern leopard frog, *Rana pipiens,* we show here that injection of noradrenaline (norepinephrine) brings about a long-term depletion of skin peptides (initial concentrations do not recover until after day 56). When peptide stores recovered, the renewed peptides were similar in composition to the initial peptides as determined by MALDI-TOF mass spectrometry and in activity against *B. dendrobatidis* as determined by growth inhibition assays. Newly metamorphosed froglets depleted of their peptide stores and exposed to *B. dendrobatidis* died more rapidly than *B. dendrobatidis*-exposed froglets with their peptides intact. Thus, antimicrobial peptides in the skin mucus appear to provide some resistance to *B. dendrobatidis* infections, and it is important for biologists to recognize that this defense is especially important for newly metamorphosed frogs in which the adaptive immune system is still immature.**

Key words: amphibian, antimicrobial peptides, *Batrachochytrium dendrobatidis*, chytridiomycosis, *Rana pipiens*, skin defenses.

Received 13 December 2012; Accepted 8 April 2013

### INTRODUCTION

Antimicrobial peptides (AMPs) are an important component of the innate immune system of vertebrates and play important roles as the first line of defense at mucosal barriers (Zasloff, 2002; Hancock et al., 2012). Like many other frog species, the skin of *Rana pipiens* has two sets of distinctive glands (Noble and Noble, 1944; Bovbjerg, 1963). Mucus glands produce a material rich in heavily glycosylated mucins (Schumacher et al., 1994) and mucopolysaccharides (Duellman and Treub, 1986), which are continuously released to keep the skin moist. AMPs and other defensive peptides are produced in granular glands (also called poison glands) within the dermal layer of the skin. The contents of the granular glands empty into the thin layer of mucus produced independently by the mucus glands (Gammill et al., 2012; reviewed in Rollins-Smith et al., 2011). We recently showed that in *R. pipiens*, the AMPs are continuously released in low amounts and persist for several hours after release. Thus, there appears to be a steady flow of low amounts of AMPs to deter skin pathogens (Pask et al., 2012).

The skin of many amphibian species has been shown to produce a diverse array of AMPs that have activity against gram-positive and gram-negative bacteria, viruses and fungi (Nicolas and Mor, 1995; Pukala et al., 2006; Rollins-Smith, 2009). Each species has its own distinctive repertoire of AMPs (reviewed by Conlon et al., 2004). The peptides are synthesized as precursor peptides with a signal sequence and an acidic propiece that are cleaved to release the mature active peptide before or at the time of secretion (reviewed in Amiche et al., 1999). The peptides are tightly packed into the enveloped granules within the syncytial structure of the granular glands poised for release (Bovbjerg, 1963; Dockray and Hopkins, 1975; reviewed in Amiche et al., 1999). The glands are surrounded by a layer of myoepithelial cells innervated by sympathetic nerves (Sjoberg and Flock, 1976). Following alarm or injury, the sympathetic nervous system is activated, neurotransmitters engage the adrenergic receptors (Benson and Hadley, 1969; Holmes and Balls, 1978), and the contents of the gland are released onto the surface of the skin (Dockray and Hopkins, 1975). When the glands are stimulated, the granules containing peptides are released in what has been described as a ‘holocrine’ fashion (Dockray and Hopkins, 1975). However, this terminology suggests that the cell membranes of the producing cells are disrupted, and the entire contents are extruded. Our own studies suggest that most physiological responses to normal stresses would result in release of some, but not all, of the contents of the glands, and the structure of the gland would persist to enable restoration of peptide synthesis (Ramsey et al., 2010; Gammill et al., 2012). Because the myoepithelial cells surrounding the glands have alpha-adrenergic receptors, they can be experimentally induced to release their contents by injection of noradrenaline (Dockray and Hopkins, 1975).

Worldwide amphibian declines and extinctions have been linked to an emerging infectious disease, chytridiomycosis (Skerratt, et al., 2007; Collins, 2010), which is caused by the fungus *Batrachochytrium dendrobatidis* (Berger et al., 1998; Longcore et al., 1999; Pessier et al., 1999). Because *B. dendrobatidis* colonizes the keratinized epithelium of the skin, we investigated the possible role of AMPs as a mechanism to provide some protection from infection by this pathogen. Accumulating evidence suggests that AMPs released into the mucus provide the first line of defense against this pathogen (Rollins-Smith and Conlon, 2005; Rollins-Smith, 2009; Ramsey et al., 2010; Pask et al., 2012; reviewed in Rollins-Smith et al., 2011). Water-borne zoospores of *B. dendrobatidis* adhere to the skin, form a germination tube, and migrate into the stratum granulosum to mature in the protected environment of the host cell (Greenspan et al., 2012; Van Rooij et al., 2012). After maturing into a zoosporangium, a discharge papillus opens, and new zoospores move out to infect a new individual or the same individual at other sites (Berger et al., 1998; 2005; Longcore et al., 1999; Pessier et al., 1999). During infection or re-infection, zoospores would encounter the chemical defenses of the host mucus. Those defenses include AMPs, lysozymes, mucosal antibodies and bacterial metabolites (reviewed in Rollins-Smith et al., 2011).

*Batrachochytrium dendrobatidis* not only infects adult frogs but also colonizes the mouthparts of tadpoles (Berger et al., 1998; Rachowicz and Vredenburg, 2004). When a tadpole undergoes metamorphosis, the newly developing adult-type skin provides an appropriate environment for *B. dendrobatidis* at the same time that the adaptive immune system is suppressed (reviewed in Rollins-Smith, 1998; Rollins-Smith et al., 2011) and much of the organism's energy is spent completing metamorphosis. Metamorphosis is driven by the concerted actions of thyroid hormones and corticosteroid hormones (reviewed in Denver, 2009; Denver, 2013). The elevated corticosteroid hormones at metamorphosis induce apoptosis of thymocyte and splenocyte populations (Rollins-Smith et al., 1997; Barker et al., 1997), resulting in a temporary suppression of immune responses during the climax of metamorphosis (reviewed in Rollins-Smith, 1998). At the same time, the thyroid hormones drive differentiation of larval skin to the adult pattern of keratinized epithelium (reviewed in Miller, 1996). Only the keratinized epithelium of the tadpole mouthparts and the adult skin are colonized by *B. dendrobatidis* (Berger et al., 1998).

