THE ROLE OF ANTIMICROBIAL SKIN PEPTIDES IN DEFENSE OF LEOPARD FROGS AGAINST CHYTRIDIOMYCOSIS

By

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DEDICATION

To my wife, Megan Pask, my parents, Mark and Jan Pask, and my brother, Greg Pask for all of your love, support, and encouragement during my journey.

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TABLE OF CONTENTS

Page

Appendix

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AMPs	antimicrobial peptides
APBS	amphibian phosphate buffered saline
BCA	bicinchonic acid
Bd	*Batrachochytrium dendrobatidis*
CHCA	α-Cyano-4-hydoxycinnamic acid
CLR	C-type lectin receptor
h	hour
HPLC	high performance liquid chromatography
m	meters
MALDI-TOF	Matrix-associated laser desorption time-of-flight
MIC	minimal inhibitory concentration
min	minute
MS	mass spectrometry
m/z	mass to charge
NE	norepinephrine
OD	optical density
s	second
TLR	Toll-like receptor
Zsp	zoospore

CHAPTER I

INTRODUCTION AND RESEARCH GOALS

Global Amphibian Declines

In recent years, amphibian populations around the world have been suffering significant declines and some species extinctions. Data collected from the United States, Puerto Rico, and Australia showed massive die-offs beginning in about the 1970s (27,54,103). In the next decade, amphibian declines were well documented in Central America and South America (111,136,137,202). Loss of amphibian populations was seen in populated areas as well as those that were considered uninhabited by humans (27,54). By the 1990's amphibian declines were recognized as a worldwide problem (37). These declines have been attributed to a variety of factors including the commercial use of amphibians, the introduction of non-native species, destruction of habitats, climate change, environmental chemicals, and infectious diseases.

Commercial use

Amphibians are used as a food source in many different countries. A recent report (177) revealed four major conclusions about the frog leg trade: 1) 95% of the world demand is supplied by wild populations. 2) The international trade of frog legs involves 30 countries and was valued at 49 million dollars in 1998. 3) The supply burden for frog legs falls on 11 species. 4) The average amount of frogs harvested annually from 1987 to 1997 was estimated to be 4,716 metric tons without including data from two

large frog leg exporting countries, China and Vietnam. It was reported that 15 million frogs that were imported and declared wild were used as pets or food from 1998 to 2002 (163). Over time, collecting this large volume of animals can deplete wild stocks (38).

Non-native species

Introduction of non-native species can cause amphibian declines or even extinctions in a direct or indirect manner in three ways. First, non-native species prey on amphibian species at both the tadpole and adult stage of the life cycle, decreasing the overall population. This predation most commonly occurs due to introduced fish, which feed on the eggs of amphibian populations. Secondly, native species and non-native species compete for the limited resources of the habitat. The third way by which non-native species affect amphibian populations is when non-native species interbreed with native species, which in turn, can cause the loss of the native species (37). A specific example of a non-native species introduction that led to amphibian declines is the case of the Mountain yellow-legged frog, *Rana muscosa*. *R. mucosa* were plentiful in the high elevation lakes of the Sierra Mountains, but they declined rapidly after the lakes were stocked with trout. When predatory fish were removed from some lakes, the *R. mucosa* populations recovered. Evidence was presented that the fish preyed on the *R. mucosa* tadpoles (188). In addition, the introduction of bullfrogs (*Rana catesbeiana)* and mosquitofish caused declines in the California red-legged frog (*Rana aurora draytonii*) (82,104,172).

Destruction of habitats

The destruction of habitats can lead to amphibian declines through human interventions that result in a change in land type (120). Canadian studies show that deforestation and wetland drainage reduced the amphibian biodiversity from 13 species to 3 species in southwestern Ontario (82). This finding suggests that a change in human land use or land type selected for the surviving species (37,172). Rainforests also house a large amount of amphibian biodiversity, but they are being lost from slash and burn farming with other rainforest land area being converted to other land types due to climate change (2). Amphibian species unable to adapt to the new habitats would have to be relocated or face die-offs.

Climate change

The world's climate is constantly changing. Two factors, temperature change and precipitation change, have been shown to have some correlation with amphibian declines. A study from Costa Rica examined the decline and extinctions in the *Atelopus* genus and concluded that increased cloud cover and misting conditions caused stability between day and night temperatures. These steady conditions favored the growth of the pathogen *B. dendrobatidis* and are referred to as the chytrid thermal optimum hypothesis (136). Several groups challenged the chytrid thermal optimum hypothesis citing the introduction of *B. dendrobatidis* and spread into naïve hosts as the most likely explanations of the declines (38,109,149,189,192). Because amphibians are ectothermic organisms, temperature can have profound effect on immunity and breeding patterns (28). Overall it is very difficult to prove that any of these factors is solely responsible for amphibian

declines. It is much more reasonable that they are working in concert to have a detrimental impact on amphibians (38).

Environmental chemicals

Pesticides, herbicides, and fertilizers all fall under the category of environmental chemicals. Amphibians are thought to serve as environmental sentinels because large impacts on an ecosystem are first experienced by these animals. Although pesticides are important for protecting vital crops from insects, these chemicals can seep into aquatic habitats following rains. These compounds can also lead to growth deficiencies and limb abnormalities (14). Industrial pollution has increased the environmental concentrations of heavy metals, and acidification of water sources has had detrimental effects on amphibian populations to the point of extinction (79,105). Environmental contaminants may affect the reproductive system indirectly by altering the endocrine system. The herbicide atrazine is reported to cause male frogs to be feminized and hermaphroditic (80). Environmental contaminants can cause elevated corticosterone levels that in turn lower reproduction (65,86). Pesticides carried upwind have been suggested to be the cause of some amphibian declines in regions of California (48).

Emerging infectious diseases

Emerging infectious diseases are a growing concern as previously pristine areas have suffered major amphibian declines (30). Ranaviruses, double-stranded DNA viruses, infect a variety of aquatic animals including amphibians, reptiles, and fish (47). Ranaviruses have been suggested as a cause for declines in North America, South

America, Australia, and Europe (15,43,44,47,52,92,205). Although these viruses did cause mortality through systemic hemorrhage or skin ulceration, there is not much evidence to suggest that ranaviruses or another suspected pathogen, *Aeromonas hydrophila*, are responsible for extinctions (44,47). The leading pathogen linked to amphibian declines, however, is the fungus *Batrachochytrium dendrobatidis.*

Batrachochytrium dendrobatidis

Batrachochytrium dendrobatidis was first isolated from a blue poison dart frog, *Dendrobates azureus* (114). *B. dendrobatidis* is in the phylum chytridiomycota and is the only member of the phylum that is pathogenic to vertebrates (84,90,91). Although it is unknown where the pathogen originated, the most reasonable hypothesis is that it spread out of Africa or Asia (5,190). The two life stages of *B. dendrobatidis* are the immature zoospore and the mature zoosporangium. The flagellated zoospore is motile for about 24 hours in which it must find a suitable substrate or die (9,114,185). When an amphibian host is found, the zoospore resorbs its flagellum and moves into the protective intracellular environment of the skin cells. Here, the zoospore develops a thallus and thread-like rhizoids and further matures into a zoosporangium. Within the zoosporangium, new zoospores differentiate. The new zoospores can be released through a discharge tube as the skin reaches the surface, and then can re-infect the same individual at a different site or a different individual. The entire life cycle takes approximately 4-5 days at the optimal growth temperature of 22°C (Fig 1-1) (9,185,192).

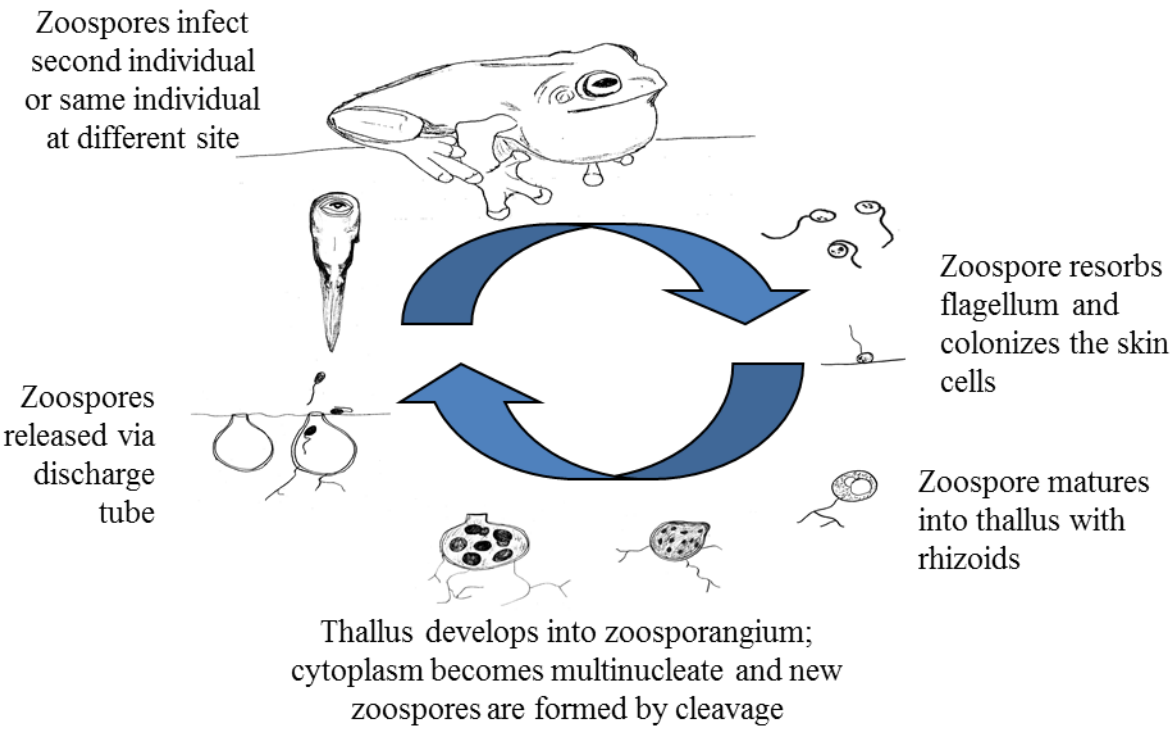

Figure 1-1. *B. dendrobatidis* life cycle. At the optimal growth temperature of 22°C it takes 4-5 days to complete the *B. dendrobatidis* life cycle (192).

Chytridiomycosis and B. dendrobatidis pathogenesis

Amphibians infected with *B. dendrobatidis* may develop clinical signs of the disease and die or, depending on their level of resistance, may remain relatively unharmed. The disease caused by *B. dendrobatidis* is called chytridiomycosis. The clinical signs observed after infection include excessive skin sloughing, skin reddening, loss of appetite, loss of righting reflex, lethargy, and abnormal posture (184,185,187). It has been postulated that the excessive skin sloughing is an adaptation to skin infections and is not directly initiated by the pathogen itself (184). The mechanism by which *B. dendrobatidis* kills amphibians is not well understood. The leading hypothesis is that colonization of the skin interferes with transport of essential ions from the environment and the amphibian (184,187). Amphibians with chronic infections have decreased calcium, potassium, and sodium concentrations in the blood compared to uninfected individuals and eventually succumb to asystolic cardiac arrest (184,186,187).

Because amphibians are ectothermic animals, temperature can have a drastic impact on their immunity to *B. dendrobatidis*. *B. dendrobatidis* thrives at temperatures ranging from 17-23°C (135,192). *B. dendrobatidis* cannot tolerate temperatures above 30 °C (136). Extreme low temperatures (7-10°C) slow maturation, but zoospores are still infectious (192). Field studies support the idea that temperature affects immunity to *B. dendrobatidis*. Amphibians found at lower temperatures had a higher prevalence of *B. dendrobatidis* compared to amphibians found at higher temperatures (194). Lower temperatures were also associated with more frequent population die-offs (11).

Not all amphibians are susceptible to chytridiomycosis. *Xenopus laevis* is relatively resistant with only 3% prevalence in wild populations (190). These finding

were recapitulated in the captive setting. Captive *X. laevis* did not show any clinical signs of infection (130). On the other hand some amphibian species, such as *Bufo boreas*, are very susceptible to chytridiomycosis. This species has suffered significant declines during the 1970s-1980s (29,31). Experimentally *B. boreas* can be exposed to as few as 10,000 zoospores for 1 day and the exposure results in 100% mortality (29).