In *B. dendrobatidis*-infected populations, metamorphosing frogs appear to be the most susceptible stage for infection (Berger et al., 1998; Bosch et al., 2001; Rachowicz and Vredenburg, 2004; Rachowicz et al., 2006; Tobler and Schmidt, 2010; Walker et al., 2010). Thus, we hypothesized that during the period around the time of metamorphosis, AMP defenses in the skin would be crucial to provide some degree of protection against pathogens such as *B. dendrobatidis*. Here, we describe an experimental treatment to deplete skin peptides in adults and metamorphosing juveniles and show that recovery of peptides is very slow after maximal depletion. Recovered AMPs were identical in composition to previously secreted AMPs and retained activity against *B. dendrobatidis*. Depletion of AMPs in newly metamorphosed juveniles negatively impacted survival following exposure to *B. dendrobatidis*.

## MATERIALS AND METHODS

### Frogs

Adult *Rana pipiens* (Schreber 1782), measuring between 5 and 6.5 cm, were purchased from Connecticut Valley Biological Supply (Southampton, MA, USA). Frogs were housed in groups of five to six in polystyrene containers measuring 44.4×24×20.3 cm containing dechlorinated tap water at 20–24°C. Juvenile *R. pipiens* were reared to the age of 3–6 months post-metamorphosis in chytrid-free conditions by T.L.C. from embryos provided by Nasco (Fort Atkinson, WI, USA) and transported to Vanderbilt University. At Vanderbilt, these frogs were housed in groups in the same polystyrene containers used for adults. For the *B. dendrobatidis* infection studies, the juveniles were housed individually in sterile 280 ml plastic Gladware containers in dechlorinated tap water at 20–24°C. Containers were placed at an incline, allowing the frogs to choose a wet or dry area. Adults and juveniles were fed live crickets (pinhead crickets and mini mealworms for juveniles), and their water was changed three times weekly (adults) or five times weekly (juveniles). All protocols were approved by the Vanderbilt University Medical Center Institutional Animal Care and Use Committee.

### Determination of the dose of noradrenaline necessary to deplete skin peptides stores

To determine the amount of noradrenaline (norepinephrine) necessary to maximally deplete skin peptides, groups of adult frogs were injected with 0 ($N$=8), 2 ($N$=8), 20 ($N$=6), 40 ($N$=9) or 80 nmol $g^{-1}$ ($N$=9) of noradrenaline-HCl (Sigma-Aldrich, St Louis, MO, USA) dissolved in amphibian phosphate buffered saline (APBS) as previously described (Rollins-Smith et al., 2006; Ramsey et al., 2010; Pask et al., 2012). Peptides released into collection buffer were collected and quantified as described below.

### Kinetics of skin peptide renewal

To determine the time necessary for recovery of skin peptides following noradrenaline-induced peptide depletion, a large group of frogs was injected with 40 ($N$=25) or 20 nmol noradrenaline $g^{-1}$ [$N$=26], and a subset of the animals was injected with the same dose at days 3, 10, 20, 30, 40 and 50 ($N$=4 or 5 at each time point). Peptides were collected and quantified at each time point.

### Peptide collection, partial purification and quantification

Frogs were injected in the dorsal lymph sac with APBS or noradrenaline dissolved in APBS and placed in collection buffer for 15 min as previously described (Rollins-Smith et al., 2006; Ramsey et al., 2010; Pask et al., 2012). After 15 min, the frog was removed and the buffer was acidified by adding trifluoroacetic acid to a final concentration of 1%. Peptides were partially enriched over C18 Sep-Paks (Waters Corporation, Milford, MA, USA) as previously described and quantified using the MicroBCA (bicinchoninic acid) assay (Pierce, Rockford, IL, USA) according to the manufacturer's instructions, except that the peptide bradykinin (RPPGFSPFR) (Sigma-Aldrich) was used as a standard (Rollins-Smith et al., 2006; Ramsey et al., 2010; Pask et al., 2012). The mass of each frog was determined at the time of the injection, and total induced peptides were quantified. To estimate the total amount of peptides recoverable in the mucus, the surface area was calculated according to the method of McClanahan and Baldwin (McClanahan and Baldwin, 1969): [Surface area=9.9(mass in grams)$^{0.56}$]. The thickness of the mucus was assumed to be 50 µm (Brucker et al., 2008), and therefore the volume of mucus covering 1 $cm^2$ of skin would be 5 µl. As a result, the total peptides (µg) per $cm^2$ × 200=total µg $ml^{-1}$ in mucus (Ramsey et al., 2010; Pask et al., 2012).

### Skin histology

A section of dorsal skin, ventral skin and the region of skin termed the dorsal plicae (Bovbjerg, 1963) from three peptide-depleted and

three APBS-injected frogs was removed after euthanasia and fixed in 10% buffered formalin for 48 h. The skin sections were processed, embedded in paraffin and stained with hematoxylin and eosin (H&E) by the Vanderbilt Immunohistochemistry Core in order to visualize granular glands. Approximately 10 sections from each skin sample (60 total) were examined. Stained slides were photographed using an Olympus BX41 microscope with an Olympus DP71 camera and DP Controller software, version 3.1.1.267 (Olympus Corporation, Center Valley, PA, USA).