Innate immunity and skin AMPs

The skin is the largest organ in the human body and serves as a physical and chemical barrier against microbes. The skin serves as a physical barrier by tightly joining the keratinocytes with desmosomes, impeding the infiltration of microbes (42,139). Components of the chemical barrier in the skin are cytokines, the complement cascade, leukocytes, and AMPs.

Skin AMPs

The cell responsible for making a majority of the AMPs in human skin is the keratinocyte, which constitutively produces low levels of AMPs to control microbial colonization (61). After AMPs are synthesized and packaged into lamellar bodies in the stratum granulosum, they are transported to the stratum corneum (1). Another rich source of AMPs in the skin are sebocytes, mast cells, and neutrophils (61). Secretions such as saliva and sweat have been shown to contain AMPs (12).

AMPs and infection

Following an injury to the skin, keratinocytes increase AMP production. AMPs have chemotaxic properties and recruit neutrophils, mast cells, and leukocytes to the site of infection as another source of AMPs (61). The invading pathogens can in turn trigger the transcription of AMPs through Toll-like receptor signaling (12). TLR stimulation serves a secondary response when the constitutive expression of AMPs from the keratinocytes fails (61).

Skin AMPs specically target microbes

Microbial surfaces of Gram-postive and Gram-negative bacteria have negatively-charged phospholipids and are naturally attracted to the overall positive charge of AMPs (93). Viral and fungal surfaces have negatively-charged components (93). After insertion into the lipid bilayer the cell membrane becomes unstable, and the cell is killed by lysis (83). Recent evidence has shown AMPs have the ability target bacteria undergoing cellular division (99).

Overview of fungal immunity

Fungi are eukaryotic organisms, and animals are exposed to them on a daily basis. Despite encountering potentially infectious fungal species frequently, most fungi do not cause disease unless they infect immunocompromised individuals (24). The worldwide prevalence of dermatological fungal infections (skin, nails, and mucosa) is estimated to be approximately 25% (24). Although fungal infections are rarely invasive, cryptococcal meningitis, disseminated candidiasis, and invasive pulmonary aspergillosis still have a

30-80% mortality rate during treatment and 100% mortality if untreated (24). The innate immune cells (macrophages and neutrophils) are considered to be central to an effective recognition of fungal pathogens. However, effective immunity also requires adaptive T-cell mediated responses (24,25) These points will be described in greater detail in the subsequent paragraphs.

Pattern recognition receptors and fungal immunity

Pattern recognition receptors can be grouped into four families: Toll-like receptors (TLRs), Nod-like receptors (NLRs), RIG-I-like receptors (RLRs), and C-type lectin receptors (CLRs). All four of these families recognize pathogens, but only TLRs and CLRs contribute directly to fungal immunity. MyD88 is an important signaling molecule in the TLR signal transduction pathways. Mice lacking this signaling molecule showed increased susceptibility to the fungal pathogens *Candida albicans, Paracoccidioides brasiliensis, Aspergillus fumigatus* and *Cryptococcus neoformans* (18,25). Specific TLRs that have been implicated in recognizing fungi are TLR1, TLR2, TLR4, TLR6, TLR7, and TLR9 (18,25). The specific role of TLRs is in fungal immunity is controversial. Mice infected with different isolates of *C. albicans* showed varying results of TLR participation (124). In addition, humans that lacked MyD88 but were immunocompetent did not show a predisposition to fungal diseases (13).

CLRs and fungal immunity

CLRs are the family of patter recognition receptors that are critical for fungal immunity. CLRs are characterized by having a C-type lectin domain. They exist as

soluble and transmembrane proteins and have been shown to bind to most disease-causing fungi (204). CLRs bind specifically to fungal cell wall carbohydrates (123). The three major components of the fungal cell wall are mannan, β-glucan, and chitin (123). Dectin-2, mannose receptor, C-type lectin receptor DC-SIGN and Mincle bind structures found in the mannan layer, and Dectin-1 binds β-glucan (55). After binding to fungal structures, these receptors are able to trigger a number of responses such as phagocytosis, and the induction of anti-fungal mediators, which will be discussed in further detail in the next section.

Signal transduction by CLRs

When activated, Dectin-1, Dectin-2, and Mincle induce intracellular signaling through tyrosine-based activation motifs that are able to recruit and activate Syk kinase. Syk kinase is able to activate proteins including MAP kinase, NFAT, and NF-κB (69,73,107,161,173). Dectin-1 and DC-SIGN can also activate NF-κB through the Raf1 pathway (71,72). Mice lacking CLRs or the downstream signaling components have defective immunity against fungal pathogens (45,73,148,159,160,173,176,200). Human polymorphisms in CLRs have been shown to increase susceptibility to fungal infections (58). Each CLR can recognize a variety of fungal pathogens, but it is suspected that there is some overlap. On the other hand, there is evidence of specificity; Dectin-1 can only recognize β-glucans in the yeast form of *Candida* because in the other forms (pseudohyphae and hyphae) mannan blocks exposure (63).

CLRs and the adaptive response

Macrophages and neutrophils have been identified as critical cell types in antifungal immunity (24,25). Loss of macrophages or neutrophils or impairment has been shown to increase susceptibility to fungal infections (25). Activation of CLRs moderate effector functions and trigger the inflammatory response (55). Dectin-1 activation has been show to produce a respiratory burst, inflammatory mediators, cytokines (TNF, IL-1β, IL-6, and IL-23), and chemokines (CCL2, CXCL1, and CCL3) (55,175,181). When mice deficient in Dectin-1 were infected with *C. albicans* and *A. fumigatus*, they were unable to control fungal growth and also showed impairment in neutrophil and monocyte recruitment and production of IL-6 (176,191).

CLR signaling can induce TH1 and TH17 responses, and it has been shown that the TH17 responses play a critical role in the antifungal immune response (3). TH1 responses are able to control systemic fungal infections by activating phagocytes through IFN-γ. TH17 responses are essential for the mucosal immune response against fungi (41). A number of defects in TH17 responses have led to increased susceptibility to fungal infections including mutations in IL-17, IL-17RA, STAT1, and STAT3 (112,140,183). Although the exact mechanism of how TH17 responses direct immunity at the mucosa is still undetermined, it is thought that IL-17 mediated neutrophil recruitment and IL-22-mediated production of antimicrobial peptides in the epithelial cells play important roles (41).

CLR signaling can also stimulate adaptive immunity against fungal pathogens. After CLRs recognize fungi, the pathogen is phagocytosed and killed by phagocytes triggering TH1 and TH17 responses. CLR-mediated signaling induces IL-12 production

by antigen presenting cells, which in turn can drive IFN-γ production by iNKT cells and TH1 cells activating more phagocytes (36,72). Through CLR-mediated signaling, antigen presenting cells can also produce the cytokines IL-1β, IL-6, and IL-23 creating an environment encouraging TH17 differentiation (107). The differentiated TH17 cells are able to produce IL-22, which drives AMP production of epithelial cells and IL-17, which recruits neutrophils (41).

Antimicrobial peptides

Antimicrobial peptides (AMPs) are an evolutionarily conserved innate immune defense. AMPs are a diverse and abundant set of molecules that are found in a variety of tissues and cell types in bacteria, invertebrates, plants, and animals (203). AMPs have been shown to have activity against a range of pathogens including bacteria, fungi, viruses, and protozoa (203). AMPs can be grouped based on sequence and structure into anionic peptides, linear cationic α-helical peptides, cationic peptides enriched for specific amino acids, and anionic and cationic peptides that contain cysteine and form disulfide bonds (23).

Anionic peptides

Anionic peptides are small negatively-charged peptides rich in glutamic and aspartic acids and range from about 721.6-823.8 Da. This group of AMPs is commonly found in bronchoalveolar fluid and airway epithelial cells (21,22). Specific members of this group of AMPs are dermicidin from humans and maximin H5 from amphibians

(101,162). This group of AMPs require zinc as a cofactor for antimicrobial activity and have activity against Gram-positive and Gram-negative bacteria (21,22).

Linear cationic α-helical peptides

This second group of AMPs contain less than 40 amino acids and lack cysteine residues (66,180). In the presence of aqueous solutions, these peptides appear disordered through their circular dichroism, but adopt an α-helical conformation in micelles and vesicles (66,94). Members of this group include mellitin from insects, magainin from amphibians and LL-37 from humans (23). When adopting the α-helical conformation, this group has activity against Gram-positive and Gram-negative bacteria (129).

Cationic peptides enriched for specific amino acids

AMPs abaecin and drosocin from honeybees and *Drosophila* respectively belong to this group for their high abundance of proline and arginine residues (15). This group also contains the bactenecin family of peptides found in cattle, sheep, and goats and PR-39 from pigs, which have high amounts of proline (33-49%) and arginine (13-33%) residues (66,127). Humans express small histidine-rich salivary polypeptides (96). These peptides lack cysteine residues.

Anionic and cationic peptides that contain cysteine and form disulfide bonds

The key characteristics of this group are the presence of cysteine residues, the formation of disulfide bonds, and stable β-sheets. The brevinin and ranatuerin AMP families from ranid frogs belong to this particular group of AMPs because they each

contain one disulfide bond (7). The α- and β-defensins found in humans belong to this group. They contain three disulfide bonds and are conserved in rats, rabbits, mice, cattle, pigs, goats, and poultry (106) Of particular note are the AMPs in this group that have antifungal properties such as drosomycin from *Drosophila* and plant antifungal defensins in addition to the brevinin and ranatuerin families (7,57).

AMP attachment

Many AMPs are attracted and bind to the bacterial membrane due to the anionic and cationic binding properties of the peptides and the cell surface molecules. Many of the molecules on Gram-negative bacteria, such as lipopolysaccharide, and Gram-positive bacteria, such teichoic acids are negatively charged. In vitro studies have determined that α-helical peptides and β-sheet peptides embed into the lipid head group which stretches the membrane (33). For other peptides interacting with membranes, such as magainin-2, the stress of the membrane is minimal (116).

AMPs and membrane insertion

Membrane insertion is governed by the peptide to lipid ratio. At low peptide to lipid ratios, peptides are oriented parallel to the membrane; however, as more peptides bind and the peptide lipid ratio increases the peptides reorganize into a perpendicular orientation eventually inserting into the membrane (201). After a membrane insertion, a number of models have been proposed to explain membrane permeabilization.

In the "barrel-stave model," helical peptides arrange themselves in a bundle forming a pore (201). The hydrophobic residues of the AMPs bind to the lipid core and

the hydrophilic residues are on the inside of the pore. The amount of peptides needed for pore formation ranges from 3-11 peptides with diameters ranging from 1.8 nm-4.0 nm (81,170). This model so far has only been supported by the AMP alamethicin.

The next model is called the "carpet model" because peptides continue to bind to the anionic phospholipid head group in a parallel fashion (138). After a high number of peptides are bound to membrane, the peptides disrupt the bilayer and form micelles similar to the action of a detergent (100,165). As a result, transient holes form, increasing exposure of the bilayer to AMPs and the eventual disintegration of the bilayer. This model is supported for the mechanism of the peptide dermaseptin (138).

In the last model, called the "toroidal-pore model," helices aggregate and cause the lipid monolayers to bend in on themselves forming a pore (117). This bending causes the two leaflets to become one because the peptides still only associate with the head groups of the bilayer (117). This particular model differs from the barrel-stave model because the peptides never dissociate from the polar head groups (201). The hydrophobic regions are bound to the membrane while the hydrophilic regions form the inner part of the pore. Magainin-2 appears to function by this mechanism of action (117).

Amphibian AMPs

One of the important dermal glands in amphibian skin is the granular gland (Figure 1-2) (19). The primary role of the granular gland is to synthesize defensive peptides including AMPs and then package and store them into secretory granules (53). The granular gland is surrounded by myoepithelial cells that contract following a signal from the α-adrenergic receptor. This action pushes the granules through the duct lumen

and onto the surface of the skin. After secretion, these AMPs that are present in the mucus layer are thought to provide a protective barrier against potential pathogens. We and others have hypothesized that antimicrobial skin peptides may play a role in protection of amphibians from skin pathogens. The focus of my research was the investigation of the role of antimicrobial peptides (AMPs) produced in granular glands of the skin in defense against chytridiomycosis.

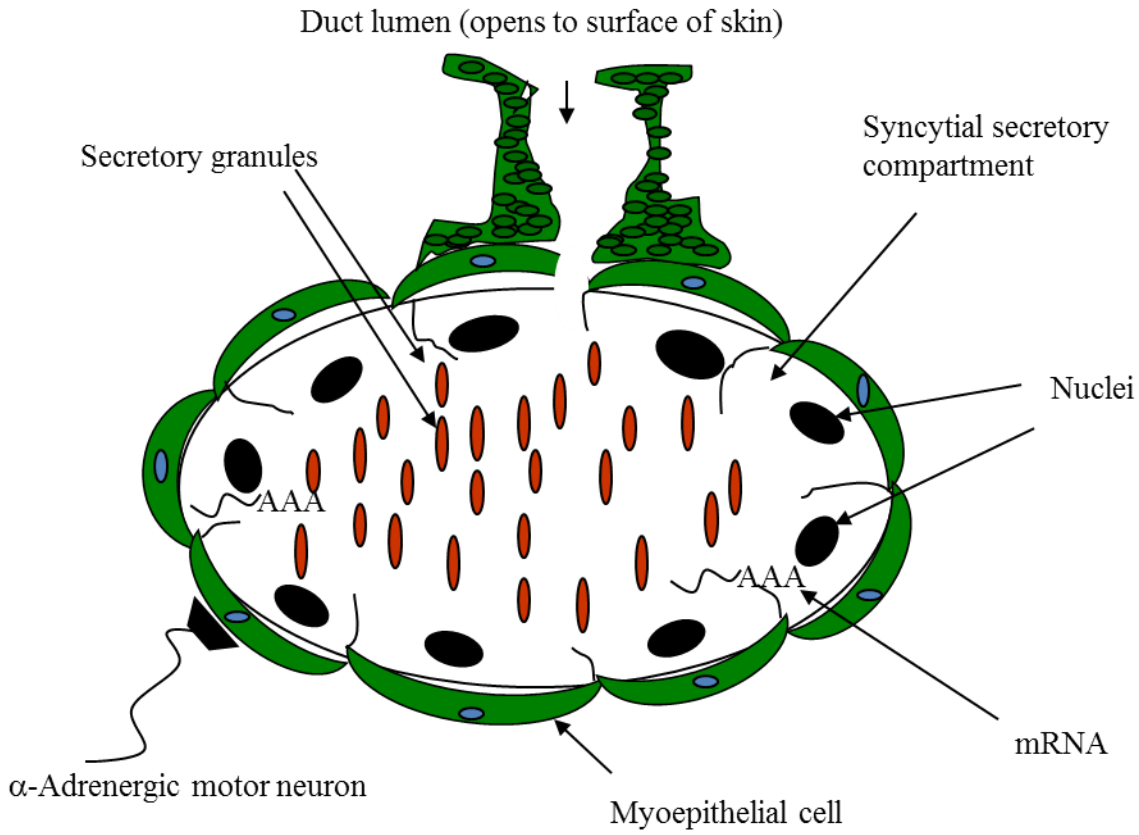

Figure 1-2. The amphibian granular gland. Amphibian granular glands synthesize defensive peptides including AMPs and secrete them onto the surface of the skin (115). This image was modified from Dockray and Hopkins (53).

Research Goals

Because *B. dendrobatidis* colonizes the skin, AMPs that are stored in the granular glands of the skin may play a role in *B. dendrobatidis* resistance. The goal of this research was to investigate the role of AMPs in the skin secretions of northern leopard frogs (*Rana pipiens*) in protection against the fungal pathogen *B. dendrobatidis*. The goals of my research are listed below.

1) To determine if AMPs were detectable on the skin of resting and active frogs. It was previously thought that proteases from the host or resident skin microbiota would rapidly digest AMPs before they could act effectively. Using MALDI-TOF MS and growth inhibition assays, I evaluated the presence and activity of AMPs from resting and active frogs. The pattern of skin peptides and effectiveness in resting and active was compared to frogs injected with norepinephrine to induce peptide release.

2) To evaluate the rate of loss of AMPs on the skin following secretion. *R. pipiens* were injected with norepinephrine to induce AMP secretion. Secretions remained on the skin for 0, 15, 30, 60, and 120 minutes before being washed off the animal. Expression of AMPs over time was examined using MALDI-TOF, and activity was examined using growth inhibition assays. MALDI-TOF MS profiles was examined for peptide cleavage products. In addition the relative concentration of individual AMPs was quantified over time by "spiking" samples with a known amount of an alkylated peptide standard.

3) To determine the role of AMPs in protecting *R. pipiens* from *B. dendrobatidis* infection. For these experiments, frogs were injected with increasing concentrations of norepinephrine to determine the necessary dose for AMP depletion. Next, *B. dendrobatidis* naïve juvenile *R. pipiens* were depleted of AMPs and experimentally

infected with *B. dendrobatidis*. Frogs were monitored for zoospore load and mortality. Controls included uninfected animals and peptide-intact animals.

4) To develop a better way for monitoring *B. dendrobatidis* infection status. The typical method for quantifying pathogen load on infected amphibians is to swab animals on the most infected areas including the ventral abdomen, legs and footpads. Although this method is optimal for the field, the main drawback is that it does not take into account the pathogen load on the whole animal. A new method was developed to take in to consideration the entire skin surface of the frog. In this method a frog was placed in a finite volume of sterile water. After a short time, the frog was removed, and the water was filtered. The filter was treated to extract DNA and qPCR was performed. This method was tested side-by-side with accepted methods of measuring infection status.

CHAPTER II

THE EBB AND FLOW OF ANTIMICROBIAL PEPTIDES ON THE SKIN OF *RANA PIPIENS*

Abstract

Many amphibian species are threatened with extinction by the emerging infectious disease, chytridiomycosis, caused by the fungus *Batrachochytrium dendrobatidis*. This unprecedented global crisis threatens to reduce the biodiversity of the entire amphibian class. The fungus invades the skin and impairs the uptake and retention of essential ions leading to cardiac arrest. Antimicrobial peptides (AMPs) secreted into the mucus of some amphibians are thought to be an important defense against chytridiomycosis. However, little is known about the quantities of AMPs secreted under natural conditions, whether they are sufficient to protect against this pathogen, and how they interact with commensal microbes. To understand how defensive peptides and skin microbes may interact, it is essential to know the precise quantities of AMPs present under natural conditions. Using matrix-assisted laser desorption time-of-flight mass spectrometry and growth inhibition assays, we show that northern leopard frogs (*Rana pipiens*) at rest constitutively release low amounts of AMPs that inhibit *B. dendrobatidis in vitro*, and AMP defenses are elevated following a simulated predator attack. Using a synthetic peptide analogue of brevinin-1Pb as an external control, we quantified the amounts of four previously described AMPs (brevinin 1Pa, brevinin-1Pb, brevinin-1Pd, and ranatuerin-2P) at several time points after secretion.

Once secreted onto the skin, the peptides are most active for 15 min, and small quantities persist for at least 2 h. Taken together, our data suggest that small amounts of AMPs are rapidly available and quite stable on the skin of *R. pipiens*. They are effective inhibitors of *B. dendrobatidis* at these low constitutive concentrations but degrade within 2 h, protecting the integrity of the skin and commensal bacteria.

Introduction

Antimicrobial peptides (AMPs) are produced by a diverse array of organisms including plants, insects, and vertebrates (203). Their presence in so many species suggests that they play a fundamental role in the innate defense of each species against microbial pathogens. However, whether they provide a 'constitutive' defense critical for survival or an 'induced' defense is somewhat controversial (40,75). The skin of many amphibians is a rich source of AMPs (40,157,203). If AMPs are part of the constitutive barrier defense against pathogenic organisms in amphibians, it is expected that the concentrations secreted would be sufficient to inhibit pathogens, but beneficial symbiotic microbes would be tolerant of the same concentrations of the peptides. The emerging infectious disease, chytridiomycosis, caused by *Batrachochytrium dendrobatidis*, continues to cause massive amphibian population declines and extinctions in many parts of the world (10,17,108,114,134,142); reviewed in (30,98,168). This loss of amphibian species will reduce the biodiversity of many ecosystems in which amphibians play an important role (168,174). While it is well known that amphibian populations vary in their susceptibility to chytridiomycosis (10,110,146,196–198), the reasons for this variability are not well understood. We hypothesize that AMPs synthesized and stored in the

granular glands of the skin of many species are an important innate defense against *B. dendrobatidis*. The pathogen is spread by water-borne flagellated zoospores that settle on the frog, enter the cells of the stratum granulosum of adult skin, and replicate in the protected environment of the host cells. The zoospores mature into zoosporangia that release new zoospores able to re-infect the same individual at a different site or a new host (9,10,114,134,185). Thus, initial infection and re-infection would require that zoospores overcome chemical defenses, including AMPs, in the mucus. Some amphibian species are resistant to severe *B. dendrobatidis* infections, but other, more susceptible species, succumb to lethal chytridiomycosis (10,108,196–198). Present evidence suggests that death due to severe chytridiomycosis results from disruption of the skin and failure to maintain a balance of essential ions (184,187). Previous studies have shown a good correlation between the in vitro effectiveness of skin peptides against *B. dendrobatidis* and the survival of amphibian species in the wild or when experimentally infected (157,196–198). Other studies have described a variety of skin bacteria isolated from *B. dendrobatidis*-resistant hosts that inhibit growth of the pathogen (195,199) and can protect young frogs from chytridiomycosis when the skin is bioaugmented with protective bacteria (78). Thus, many species of beneficial bacteria persist in the mucus where AMPs would be secreted. Although AMPs have been isolated from skin secretions of many amphibian species, there is little information about the availability of these peptides on the skin of undisturbed resting frogs or naturally active frogs. The central questions addressed by this study are whether a suite of natural AMPs can be detected in resting and active frogs and are they inhibitory to *B. dendrobatidis* at these

constitutive concentrations. Once secreted onto the skin, how stable are the peptides and how long are they effective?

Materials and Methods

Frogs

Rana pipiens measuring between 2 and 2.5 inches were purchased from Connecticut Valley Biological (Southampton, MA, USA). Frogs were housed in groups of 5 or 6 in covered polystyrene containers in dechlorinated tap water at a temperature of 20–24 °C. Containers were placed at an incline, allowing the frogs to choose a wet or dry area. They were fed live crickets, and their water was changed three times weekly. All protocols were approved by the Vanderbilt University Medical Center Institutional Animal Care and Use Committee.

Peptide collection and quantification

To collect AMPs from the skin, frogs were injected in the dorsal lymph sac with amphibian phosphate buffered saline (APBS; 6.6 g NaCl, 1.15 g Na_2HPO_4, and 0.2 g KH_2PO_4 per 1 liter distilled water) or norepinephrine-HCl (Sigma, St. Louis, MO, USA) dissolved in APBS and were placed in collection buffer for 15 min (Figure 2-1). After the frog was removed, the buffer was acidified, and the peptides were enriched by passage over C18-Sep-Paks (Waters Corporation, Milford, MA, USA). The eluted peptides were quantified using the microBCA assay, concentrated by centrifugation under vacuum, and resuspended in HPLC water at a known concentration.

To collect skin secretions from resting frogs, they were placed directly into collection buffer without norepinephrine injection. Frogs that were given a simulated 'alarm' stress were removed from their holding tank, rinsed with clean water, placed in the dry container, and chased in a large polypropylene box for 5 min. The investigator reached in with a gloved hand and forced each frog to move around the box for 5 min, and then the frog was placed in collection buffer for an additional 10 min while the peptides accumulated. Peptides were concentrated and quantified as described above.

The weight of each frog was determined at the time of peptide collection, and total peptides were quantified. To estimate the amount of peptides in mucus, the surface area of the skin was determined according to the method of McClanahan and Baldwin [Surface area = 9.9 (weight in grams)$^{0.56}$] (119). We assumed the thickness of the mucus to be 50 µm (26), and therefore the volume of mucus covering one cm^2 of skin would be 5 µl. One ml (1000 µl) = 5 µl X 200. Thus, total peptides (µg) per cm^2 X 200 = total µg ml^{-1} in mucus.

To determine the stability of AMPs on the skin, frogs were induced to secrete peptides by injection of APBS or norepinephrine. The secretions remained on the skin of norepinephrine-injected frogs for 0, 15, 30, 60, or 120 min before they were washed off by immersion in collection buffer, enriched over C-18 Sep-Paks, and quantified. For the 0-time point, frogs were injected with norepinephrine and immersed in collection buffer within 10 s of injection.