### Matrix-assisted laser desorption time of flight mass spectrometry

After partial purification, the recovered peptides were concentrated to dryness by centrifugation under vacuum at 70°C and prepared for matrix-assisted laser desorption time of flight (MALDI-TOF) analysis as previously described (Rollins-Smith et al., 2006; Ramsey et al., 2010; Pask et al., 2012). The peptides were resuspended at a concentration of 1 mg ml$^{-1}$ in highly pure water suitable for high performance liquid chromatography (HPLC), and a mixture containing 0.6 µl of resuspended peptides and 0.6 µl of α-cyano-4-hydroxycinnamic acid matrix (Sigma-Aldrich) was spotted onto the target plate and air-dried. MALDI-TOF mass spectrometry (MS) was performed using the Bruker Daltronics Ultraflex III time-of flight mass spectrometer (Billerica, MA, USA) operated in reflector, delayed extraction and positive ion mode. The instrument was calibrated using a mixture of standard peptides including leucine encephalin with a mass to charge ratio (*m*/*z*) of 556.277, human angiotensin II (*m*/*z* 1046.542), human [Glu1]-fibrinopeptide B (*m*/*z* 1570.677) and bovine oxidized insulin chain B (*m*/*z* 3494.651). Spectra were acquired from the 500 to 5000 *m*/*z* range. Automated data acquisition was performed by averaging 250 laser shots. Samples from greater than 30 adult and 12 juvenile frogs were examined, and representative profiles are shown.

### Growth inhibition assays

The growth inhibition assays were performed as previously described (Rollins-Smith et al., 2006; Ramsey et al., 2010; Pask et al., 2012). Briefly, *B. dendrobatidis* zoospores were grown on 1% tryptone agar for 1 week at 23°C. Freshly isolated zoospores were plated ($5\times10^4$ 50 µl$^{-1}$, five replicates for each concentration to be tested) in tryptone broth in a 96-well flat-bottom microtiter plates with 50 µl of a serially diluted mixture of skin peptides dissolved in HPLC-grade water. Positive control wells (five replicates) contained zoospores in 50 µl tryptone broth and 50 µl HPLC water. Negative control wells (five replicates) contained heat-killed zoospores (60°C for 10 min) in 50 µl of tryptone broth and 50 µl HPLC water. Plates were incubated at 23°C for 1 week, and growth was measured as an increased optical density at 490 nm ($OD_{490}$) using an MRX Microplate Reader (Dynex Technologies, Chantilly, VA, USA). Peptide effectiveness was defined as the percent inhibition at a peptide concentration of 12.5 µg ml$^{-1}$ multiplied by the total amount of peptides recovered from the individual frog (Pask et al., 2012). For the comparison of peptide effectiveness, growth inhibition was determined over a series of peptide concentrations for five frogs sampled at day 0 and five frogs sampled at day 3.

### Quantification of *B. dendrobatidis* zoospores on the skin

Frogs were swabbed with a sterile cotton swab 10 times on the abdomen, legs and each foot according to established protocols (Boyle et al., 2004; Hyatt et al., 2007). Swabs were placed into tubes and DNA was extracted and amplified by probe-based quantitative polymerase chain reactions (qPCR) as previously described (Ramsey et al., 2010). For this assay, there was a 'no template control' containing all reaction components except the DNA template, a positive control containing DNA extracted from a swab loaded with a known number of zoospores, and a negative control containing DNA extracted from a sterile swab. All skin swab samples and controls were run in triplicate, and cycle threshold ($C_t$) values were averaged between the three wells.

### Exposure of post-metamorphic froglets to *B. dendrobatidis*

*Batrachochytrium dendrobatidis*-free *R. pipiens* at 3–6 months post-metamorphosis were reared from embryos and were thus naïve to *B. dendrobatidis*. Frogs were swabbed before the experiment to ensure they were *B. dendrobatidis*-free. All 27 juveniles used in this experiment were negative. The experiment consisted of four experimental groups: (1) frogs injected with 40 nmol noradrenaline g$^{-1}$ and again 2 days later with 20 nmol noradrenaline g$^{-1}$ to deplete their granular glands and exposed to $10^4$ zoospores of isolate JEL275 (Carey et al., 2006) (*N*=8); (2) frogs injected with an equivalent volume of APBS and exposed to $10^4$ zoospores (*N*=8); (3) frogs injected with 40 nmol noradrenaline g$^{-1}$ and again 2 days later with 20 nmol noradrenaline g$^{-1}$ and maintained as groups 1 and 2 without further manipulation (*N*=3); and (4) frogs injected with APBS and maintained as groups 1–3 without further manipulations (*N*=8). *Batrachochytrium dendrobatidis* exposure lasted for 2 days until the water on all frogs was changed and zoospores were flushed out. After exposure, the frogs were examined for signs of illness or death every day and swabbed at 10 days post exposure.

### Statistical comparisons

Peptide concentrations, optical density determinations of *B. dendrobatidis* growth ($OD_{490}$), relative peptide effectiveness and zoospore equivalents determined by qPCR were averaged for each group, and the means ± s.e.m. were compared by one-way ANOVA after log transformation (Tukey *post hoc* test) or by Student's *t*-test following log transformation to meet the assumptions of a normal distribution and homogeneity of variances for parametric statistics. Percent survival of juveniles at 10 days post exposure to *B. dendrobatidis* was compared among experimental groups using a chi-square test. A *P*-value of ≤0.05 was considered statistically significant. The number of animals or replicates is shown in the figure legends.

## RESULTS

### Determination of the dose of noradrenaline necessary to deplete skin peptide stores

To determine the importance of AMPs in *B. dendrobatidis* infection, it was necessary to deplete the stores of AMPs in granular glands. Prior to this study, the concentration necessary to fully deplete glands had not been determined. With increasing concentrations of noradrenaline, greater concentrations of total peptides were recovered in a dose-dependent fashion. The concentration required to maximally deplete peptides was 40 nmol noradrenaline g$^{-1}$ (Fig. 1). Although injection of 80 nmol noradrenaline g$^{-1}$ induced slightly more peptides than the 40 nmol g$^{-1}$ dose, the difference was not statistically significant.