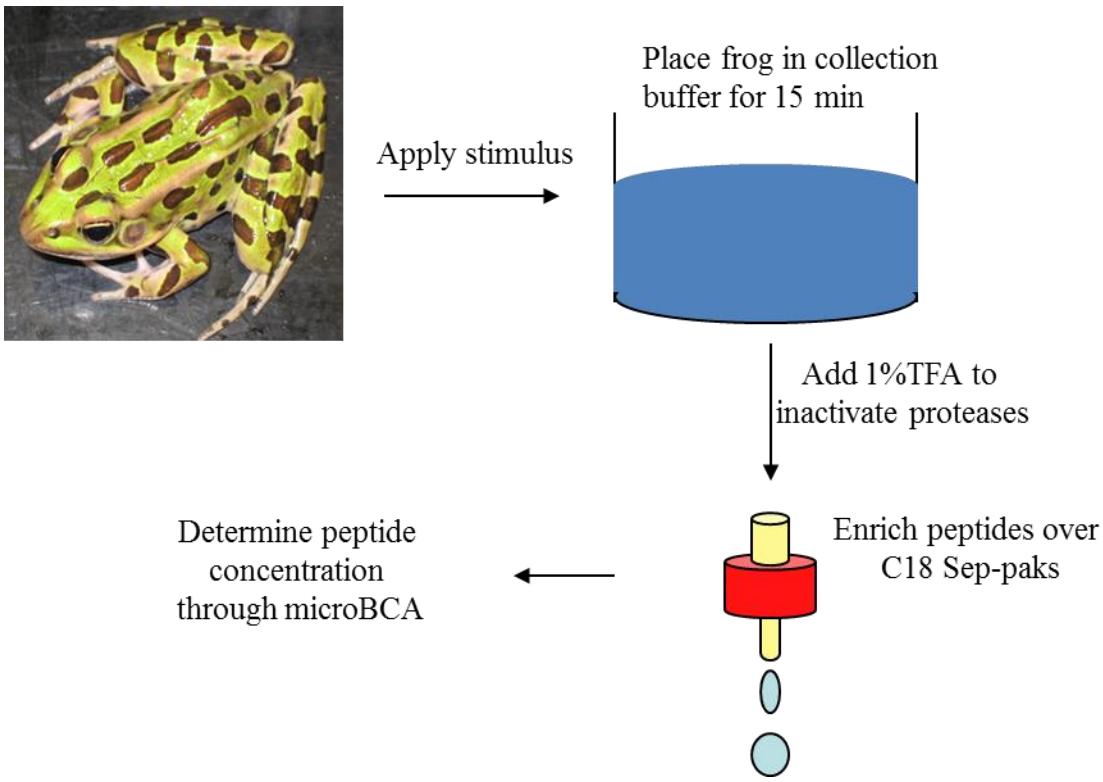

Figure 2-1. Peptide collection protocol. The stimulus applied to the frog is either, chasing with a gloved hand or injecting with norepinephrine or APBS.

Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (MS)

Following partial purification, the recovered peptides were concentrated to dryness by centrifugation under vacuum at 70 °C, resuspended in HPLC-grade water at 1 mg ml^{-1}, mixed with α-Cyano-4-hydroxycinnamic acid matrix (CHCA) (LaserBio Labs, Sophia-Antipolis, France) and spotted onto the target plate. MALDI-TOF MS was performed using a Bruker Daltonics 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 enkephalin 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 500 to 5,000 *m/z* ratio range. Automated data acquisition was performed by averaging 250 laser shots.

For direct MALDI-TOF analysis, carbon embedded film (Goodfellow Cambridge Ltd., Cambridge, England) was cut into 1 cm squares and activated by dipping in 100% methanol (Figure 2-2) (199). After air drying, the film was applied to the frog for 10 s on each side of the frog. Film was attached to a sample plate with electrically conductive adhesive transfer tape (3M Co., St. Paul, MN, USA), spotted with CHCA, and mass signals collected as described above.

To determine the relative intensity of each peptide in a sample at a given time point, we added a synthetic external standard peptide, cys-iodoacetamide-modified brevinin-1Pb (1.25 nM), in which the two cysteine residues were alkylated. The sequence and mass to charge ratio of the modified peptide is

FLPIIAGIAAKVFPKIFCAISKKC, *m/z* 2693.30. The mass of modified brevinin-1Pb is shifted by 117.9 mass units in the MALDI-TOF profiles. For each peptide the relative intensity in arbitrary units was compared to the relative intensity of alkylated brevinin-1Pb in the same sample, and the ratios for all of the samples were averaged.

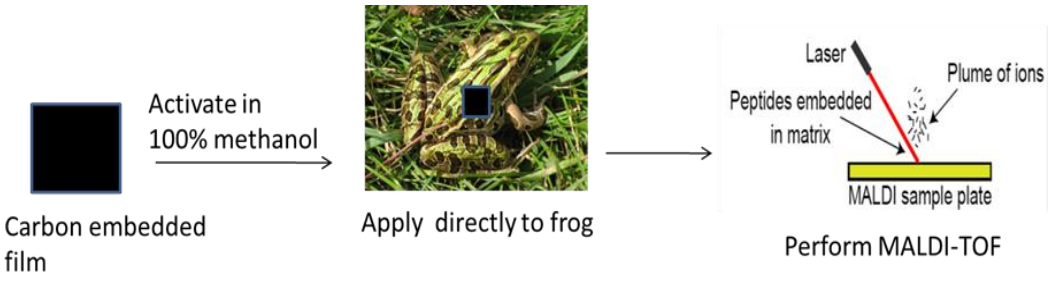

Figure 2-2. Direct MALDI-TOF protocol. Using this method, skin peptides can be sampled directly and detected without purification.

Growth inhibition assays

Briefly, freshly isolated *B. dendrobatidis* zoospores (isolate JEL 197) were plated (5×10^4/50 µl) in 1% tryptone broth with 50 µl of a serially diluted mixture of skin peptides. Positive control wells (no peptide addition) contained zoospores and 50 µl of HPLC water. Negative control wells contained 50 µl of heat-killed zoospores (60 °C, 10 min) and 50 µl of HPLC water. Plates were incubated at 23 °C for 1 week and growth was measured as an increase in optical density at 490 nm (OD_{490}). For most peptide inhibition assays, we determined a minimal inhibitory concentration (MIC) defined as the lowest concentration of peptides at which no growth could be observed. Relative peptide effectiveness is percent inhibition of each sample at a concentration of 12.5 µg ml^{-1} multiplied by the total peptides recovered (µg ml^{-1} of mucus).

Statistical comparisons

All parameters compared in this study (*B. dendrobatidis* growth as OD_{490}, peptide concentrations as µg ml^{-1} of mucus, relative intensity of peptide signals in comparison with alkylated brevinin-1Pb, and relative peptide effectiveness) were averaged and the mean values ± standard errors were compared by a one-tailed or two-tailed Student's *t*-test or one-way ANOVA with planned comparisons and Tukey *post hoc* tests as indicated in figure legends. Peptide concentrations, OD values, and relative peptide effectiveness values were log transformed as indicated in figure legends to meet the assumptions of normal distribution and homogeneity of variances for parametric statistics. A *P*-value of ≤ 0.05 was considered to be statistically significant. Error bars shown in all figures represent standard errors. If no error bar is visible, the standard error was less than the

diameter of the symbol. In *B. dendrobatidis* growth inhibition assays, each data point represents the mean ± standard error of five replicate wells. For other parameters, the number of animals or samples is shown in the figure legends.

Results

Expression of AMPs in resting, chased, and norepinephrine induced frogs

The MALDI-TOF MS profile of skin peptides in resting and chased frogs determined by direct sampling with carbon-embedded film in comparison with norepinephrine-injected frogs shows that both resting and active (chased) frogs express detectable amounts of several AMPs (brevinin-1Pa, brevinin-1Pb, brevinin-1Pd, and ranatuerin-2P) characteristic of frogs from the northeastern USA (178) (Fig. 2-3A-C). Comparison of the amount of total hydrophobic (C-18 enriched) skin peptides secreted by resting versus chased frogs shows that the amounts recovered from the chased frogs (6,411.2 ± 1,672.3 μg ml^{-1} of mucus) was significantly greater than that of the resting frogs (1,795.7 ± 266.8 μg ml^{-1} of mucus) (Fig. 2-3D). The mixture of recovered skin peptides from both the resting frogs (Fig. 2-3E) and chased frogs (Fig. 2-1F) showed significant antimicrobial activity against *B. dendrobatidis* zoospores, but it was less than that observed when peptides were induced by norepinephrine (20 nmol/g) (Fig. 2-3G). The MICs for peptide samples from resting frogs ranged from 100 to >200 μg ml^{-1} (N = 4); whereas those of peptides from chased frogs were 100–200 μg ml^{-1} (N = 4), and those of peptides from norepinephrine-induced frogs were usually 100 μg ml^{-1} or less (range 25–250 μg ml^{-1}, N = 9). This suggests that both resting and chased frogs have significant

amounts of AMPs capable of inhibiting skin pathogens (178), but they express less than norepinephrine-injected frogs.

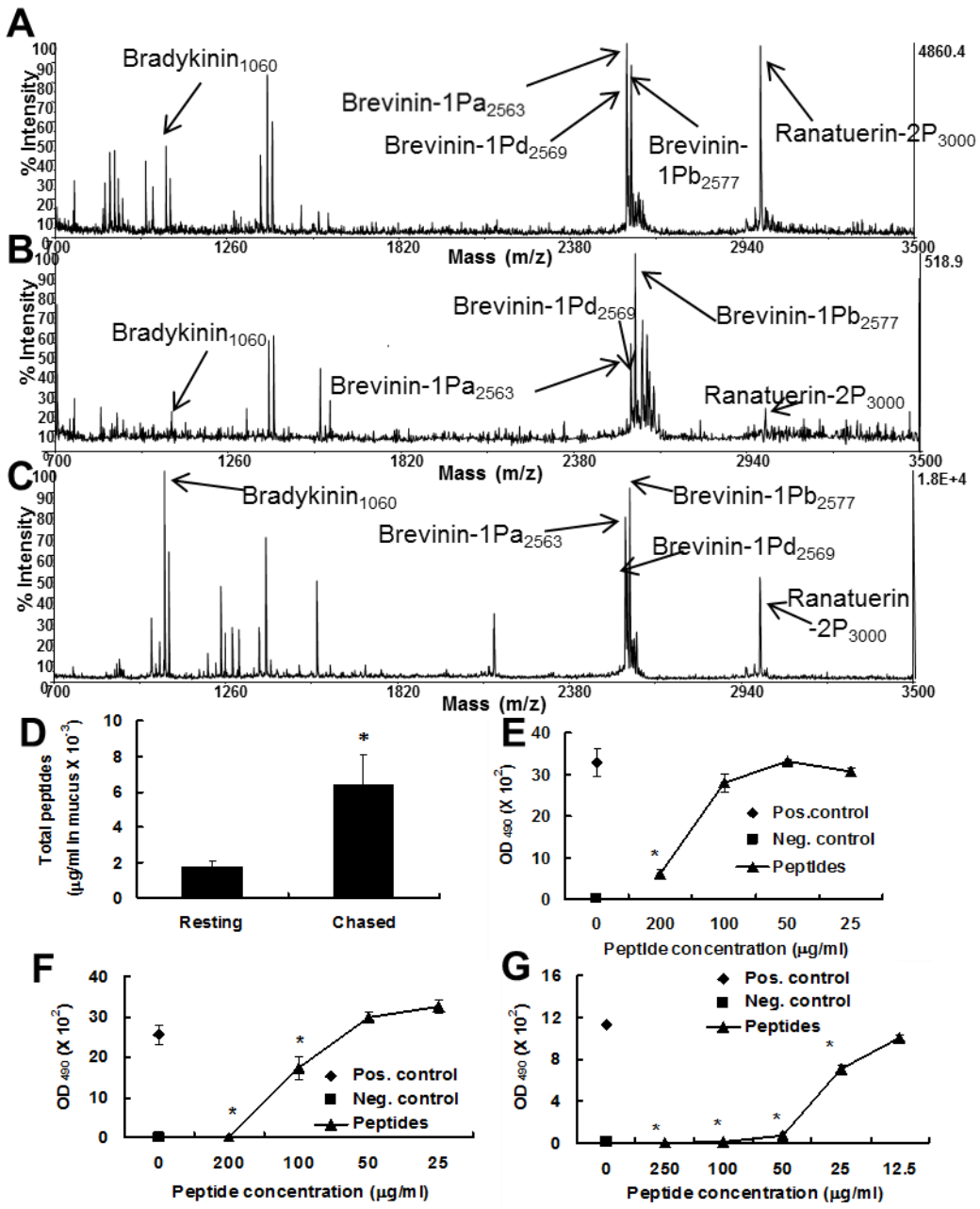

Figure 2-3. MALDI-TOF profiles and *B. dendrobatidis* growth inhibition by skin peptides from resting, chased, and norepinephrine-stimulated frogs. Representative skin peptides of (A) resting, (B) chased, and (C) norepinephrine-induced frogs. The *m/z* for each antimicrobial peptides (AMP) is shown. (D) Total hydrophobic peptides recovered from resting (N = 5) and chased (N = 5) frogs. *Significantly greater than those of resting frog by two-tailed Student's *t*-test after log transformation, $P \leq 0.05$. Growth inhibition of *B. dendrobatidis* zoospores by skin secretions from (E) resting, (F) chased, and (G) norepinephrine-induced frogs (20 nmol/g). *Growth was significantly less than that of positive control wells (N = 5 replicates) by two-tailed Student's *t*-test after log transformation, $P \leq 0.05$.