### Renewal of depleted skin peptides

In preparation for studies to determine the effects of skin peptide depletion on susceptibility to experimental infections with *B. dendrobatidis*, it was important to determine how long peptide contents of granular glands remained depleted. When peptides were

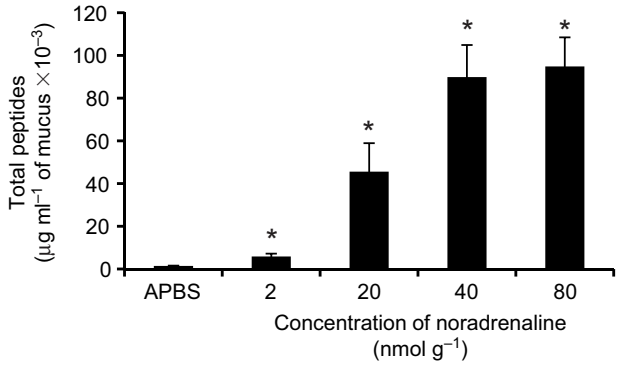
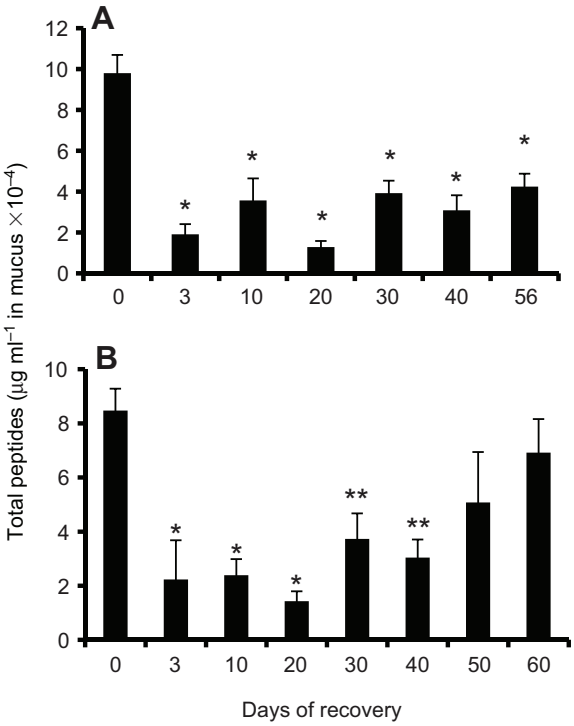
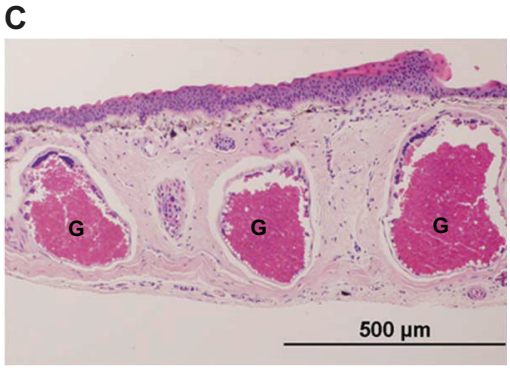



Fig. 1. Dose–response to noradrenaline injections. Frogs (*Rana pipiens*, *N*=6 to 9 per group) were injected with amphibian phosphate buffered saline (APBS) (*N*=8) or 2 (*N*=8), 20 (*N*=6), 40 (*N*=9) or 80 nmol noradrenaline $g^{-1}$ (*N*=6). *All concentrations of noradrenaline induced more peptides than those recovered from APBS-injected frogs by one-way ANOVA after log transformation, Tukey *post hoc* test, $P \leq 0.01$. Concentrations of peptides recovered from frogs injected with 2, 20 and 40 nmol noradrenaline $g^{-1}$ were significantly different from each other and from APBS controls by one-way ANOVA after log transformation, Tukey *post hoc* test, $P \leq 0.05$.



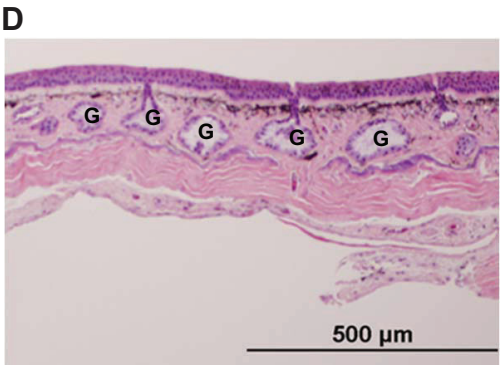




Fig. 2. Recovery of peptides following injection of noradrenaline. (A) Injection of 40 nmol noradrenaline $g^{-1}$ at each time point (*N*=25 at day 0; *N*=4 or 5 at other time points). *Peptide concentrations were significantly reduced in comparison with those collected at day 0 by two-tailed Student's *t*-test after log transformation, $P<0.02$. (B) Injection of 20 nmol noradrenaline $g^{-1}$ at each time point (*N*=26 at day 0; *N*=4 or 5 at other time points). *Peptide concentrations were significantly reduced in comparison with those collected at day 0 by two-tailed Student's *t*-test after log transformation, $P<0.025$; **significantly reduced by one-tailed Student's *t*-test, $P \leq 0.05$. (C) Skin from APBS-injected control frog showing intact granular glands (G). (D) Skin from frog injected with 40 nmol noradrenaline $g^{-1}$ showing intact but largely empty granular glands.

depleted by one injection of 40 nmol noradrenaline $g^{-1}$, some residual peptides could be recovered at each time point tested, but peptide stores had not recovered to initial levels as late as 56 days after the first injection (Fig. 2A). Following one injection of 20 nmol noradrenaline $g^{-1}$, peptide stores were still significantly depleted until day 40 but recovered to initial levels after 50 days (Fig. 2B). To determine the effects of the noradrenaline injection on gland morphology, histology was performed. Dorsal skin sections were the most informative because animals were injected in an area adjacent to the complex of skin glands termed the dermal plicae (Bovbjerg, 1963). The morphology of intact glands is shown in a skin section from an APBS-injected frog. The glands are full of bright red granules by H&E staining (Fig. 2C). In contrast, gland morphology following an injection with 40 nmol noradrenaline $g^{-1}$ was quite different. The granular glands were empty of the eosinophilic granules, but they did not show significant structural damage (Fig. 2D).