Stability of AMPs secreted onto the skin

Frogs were induced to secrete their peptides by injection of 20 nmol g^{-1} norepinephrine, and the peptides were allowed to remain on the skin for various periods of time until the frogs were immersed in buffer to collect the peptides. Total peptides recovered from secretions that had been left on the skin for 0 min (less than 10 s between injection of norepinephrine and immersion in buffer) or 15 min were not significantly different from each other, and the amounts were maximal at these time points (67,928 ± 12,773 μg ml^{-1} of mucus at 0 min and 86,401 ± 11,957 μg ml^{-1} of mucus at 15 min). After 30 min on the skin, the total amount of peptides washed from the skin had decreased significantly from the amount recovered after 15 min on the skin, and the amounts of peptides recovered at 60 and 120 min after secretion were very low (11,336 ± 2,037 μg ml^{-1} of mucus at 60 min and 5,265 ± 1,474 μg ml^{-1} of mucus at 120 min). Control frogs injected with APBS consistently secreted a very low concentration of peptides (1506 ± 146 μg ml^{-1} of mucus) (Fig. 2-4).

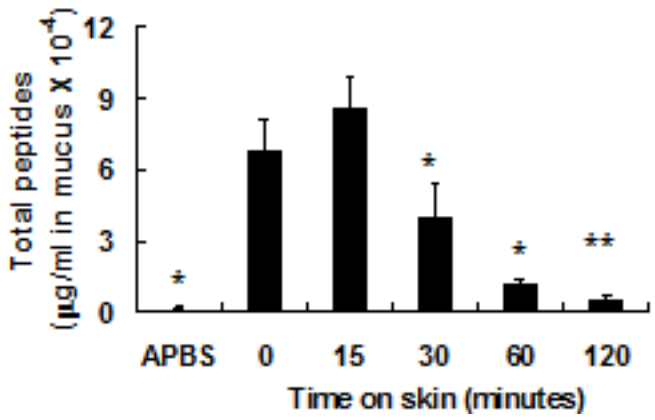

Figure 2-4. Total peptide stability on the skin. (A) Peptides recovered from frogs injected with amphibian phosphate buffered saline (APBS) (*N*=8) or 20 nmol/g norepinephrine (*N* = 28). Secretions remained on the skin of norepinephrine-injected frogs for 0, 15, 30, 60, and 120 min before collection (*N* = 5 or 6 samples at each time point).*Significantly less peptides in comparison with the amounts recovered at 15 min, $P < 0.01$;**Significantly less peptides in comparison with the amounts recovered at 0, 15, or 30 min, $P \leq 0.01$ by one-way ANOVA after log transformation and Tukey *post hoc* test.

By MALDI-TOF MS, we examined the relative intensity of four AMPs in comparison with an external standard (a synthetic alkylated-brevinin-1Pb). Shown in Figure 2-5, is the mechanism to alkylate the cysteine residues creating a mass shift to distinguish it from the native brevinin-1Pb. The alkylated form was assessed for purity by diluting to a concentration of 100 nM and performing MALDI-TOF MS. Next, the external standard was added to every peptide samples from the different time points. Shown in Figure 2-6A-B are representative plots from the 0 and 60 min time point. The peak intensity of the external standard increases over time.

The pattern of relative intensities for individual brevinin-1 peptides (brevinin-1Pa, brevinin-1Pb, and brevinin-1Pd) and ranatuerin-2P in comparison with the external standard showed that these peptide signals were most stable at 15 min on the skin. Although reduced in intensity, they remained quite stable for up to 60 min on the skin but were significantly reduced by 120 min on the skin (Figs. 2-7A-D).

Enriched peptide samples from the above time points were incubated with *B. dendrobatidis* zoospores to assess their activity in growth inhibition assays. Peptides from the 0- and 15-min time points were strongly inhibitory. The MICs for the 0-time point samples ranged from 25 to 250 μg ml^{-1} (average 69.4 μg ml^{-1}). One representative sample of peptides from the 15-min time point is plotted in Fig 2-8A. Peptides from the 30-min time point were less effective with an MIC of 50 μg ml^{-1} (one representative sample of peptides plotted in Fig 2-8A). The peptides recovered at later time points did show some inhibition but failed to achieve an MIC at concentrations up to 500 μg ml^{-1}. One representative of a sample of peptides collected at 60 min is plotted in Fig. 2-8A.

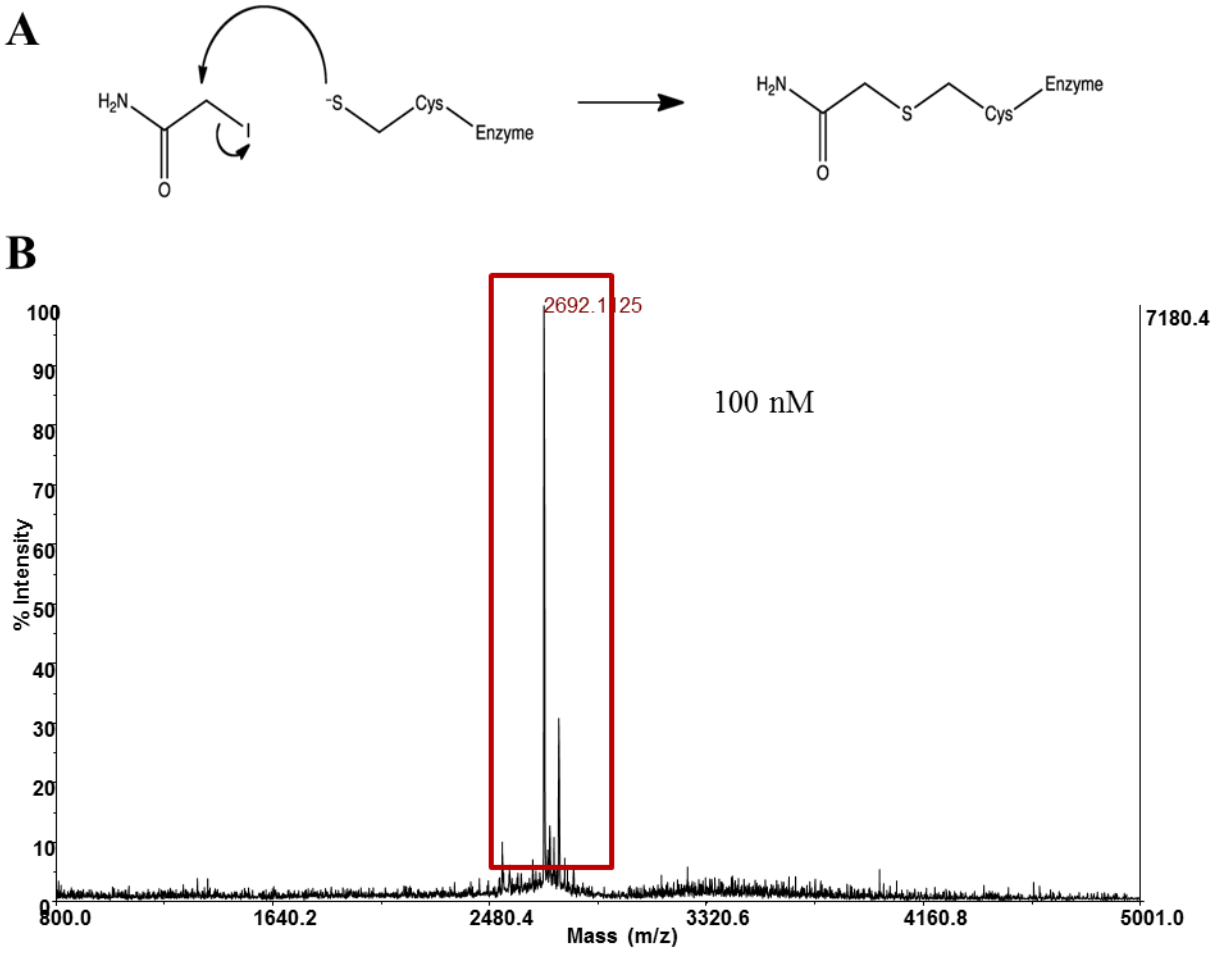

Figure 2-5. Mechanism of irreversibly alkylating cysteine residues and external standard purity. (A) By treating with iodoethamide, cysteines become alkylated creating a mass shift and peak that is not normally present in *R. pipiens* skin peptide samples. The alkylated form of brevinin-1Pb has a mass of 2693 *m/z*. (B) The alkylated form of brevinin-1Pb was assessed for purity by MALDI-TOF MS.

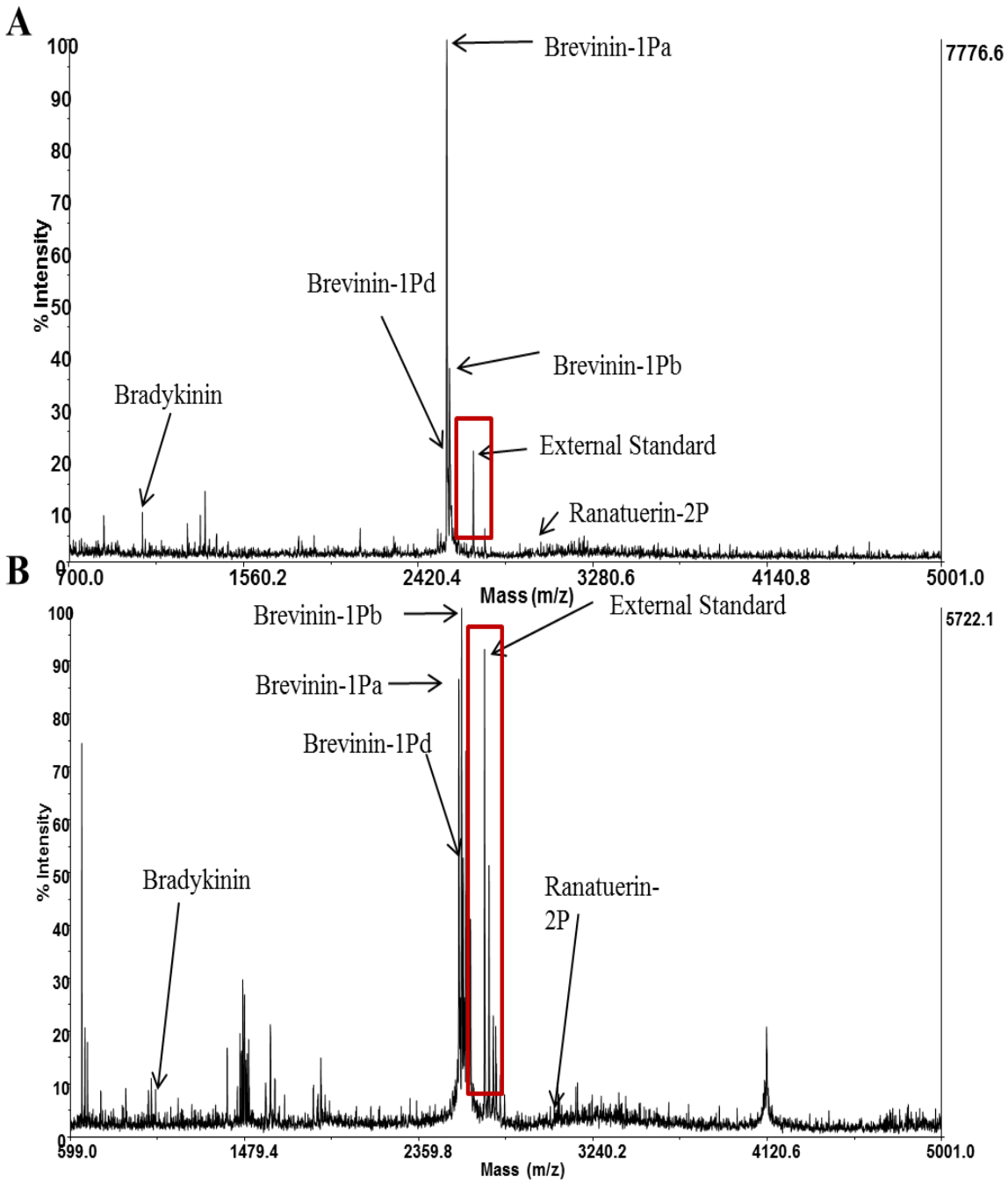

Figure 2-6. Representative MALDI-TOF MS profiles with the external standard at the 0 (A) and 60 (B) minute time points. The peak of the external standard is boxed.

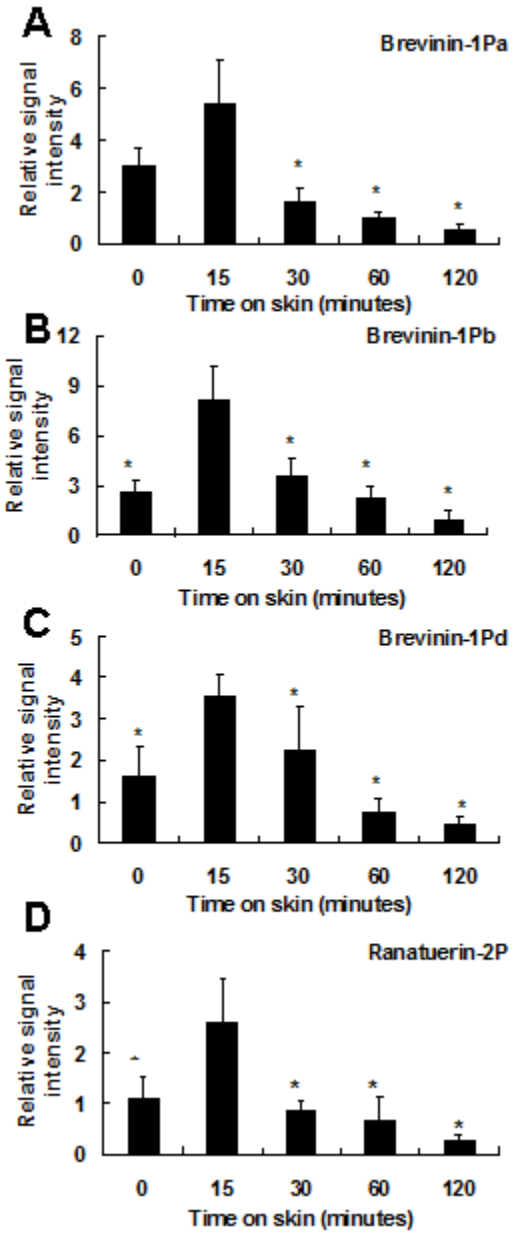

Figure 2-7. Individual peptide stability. (A-D) Relative peptide signal intensity (arbitrary units) of (A) brevinin-1Pa, (B) brevinin-1Pb, (C) brevinin-1Pd, and (D) ranatuerin-2P in comparison with the external standard (1.25 nM alkylated-brevinin-1Pb). *Significantly less than the signal intensity at 15 min by one-tailed Student's *t*-test after log transformation, $P \leq 0.05$.

Peptide effectiveness was calculated by multiplying the percent inhibition of each individual sample at 12.5 µg/ml by the total amount of peptides recovered at each time point. There was no significant difference in peptide effectiveness between the 0- and 15-min time point, but the peptide effectiveness significantly decreased at the 30, 60, and 120 min time points (Fig. 2-8B). The peptide effectiveness of mixtures of peptides from resting and chased frogs is not shown here because there was no inhibition at 12.5 µg ml^{-1}.

Detection of peptide fragmentation ions over time

To further understand the possible degradation of AMPs on the skin after secretion, we studied the MALDI-TOF peptide profiles of representative samples which remained on the skin for 60 or 120 min. Not only did the signals for the intact peptides decrease in intensity, but a number of smaller peptide fragments that would be the expected products of proteolytic cleavage were detected at 60 and 120 min. For example, at 60 and 120 min after secretion onto the skin, all of the brevinin-1P signals and the mass signal for ranatuerin-2P were reduced and peptide fragments of mass 752, 1139, 1456, 1610, and 1972 could be detected (Table 2-1). These fragments were absent from the time 0 samples. The smaller peptide fragments would occur if a protease that cleaves at a position preceding lysine residues was released at the time of granular gland discharge. These fragments can also be observed in the MALDI-TOF spectra of resting and chased frogs (Table 2-1).

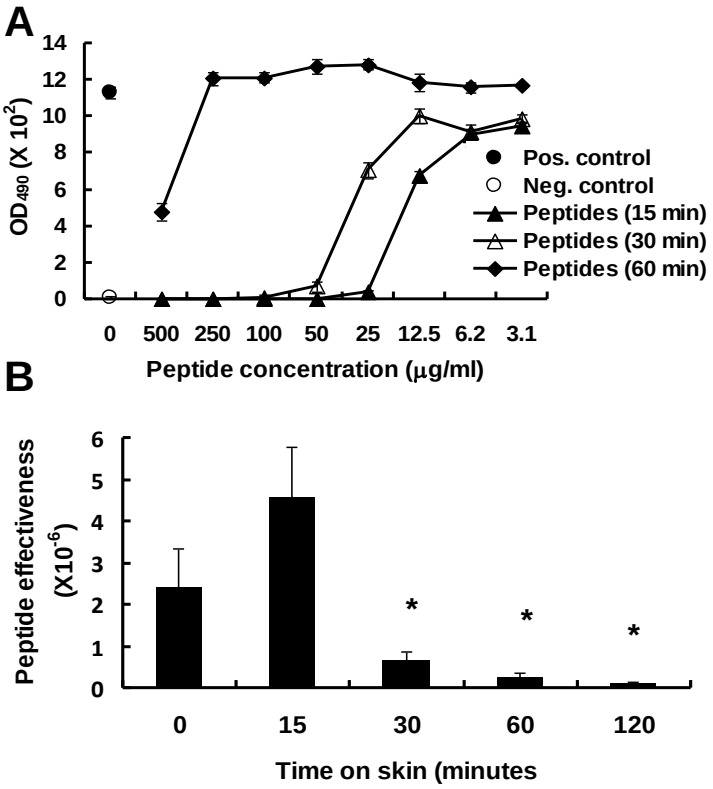

Figure 2-8. Inhibition of *B. dendrobatidis* growth by peptides recovered after various periods on the skin and peptide effectiveness. (A) Growth inhibition by a representative sample of peptides collected after 15, 30, and 60 min on the skin. The positive control was living zoospores in broth and the negative control was heat-killed zoospores in broth. (B) Average peptide effectiveness of all samples (N = 5 or 6 at each time point except at 120 min N = 3 due to insufficient quantities to test in the remaining samples). Peptide effectiveness is percent inhibition at a concentration of 12.5 μg ml^{-1} multiplied by the total peptides recovered (μg ml^{-1} of mucus).*Significantly less effective than peptides on the skin for 15 min by two-tailed Student's t-test, $P \leq 0.005$ after log transformation.

Table 2-1. Antimicrobial peptides and fragment ions detected by matrix-assisted laser desorption time-of-flight mass spectrometry.

Peptide	Sequence	Monoisotopic mass (*m/z*)	Observed mass in resting or chased	Observed mass at 60 or 120 min
Bradykinin	RPPGFSPFR	1060	X	X
Brevinin-1Pa	FLPIIAGVAAKVFPKIFCAISKKC	2563	X	X
Brevinin-1Pb	FLPIIAGIAAKVFPKIFCAISKKC	2577	X	X
Brevinin-1Pd	FLIPIIASVAANVFSKIFCAISKKC	2569	X	X
Brevinin-1Pa or Brevinin-1Pb fragment	KVFPKIFCAISKKC	1610	X	X
Brevinin-1Pa, Brevinin-1Pb, or Brevinin-1Pd fragment	KIFCAISKKC	1139	X	X
Brevinin-1Pb fragment	FLPIIAGIAAKVFP	1456	X	X
Ranatuerin-2P	GLMDTVKNVAKNLAGHMLDKL KCKITGC	3000	X	X
Ranatuerin-2P fragment	KCKITGC	752	X	X
Ranatuerin-2P fragment	KNLAGHMLDKLKCKITGC	1972		X

Discussion

Expression of AMPs in resting, chased, and norepinephrine-injected frogs

Our studies of the northern leopard frog, *R. pipiens*, address the fundamental question of whether AMPs secreted onto the skin of amphibians are constitutively secreted at concentrations sufficient to inhibit skin pathogens. Using a very sensitive direct MALDI-TOF MS approach (32,199), as well as collection of skin washings, we show by MALDI-TOF MS analysis and growth inhibition assays that both resting frogs and chased frogs secrete detectable amounts of peptides that inhibit growth of *B. dendrobatidis* zoospores (Fig. 2-3). Previously, it was unclear if the peptide mixture from resting frogs would show evidence of individual AMPs. There was a concern that proteases on the skin would degrade AMPs too quickly for peptide activity to be detected on resting frogs (145,171). However, when peptide mixtures from resting and chased frogs were tested for activity against *B. dendrobatidis* zoospores (the infectious stage of the *B. dendrobatidis* life cycle), the peptide mixtures from both were inhibitory. The peptide mixtures from the chased frogs were somewhat more inhibitory than those from the resting frogs, but less effective than those from norepinephrine-induced frogs (Figs. 2-3E-G). The increased inhibition observed in samples from chased frogs may be due to a higher concentration of AMPs that have not been degraded by proteases. We assume that most peptides present on the skin of resting frogs were secreted sometime in the previous 24 h and would be degraded by putative endogenous proteases secreted from granular glands (146,172) or by possible proteases secreted by microbiota resident on the skin (199). In contrast, most of the peptides present on the skin after the frog was chased

would have been secreted within 15 min of their collection in buffer (5 min of the chase period and 10 min in the buffer). Thus, they would have had much less time for exposure to proteases.

Stability of AMPs secreted onto the skin

Although individual peptides from *R. pipiens* have been shown to have growth inhibitory activity against *B. dendrobatidis* (151,178), the stability of the AMPs once secreted onto the skin has not previously been determined. Our studies show that the greatest amount of recoverable peptides and the greatest inhibitory activity against *B. dendrobatidis* was observed in the first 15 min after secretion onto the skin (Figs 2-4 and 2-8). At later time points, the amount of peptides and antimicrobial activity declines until it is very low at 120 min after secretion onto the skin. Proteases secreted by the frog (145,171) and possible proteases secreted by skin bacteria may degrade the peptides. Gradual loss of secreted AMPs may be necessary to protect beneficial symbiotic microbes (195,199).

To determine if and when individual AMPs are being degraded, MALDI-TOF MS was performed on the secretions at different times after collection. At the 0-min time point, the same AMPs are found as observed in the resting frog samples (Fig 2-6A). Brevinin-1Pa, brevinin-1Pb, brevinin-1Pd, bradykinin, and ranatuerin-2P were observed (Figs 2-6A). When MALDI-TOF was performed on frog secretions that had been on the skin for 60 min, the relative intensities of the AMPs had decreased (Fig 2-6B). Relative intensities for each AMP were compared to that of an external standard resulting in relative intensity ratios. Compared to the external standard, the peptide signals detected

up to 60 min on the skin were quite stable for brevinin-1Pa, brevinin-1Pb, brevinin-1Pd, and ranatuerin-2P (Figs 2-7A-D).

Detection of peptide fragmentation

Further evidence that the AMPs are being degraded is the presence of breakdown products. A protease that cleaves at a position preceding lysine residues has been reported in skin secretions from *Xenopus laevis* (145). At 60 and 120 min after secretion onto the skin, the MALDI-TOF profile shows peptide cleavage fragments expected for both families of peptides based on predicted mass (Table 2-1). Additional evidence that the peptides are being degraded is shown by the decreased ability of samples that had been on the skin for a longer period to inhibit the growth of *B. dendrobatidis* zoospores. The longer the peptides were on the skin, the higher the concentration of peptides necessary to achieve complete inhibition of growth (Fig. 2-8A). In an effort to further quantify the peptide activity, peptide effectiveness was calculated. Peptide effectiveness is robust at 0 min, peaks after 15 min on the skin, and declines rapidly after 30 min (Fig. 2-8B). This finding suggests that the AMPs secreted onto the skin are highly effective for at least 15 min, and some activity persists for at least 2 h.