### Characterization of recovered peptides by MALDI-TOF MS

To determine whether the profile of AMPs that were newly synthesized following granular gland depletion was compositionally the same or different from the initial profile of peptides, peptides induced at days 0, 3 and 60 were compared by MALDI-TOF MS. Shown in Fig. 3A are representative MALDI-TOF spectra. The profiles are very similar to each other, with the three expected brevinin peptides (brevinin-1Pb, brevinin-1Pd and brevinin-1Pa) present in order of relative intensity. Also present is a strong signal for ranatuerin-2P and another defensive peptide, bradykinin (Fig. 3A). When comparing the activity of the recovered peptides from different time points, the ability to inhibit *B. dendrobatidis* growth was comparable between the initial peptides (day 0, Fig. 3B) and the peptides recovered at other time points (day 3 shown, Fig. 3C). Note that both sets of peptides showed a minimal inhibitory concentration (MIC) of approximately 50 μg $ml^{-1}$. Given that the activity of peptides from day 0 and 3 were not different, peptides recovered at day 60 were not tested for growth inhibitory activity. This finding demonstrates that

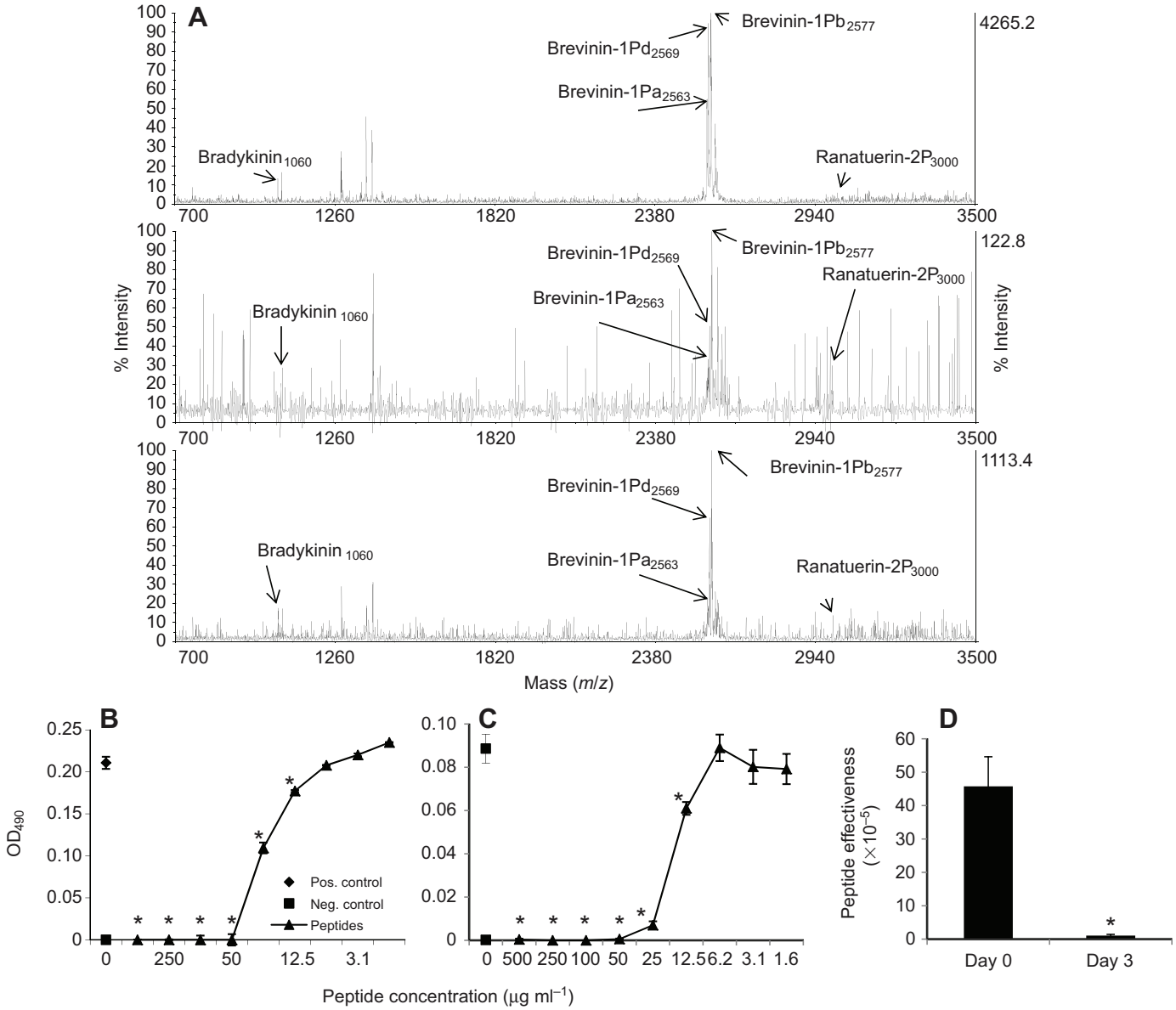



Fig. 3. Characterization of skin peptides recovered at days 0, 3 and 60 after noradrenaline induction. (A) Representative MALDI-TOF profiles of peptides recovered after injection of 40 nmol noradrenaline $g^{-1}$ at day 0 (top), day 3 (middle) and day 60 (bottom) ($N$=5 frogs examined at each time point). (B) Growth inhibition of *Batrachochytrium dendrobatidis* zoospores by peptides recovered at day 0 after noradrenaline injection. (C) Growth inhibition of *B. dendrobatidis* zoospores by peptides recovered at day 3 after noradrenaline injection. For B and C, MIC=50 μg $ml^{-1}$. *Growth was significantly less than that of positive control wells by two-tailed Student's $t$-test after log transformation, $P$≤0.05 (five replicates for each concentration of peptide, negative control and positive control wells). (D) Peptide effectiveness of the mixture of peptides recovered at day 0 and day 3. *Peptide effectiveness at day 3 was significantly decreased in comparison with that observed for peptides collected at day 0 by two-tailed Student's $t$-test after log transformation, $P$≤0.05 ($N$=5 frogs sampled at day 0 and day 3).

although the amount of AMPs present in the granular glands was reduced at later time points, the same relative mixture of peptides was present and effective against *B. dendrobatidis*. The relative effectiveness of the AMP mixture at day 3 *versus* day 0 showed that the relative effectiveness at day 3 was greatly diminished, reflecting the reduced amounts of peptides that can be induced following their depletion 3 days earlier (Fig. 3D).