Collectively, our studies show that northern leopard frogs constitutively secrete small amounts of antimicrobial skin peptides that increase with frog activity or simulated predator stress. This may indicate a role for defensive peptides in wound repair or for deterrence of predators. The concentrations are sufficient to inhibit growth of *B. dendrobatidis* zoospores and may be effective deterrents of *B. dendrobatidis* colonization of the skin. Upon induction, the peptides are highly effective for at least 15 min on the

skin but degrade thereafter. We suggest that the degradation of AMPs may be necessary to limit harm to beneficial symbiotic microbes on the skin. Since *R. pipiens* is known to harbor *B. dendrobatidis* infections in the wild (128,192), constitutive AMP defenses may play a role in limiting the intensity of infection. Ongoing studies will determine how AMP expression in amphibian skin responds to infection by *B. dendrobatidis* or other pathogens and how these AMP concentrations affect resident bacterial populations. These studies illustrate the value of northern leopard frogs as a model species to study the role of skin peptides in protection from *B. dendrobatidis* and other skin pathogens. They strongly support a role for the constitutive expression of AMPs in defense of the skin.

CHAPTER III

SKIN PEPTIDES PROTECT JUVENILE LEOPARD FROGS FROM CHYTRIDIOMYCOSIS

Abstract

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 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 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 by MALDI-TOF mass spectrometry and in activity against *B. dendrobatidis* 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.

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 (76,203). Like many other frog species, the skin of *R. pipiens* has two sets of distinctive glands (19,60). Mucus glands produce a material rich in heavily glycosylated mucins (164) and mucopolysaccharides (56), 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 (62,154). 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 (131).

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 (126,141,157). Each species has its own distinctive repertoire of AMPs (39). 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 (4). The peptides are tightly packed into the enveloped granules within the syncytial structure of the granular glands poised for release (19,126). The glands are surrounded by a layer of myoepithelial cells innervated by sympathetic nerves (167). Following alarm or injury, the sympathetic nervous system is activated, neurotransmitters engage the adrenergic receptors (8,85), and the contents of the gland are released onto the surface of the skin (53). When the glands are stimulated, the granules containing peptides are released in a what has been described as a "holocrine" fashion (53). 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 (62,144). Because the myoepithelial cells surrounding the glands have alpha-adrenergic receptors, they can be experimentally induced to release their contents by injection of norepinephrine (53).

Worldwide amphibian declines and extinctions have been linked to an emerging infectious disease, chytridiomycosis, (38,168) which is caused by the fungus *Batrachochytrium dendrobatidis* (*B. dendrobatidis*) (10,114,134). 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 (131,144,152,154,157). 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 (710). 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 (9,10,114,134). 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 (154).

B. dendrobatidis not only infects adult frogs but also colonizes the mouthparts of tadpoles (10,143). 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 (154,156) and much of the organism's energy is spent completing metamorphosis. Metamorphosis is driven by the concerted actions of thyroid hormones and corticosteroid hormones (49,50). The elevated corticosteroid hormones at metamorphosis induce apoptosis of thymocyte and splenocyte populations (6,150) resulting in a temporary suppression of immune responses during the climax of metamorphosis (156). At the same time, the thyroid hormones drive differentiation of larval skin to the adult pattern of keratinized epithelium (121). Only the keratinized epithelium of the tadpole mouthparts and the adult skin are colonized by *B. dendrobatidis* (10).

In *B. dendrobatidis*-infected populations, metamorphosing frogs appear to be the most susceptible stage for infection (10,17,142,143,179,189). Thus, we hypothesized that during the period around the time of metamorphosis, AMP defenses in the skin would be critical to provide some degree of protection against pathogens like *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* measuring between 2 to 2.5 inches, were purchased from Connecticut Valley Biological Supply (Southampton, MA, USA). Frogs were housed in groups of 5-6 in polystyrene containers measuring 17.5 x 9.5 x 8 inches 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 eggs 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 9 oz 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 norepinephrine necessary to deplete skin peptides stores

To determine the amount of 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 [N= 9] nmol/g of norepinephrine-HCl (Sigma, St. Louis, MO, USA) dissolved in amphibian phosphate buffered saline (APBS) as previously described (Chapter II). 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 norepinephrine-induced peptide-depletion, a large group of frogs was injected with 40 nmol norepinephrine/g [N = 25] or 20 nmol norepinephrine/g [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 norepinephrine dissolved in APBS and placed in collection buffer for 15 minutes as previously described (Chapter II). After 15 minutes, 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) and quantified using the MicroBCA™ (bicinchonic acid) assay (Pierce, Rockford, IL, USA) according to manufacturer's instructions except that the peptide bradykinin (RPPGFSPFR) (Sigma Chemical, St. Louis, MO, USA) was used as a standard (131,144,155). The weight 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 (1969) [Surface area = 9.9 (weight in grams)$^{0.56}$]. The thickness of the mucus was assumed to be 50 µm (26), and therefore the volume of mucus covering one cm^2 of skin would be 5 µl. As a result, the total peptides (µg) per cm^2 X 200 = total µg/ml in mucus (131,144).

Skin histology

A section of dorsal skin, ventral skin, and the region of skin termed the dorsal plicae (19) from 3 peptide-depleted and 3 APBS-injected frogs was removed after euthanasia and fixed in 10% buffered formalin for 48 hours. 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 (MALDI-TOF) mass spectrometry (MS)

After partial purification, the recovered peptides were concentrated to dryness by centrifugation under vacuum at 70°C and prepared for MALDI-TOF analysis as previously described (Chapter II). The peptides were resuspended at a concentration of 1 mg/ml in 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 (CHCA) matrix (Sigma, St. Louis, MO, USA) was spotted onto the target plate and air-dried. MALDI-TOF MS was performed using the Bruker Daltonics 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 enkephalin 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 500 to 5,000 *m/z* ratio 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 (Chapter II). Briefly, *B. dendrobatidis* zoospores were grown on 1% tryptone agar for one week at 23°C. Freshly isolated zoospores were plated (5×10^4/50 µl, 5 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 (5 replicates) contained zoospores and 50 µl HPLC water. Negative control wells (5 replicates) contained heat-killed zoospores (60°C for 10 minutes) and 50 µl of HPLC water. Plates were incubated at 23°C for one week, and growth was measured as an increased optical density at 490 nm (OD_{490}) using an MRX Microplate Reader (Dynex Technologies, Inc., Chantilly, VA, USA). Peptide

effectiveness was defined as the percent inhibition at a peptide concentration of 12.5 µg/ml multiplied by the total amount of peptides recovered from the individual frog (131). For the comparison of peptide effectiveness, growth inhibition was determined over a series of peptide concentrations for 5 frogs sampled at day 0 and 5 frogs sampled at day 3.

Quantification of B. dendrobatidis zoospores on the skin

Frogs were swabbed with a sterile cotton swab ten times on the abdomen, legs, and each foot according to established protocols (20,89). Swabs were placed into tubes containing 60 µl of PrepMan Ultra (Applied Biosystems, Foster City, CA) and 30-35 mg zirconium/silica beads (0.5 mm in diameter) (Biospec Products, Bartlesville, OK). Swabs were homogenized in a Mini Beadbeater (MP Bio, Solon, OH for 45 seconds and then centrifuged for 15,000 X g for 30 seconds. The homogenization and centrifugation steps were repeated once. Next, samples were boiled for 10 minutes and cooled at room temperature for 2 minutes. Samples were centrifuged at 15,000 X g for 3 minutes and the supernatants were removed for real-time PCR.

Real-time probe-based PCR assay were performed on the Mx3000P Real-Time PCR system (Stratagene, La Jolla, CA). The thermal profile was the default conditions (95°C for 10 minutes,95°C for 30 seconds, 55°C for 1 minute, and 72°C for 1 minute) for 40 cycles. A standard curve based on the cycle threshold (Ct) values from control zoospore DNA at known concentrations. 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 Ct values were averaged between the three wells.

Exposure of post-metamorphic froglets to B. dendrobatidis

B. dendrobatidis-free *R. pipiens* at 3-6 months post-metamorphosis were reared from eggs 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 norepinephrine/g and again 2 days later with 20 nmol norepinephrine/g to deplete their granular glands and exposed to 10^4 zoospores of isolate JEL275 (30) [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 norepinephrine /g and again 2 days later with 20 nmol norepinephrine/g and maintained as groups 1 and 2 without further manipulation [N = 3]; 4) Frogs injected with APBS and maintained as groups 1-3 without further manipulations [N = 8]. *B. dendrobatidis* exposure lasted for two days until the water on all frogs was changed and zoospores flushed out. After exposure, the frogs were examined for signs for 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 mean values ± standard errors (M ± SE) were

compared by one-way analysis of variance (ANOVA) after log transformation (Tukey post hoc test) or by Student's *t* test following log transformation to meet the assumptions of 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 by 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 norepinephrine 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 norepinephrine, greater concentrations of total peptides were recovered in a dose-dependent fashion. The concentration required to maximally deplete peptides was 40 nmol norepinephrine/g (Fig. 3-1). Although injection of 80 nmol norepinephrine/g induced slightly more peptides than the 40 nmol/g 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 for how long peptide contents of granular glands remained depleted. When peptides were depleted by one injection of 40 nmol norepinephrine/g, 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. 3-2A). Following one injection of 20 nmol norepinephrine/g, peptide stores were still significantly depleted until day 40 but recovered to initial levels after 50 days (Fig. 3-2B). To determine the effects of the norepinephrine-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 (19). 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. 3-2C). In contrast, gland morphology following an injection with 40 nmol norepinephrine/g was quite different. The granular glands were empty of the eosinophilic granules, but they did not show significant structural damage (Fig. 3-2D).

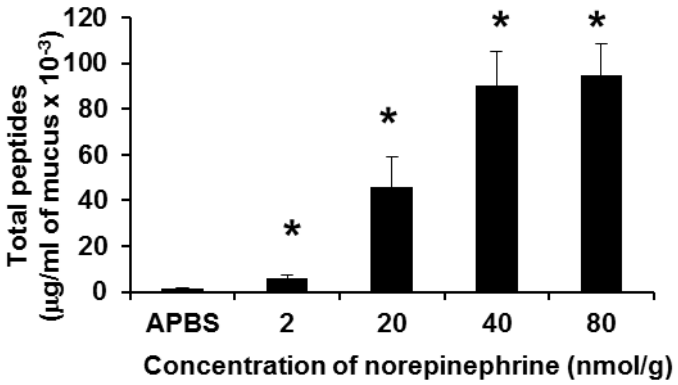

Figure 3-1. Dose-response to norepinephrine injections. Frogs (N = 6 to 9 per group) were injected with APBS [N = 8] or 2 [N = 8], 20 [N = 6], 40 [N = 9] or 80 [N = 6] nmol norepinephrine/g. *All concentrations of norepinephrine induced more peptides than those recovered from APBS-injected frogs by one-way 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 norepinephrine/g were significantly different from each other and from APBS controls by one-way ANOVA after log transformation, Tukey *post hoc* test, $P \leq 0.05$.