### Role of AMPs in protecting *R. pipiens* juveniles

Young post-metamorphic *R. pipiens* were used in these experiments because they had been reared in a laboratory free of the presence of *B. dendrobatidis* and were thus naïve to the pathogen. MALDI-TOF analysis was performed on partially purified AMPs from these juvenile frogs to confirm that they have a normal complement of AMPs at this life stage. Because the parents of these frogs were collected in Minnesota or Wisconsin, the juvenile frogs expressed a slightly different profile of AMPs characteristic of populations in this region (Tennessen et al., 2009). This profile includes brevinin-1Pa, brevinin-1Pb, brevinin-1Pe and ranatuerin-2P (Fig. 4A). The peptides recovered from the juvenile frogs showed excellent capacity to inhibit growth of *B. dendrobatidis* with an approximate MIC of 50–100 μg $ml^{-1}$ (range 25–250 μg $ml^{-1}$, mean ± s.e.m.=96±27 μg $ml^{-1}$), comparable to that of peptides from adult frogs (Fig. 4B). When these post-metamorphic frogs were depleted of their peptides by two injections of noradrenaline and exposed to *B. dendrobatidis* in their water (NA+*Bd*), they did not survive as well

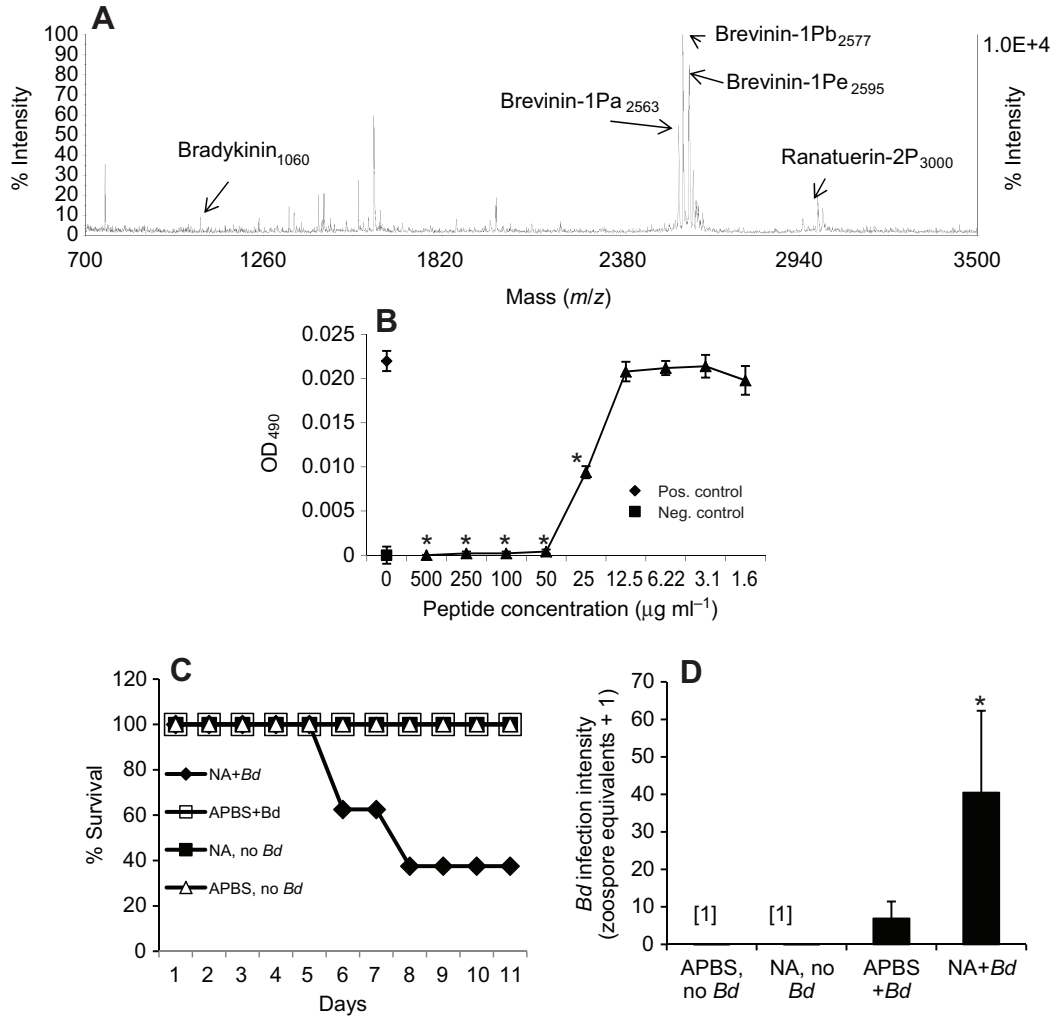



Fig. 4. Peptide depletion induced by noradrenaline increases susceptibility of post-metamorphic juveniles to *Batrachochytrium dendrobatidis*. (A) Representative MALDI-TOF profile of hydrophobic skin peptides from juvenile frogs including antimicrobial peptides brevinin-1Pa, brevinin-1Pb, brevinin-1Pe and ranatuerin-2P (representative of 12 individual frogs). (B) Growth inhibition of *B. dendrobatidis* zoospores by skin peptides from juvenile frogs (representative of 12 individual frogs tested). MIC=50 μg ml$^{-1}$. *Growth was significantly less than that of positive control wells by two-tailed Student's *t*-test after log transformation, $P \leq 0.05$ (five replicates for each concentration of peptide, negative control, and positive control wells). (C) Percent survival of post-metamorphic juveniles peptide-depleted and exposed to *B. dendrobatidis* (NA+*Bd*, $N$=8), APBS-injected and exposed to *B. dendrobatidis* (APBS+*Bd*, $N$=8), noradrenaline-injected but not exposed to *B. dendrobatidis* (NA, no *Bd*, $N$=3) or APBS injected but not exposed to *B. dendrobatidis* (APBS, no *Bd*, $N$=8). Percent survival of peptide-depleted and *B. dendrobatidis*-exposed frogs was significantly less than that of APBS-injected controls exposed or not exposed to *B. dendrobatidis* by chi-square test, $P \leq 0.05$ and also significantly less than peptide-depleted frogs not exposed to *B. dendrobatidis*, $P \leq 0.1$. (D) Infection intensity (zoospore equivalents + 1) on the skin of surviving frogs at day 10 that were APBS-injected but not exposed to *B. dendrobatidis* (*Bd*) (APBS, no *Bd*, $N$=8), injected with noradrenaline (NA) and not exposed to *Bd* (NA, no *Bd*, $N$=2), APBS-injected and exposed to *Bd* (APBS+*Bd*, $N$=7) or injected with noradrenaline and exposed to *Bd* (NA+*Bd*, $N$=3). Data represent mean + 1 s.e.m. log zoospore equivalents. *Greater number of zoospore equivalents present on the skin of NA+*Bd* frogs compared with APBS+*Bd* frogs by a one-tailed Student's *t*-test after log transformation, $P$=0.0352.

as *B. dendrobatidis*-exposed animals that had an intact set of peptides (APBS+*Bd*). Among the peptide-depleted and *B. dendrobatidis*-exposed frogs, only three of eight had survived to day 10 while eight of eight APBS-injected control frogs that were exposed to *B. dendrobatidis* were alive. Other control groups that were noradrenaline-treated (3/3) or APBS-injected and not exposed to *B. dendrobatidis* (8/8) also survived to day 10 (Fig. 4C). Among frogs surviving at 10 days post-exposure to *B. dendrobatidis*, the remaining peptide-depleted frogs had significantly higher numbers of zoospores on the skin than APBS-injected and *B. dendrobatidis*-exposed frogs (Fig. 4D). Frogs not exposed to *B. dendrobatidis* had no evidence of *B. dendrobatidis* infection determined by qPCR.

## DISCUSSION

The present study supports previous work that showed a correlation between the survival of amphibians exposed to *B. dendrobatidis* with beneficial AMPs (Woodhams et al., 2006a; Woodhams et al., 2006b, 2007). It also supports a previous study showing that depletion of skin peptides in *Xenopus laevis* (naturally resistant to chytridiomycosis) resulted in increased pathogen burden (Ramsey et al., 2010).

### Development of a method to deplete skin peptides

Using adult northern leopard frogs (*R. pipiens*) as a model, we developed a protocol to deplete skin AMPs by injection of

noradrenaline and observed the relatively slow renewal of the same set of peptides over a period of approximately 2 months. We then applied the same methods to deplete skin peptides from juvenile leopard frogs raised under laboratory conditions to insure that they were not exposed to *B. dendrobatidis*. This allowed us to examine the role of skin peptides in protection from *B. dendrobatidis* infections free of the potential complication of mucosal antibodies if the frogs had previously been exposed to *B. dendrobatidis* (Ramsey et al., 2010). Although one injection of 40 nmol noradrenaline $g^{-1}$ significantly reduced available skin peptides, some peptides were recovered at 3 days after the first injection. We previously showed in *X. laevis* that two noradrenaline injections separated by 2 days were necessary to completely deplete skin peptides (Gammill et al., 2012). Thus, for the experimental infection study with juvenile *R. pipiens*, two noradrenaline injections were used.

### Kinetics of peptide recovery and characteristics of the recovered peptides

Previous studies of peptide recovery following noradrenaline injection examined relatively mild inductions, and recovery was examined after a very short time. In *X. laevis*, recovery of peptides following a mild noradrenaline injection (0.5 to 1 nmol $g^{-1}$) was detected by fast atom bombardment MS. The full complement of expected peptides was detected within 2–6 days (Giovannini et al., 1987) suggesting that recovery is very rapid or peptides were not depleted by this injection. Following injection of a slightly higher concentration of noradrenaline (3 nmol $g^{-1}$), gland morphology was not completely restored for 2 weeks as determined by histology and electron microscopy (Dockray and Hopkins, 1975). This suggested that a higher concentration of noradrenaline is necessary to more completely deplete peptides. We have shown that in *X. laevis*, two injections of 80 nmol $g^{-1}$ within 2 days were necessary to completely deplete peptides (Gammill et al., 2012), and peptide recovery following depletion with 80 nmol $g^{-1}$ required 7 to 9 weeks (Ramsey et al., 2010). In the present study, we showed that peptide recovery after nearly complete depletion of *R. pipiens* peptides (injected with 40 nmol noradrenaline $g^{-1}$) required greater than 56 days (Fig. 2). By MALDI-TOF MS analysis, the profile of recovered peptides at days 0, 3 and 60 were identical. When concentrated, the peptides had similar growth inhibitory activity at days 0 and 3 (Fig. 3B,C). This finding demonstrates that although the amount of AMPs present in the granular glands was reduced at day 3, the same relative mixture of peptides was present and effective against *B. dendrobatidis*. The relative effectiveness of the AMP mixture at day 3 *versus* day 0 showed that the relative effectiveness at day 3 was greatly diminished, reflecting the reduced amounts of peptides that can be induced following their depletion 3 days earlier (Fig. 3D).

### Immune defenses against *B. dendrobatidis* in juvenile frogs

Our previous study of immune defenses against *B. dendrobatidis* in *X. laevis* showed that both innate immune defenses (AMPs) and adaptive lymphocyte-mediated defenses play a role in protection of this highly resistant species (Ramsey et al., 2010). The development of immune defenses in *R. pipiens* is not well studied, but we can make some informed assumptions about the maturation of the immune system based on studies of *X. laevis*. In *Xenopus*, adult-type recognition of minor histocompatibility antigens and development of high affinity antibodies develops within 1–2 months post-metamorphosis (DiMarzo and Cohen, 1982; Rollins-Smith et al., 1988; Hsu and Du Pasquier, 1984; Hsu and Du Pasquier, 1992; reviewed in Rollins-Smith, 1998). Presence of high affinity IgY antibodies in *Rana catesbeiana* juveniles at 3 months post-metamorphosis (Du Pasquier and Haimovich, 1976) suggests that the adult B cell population in ranid frogs develops during the post-metamorphic lymphocyte expansion just as it does in *Xenopus*. Thus, we are confident that the juvenile *R. pipiens* used in our studies at 3–6 months post-metamorphosis had an adult-type immune system. Much less is known about the ontogeny of expression of the antimicrobial peptide defenses. However, a previous study in *R. pipiens* showed that granular glands are completely developed in the dermal plicae of *R. pipiens* at the conclusion of metamorphosis (Bovbjerg, 1963). Thus, we assumed that the expected antimicrobial peptides would be present at 3–6 months post-metamorphosis when the chytrid-exposure experiments were conducted. To confirm that the AMP repertoire was complete, we examined the peptides present in 12 of these young frogs and found the expected AMPs in the majority of these animals (9/12) as shown in Fig. 4A. Thus, the normal complement of AMPs had developed in these young frogs, and the peptide depletion protocols developed in larger adults were effective in depleting the peptide reserves of the younger frogs.

Although the most direct effect of injection of noradrenaline at the relatively high concentrations used in our experiments is the depletion of skin peptide reserves, the noradrenaline could also have other immunosuppressive effects such as activation of corticosterone release (Shepherd and Holzwarth, 2001). Future studies will determine whether there are other immunosuppressive effects of noradrenaline treatment. This very strong noradrenaline stimulus to deplete peptides was a pharmacological treatment to impair the skin peptide defenses. It is unlikely that natural stresses in nature would result in such significant long-term depletion of skin peptides. We showed previously (Pask et al., 2012) that the stress to a frog of being chased for 10 min resulted in a total peptide release of approximately 6400 μg peptides $ml^{-1}$ mucus, comparable to that observed following the injection of 2 nmol $g^{-1}$ noradrenaline in the experiments presented here (Fig. 1). Thus, the treatment to deplete peptides (40 nmol $g^{-1}$ noradrenaline), which induced approximately 90,000 μg peptides $ml^{-1}$ mucus, far exceeds the amount that would naturally be released.

### AMP protection of naïve juvenile frogs exposed to *B. dendrobatidis*

Metamorphosis in amphibians is a time of fasting due to the extensive reorganization of the digestive tract (reviewed in Fox, 1981). In *Xenopus*, tadpoles are fasting after stage 62 but their glucose levels in body fluids do not decrease (Hanke and Leist, 1971). As suggested by Jaudet and Hatey (Jaudet and Hatey, 1984), glycogen, lipids and proteins are probably converted to glucose (Hanke and Leist, 1971; Gunesch, 1974). Elevation of corticosteroid hormones is observed during metamorphosis of all amphibian species studied (reviewed in White and Nicoll, 1981; Kikuyama et al., 1993; Denver, 2009). Therefore, it is likely that a trade-off may occur that diverts energy from other physiological processes to the tissue and organ remodeling that occurs at metamorphosis because the organism has a finite amount of energy (reviewed in Rollins-Smith and Woodhams, 2012). One of these systems affected is the immune system. Wood frogs (*Rana sylvatica*) develop in temporary ponds, which may dry up in an unpredictable fashion. In conditions that simulated quickly drying ponds, tadpoles metamorphosed earlier and appeared to experience a trade-off in decreased total leukocyte counts and diminished cellular immune response (skin swelling in response to a plant lectin) (Gervasi and Foufopoulos, 2008). Precocious metamorphosis induced by administration of thyroid hormones limited lymphocyte development and impaired

allograft rejection responses in *X. laevis* (Rollins-Smith et al., 1988). During metamorphosis, the immune system undergoes involution due to elevated corticosteroid hormones (reviewed in Rollins-Smith, 1998), and new metamorphs are particularly susceptible to diseases including chytridiomycosis (Bosch et al., 2001; Rachowicz and Vredenburg, 2004; Rachowicz et al., 2006; Tobler and Schmidt, 2010; Walker et al., 2010).

To assess the role of AMPs in protecting naïve juvenile frogs, froglets at 3–6 months post-metamorphosis, which expressed a normal complement of AMPs capable of inhibiting growth of *B. dendrobatidis* at this life stage, were depleted of their peptides and exposed to a limited number of *B. dendrobatidis* zoospores. Frogs that had their peptide stores intact survived the experimental infection significantly better than those that were peptide depleted. Peptide-depleted juveniles that survived the infection had significantly higher *B. dendrobatidis* zoospore loads compared with the peptide-intact animals. In a previous study, we showed that AMPs are constitutively expressed by adult *R. pipiens* at low levels that are sufficient to inhibit *B. dendrobatidis* growth *in vitro* (Pask et al., 2012). The present study demonstrates that those constitutively released AMPs provide critical protection for the most susceptible life stage against *B. dendrobatidis*.

## LIST OF SYMBOLS AND ABBREVIATIONS

| | |
|---|---|
| AMP | antimicrobial peptide |
| APBS | amphibian phosphate buffered saline |
| *Bd* | *Batrachochytrium dendrobatidis* |
| $C_t$ | cycle threshold (the number of cycles necessary for the fluorescence signal to exceed background) |
| H&E | hematoxylin and eosin |
| HPLC | high performance liquid chromatography |
| MALDI-TOF | matrix-assisted laser desorption time-of-flight |
| MIC | minimal inhibitory concentration |
| MS | mass spectrometry |
| *m*/*z* | mass to charge ratio |
| NA | noradrenaline |
| $OD_{490}$ | optical density at 490 nm |
| qPCR | quantitative polymerase chain reaction |

## ACKNOWLEDGEMENTS

We thank William Karasov (Department of Forest and Wildlife Ecology, University of Wisconsin-Madison) for assistance in raising *Rana pipiens* froglets in a chytrid-free environment. We acknowledge the assistance of the Vanderbilt Mass Spectrometry Core and the Vanderbilt Immunohistochemistry Core.

## AUTHOR CONTRIBUTIONS

J.D.P., T.L.C., and L.A.R.-S. designed the experiments. J.D.P. conducted most of the experiments. T.L.C. reared tadpoles in a chytrid-free environment to provide the naïve animals for the infection study. J.D.P. and L.A.R.-S. wrote the manuscript, and T.L.C. added comments and suggested edits.

## COMPETING INTERESTS

No competing interests declared.

## FUNDING

This research was supported by grants IOS-0619536, IOS-0843207 and IOS-1121758 from the National Science Foundation (to L.A.R.-S.).

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