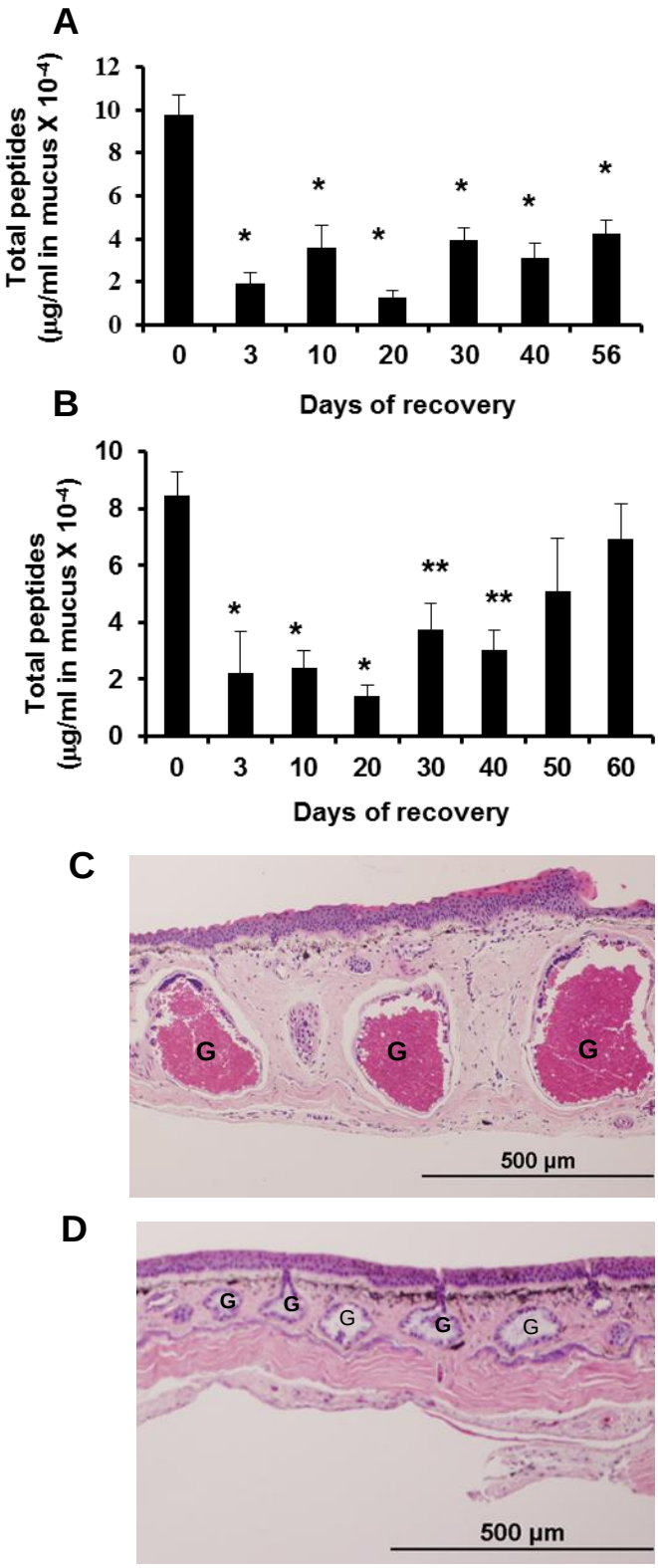

Figure 3-2. Recovery of peptides following injection of norepinephrine. (A) 40 nmol norepinephrine/g injected at each time point (N = 25 at day 0; N = 4 or 5 at other time points). *Peptide concentrations significantly reduced in comparison with those collected at day 0 by two-tailed Student's t-test after log transformation, $p < 0.02$. (B) 20 nmol norepinephrine/g injected at each time point (N = 26 at day 0; N = 4 or 5 at other time points). *Peptide concentrations 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. (D) Skin from frog injected with 40 nmol/g of norepinephrine showing intact but largely empty granular glands. Granular glands are indicated with the letter "G". significantly depleted until day 40 but recovered to initial levels after 50 days (Fig. 3-2B).

Characterization of recovered peptides by MALDI-TOF mass spectrometry

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 mass spectrometry. Shown in Figure 3-3A 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. 3-3A). 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. 3-3B) and the peptides recovered at other time points (Day 3 shown, Fig. 3-3C). Note that both sets of peptides showed a minimal inhibitory concentration (MIC) of about 50 µg/ml. 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.

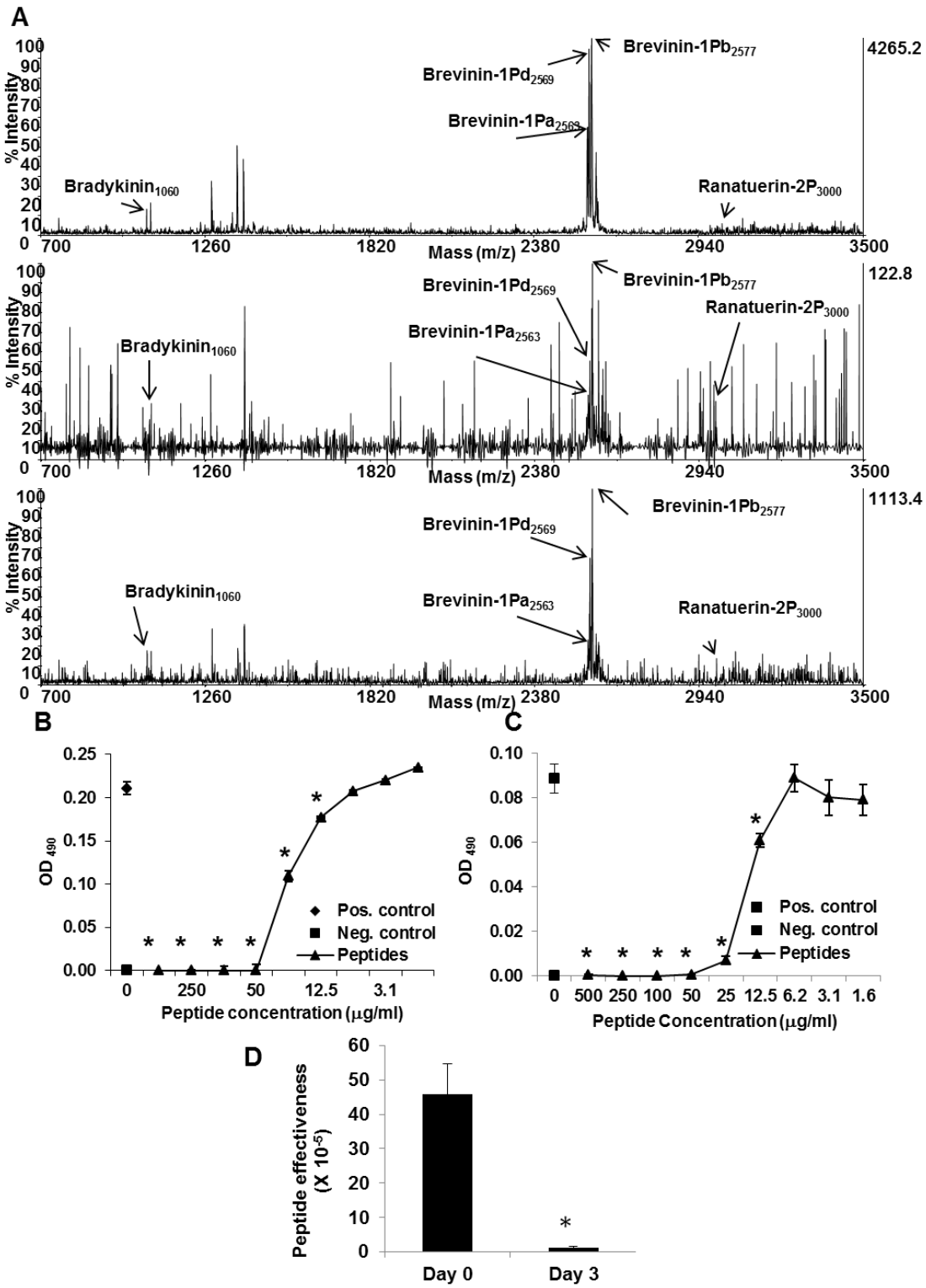

Figure 3-3. Characterization of skin peptides recovered at days 0, 3, and 60 after norepinephrine induction. (A) Representative MALDI-TOF profiles of peptides recovered after injection of 40 nmol norepinephrine/g at day 0 (top), day 3 (middle) and day 60 (bottom) (N = 5 frogs examined at each time point). (B) Growth inhibition of *B. dendrobatidis* zoospores by peptides recovered at day 0 after norepinephrine injection.

(C) Growth inhibition of *B. dendrobatidis* zoospores by peptides recovered at day 3 after norepinephrine injection. For (B, C), MIC = 50 μg/ml. *Growth was significantly less than that of positive control wells by two-tailed Student's *t*-test after log transformation, $P \leq 0.05$ (5 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, $P \leq 0.05$ (N =5 frogs sampled at day 0 and day 3).

Role of AMPs in protecting Rana pipiens juveniles

Young postmetamorphic *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 the state of Minnesota or Wisconsin, the juvenile frogs expressed a slightly different profile of AMPs characteristic of populations in this region (178). This profile includes brevinin-1Pa, brevinin-1Pb, brevinin-1Pe, and ranatuerin-2P (Fig. 3-4A). 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 (Range 25-250 µg/ml, M ± SE = 96 ± 27 µg/ml), comparable to that of peptides from adult frogs (Fig. 3-4B). Next, the juvenile frogs were depleted of their peptides and experimentally infected (Fig 3-5A).When these postmetamorphic frogs were depleted of their peptides by two injections of norepinephrine and exposed to *B. dendrobatidis* in their water (NE+*Bd*), they did not survive as well as *B. dendrobatidis*-exposed animals which had an intact set of peptides (APBS+*Bd*). Among the peptide-depleted and *B. dendrobatidis*-exposed frogs, only 3/8 had survived to day 10 while 8/8 APBS-injected control frogs that were exposed to *B. dendrobatidis* were alive. Other control groups that were norepinephrine-treated (3/3) or APBS-injected and not exposed to *B. dendrobatidis* (8/8) also survived to day 10 (Fig. 3-5B). Among frogs surviving at 10 days post-exposure to *B. dendrobatidis*, remaining peptide-depleted frogs had significantly higher numbers of zoospores on the skin than

APBS-injected and *B. dendrobatidis*-exposed frogs (Fig. 3-5C). Frogs not exposed to *B. dendrobatidis* had no evidence of *B. dendrobatidis* infection determined by qPCR.

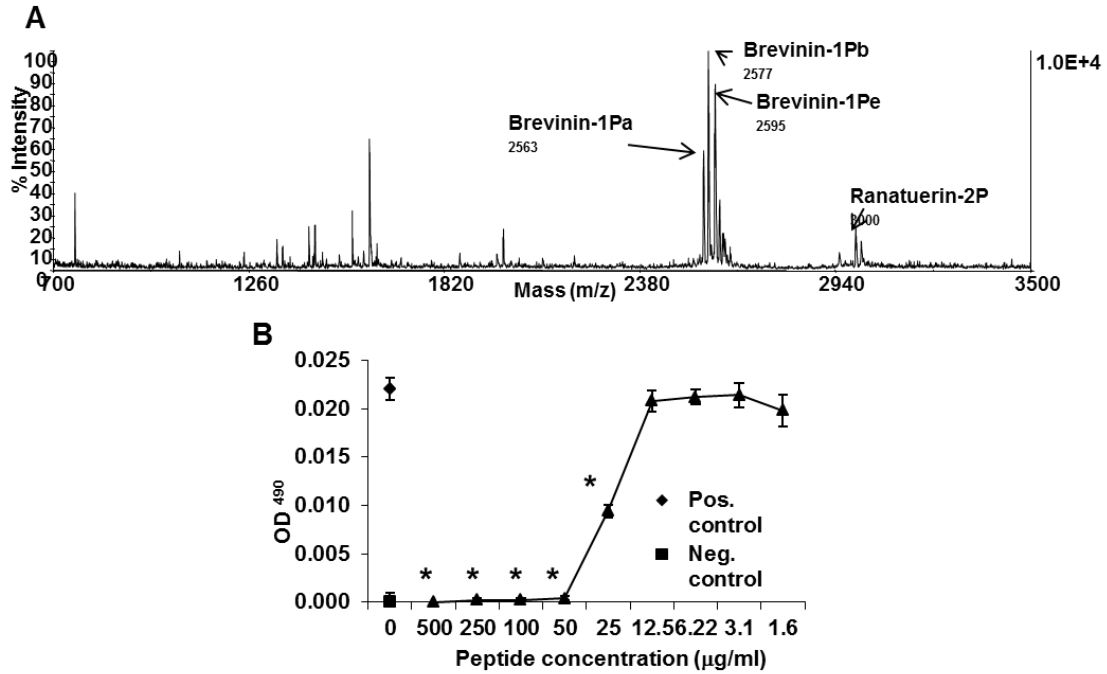

Figure 3-4. Characterization of AMPs from postmetamorphic juveniles. (A) Representative MALDI-TOF profile of hydrophobic skin peptides from juvenile frogs including antimicrobial peptides brevinin-1Pa, brevinin-1Pb, brevinin-1Pe, and ranatuerin-2P (representative of 12 individual frogs). (B) Growth inhibition of *B. dendrobatidis* zoospores by skin peptides from juvenile frogs (representative of 12 individual frogs tested). MIC = 50 μg/ml. *Growth was significantly less than that of positive control wells by two-tailed Student's *t*-test after log transformation, $P \leq 0.05$ (5 replicates for each concentration of peptide, negative control, and positive control wells).

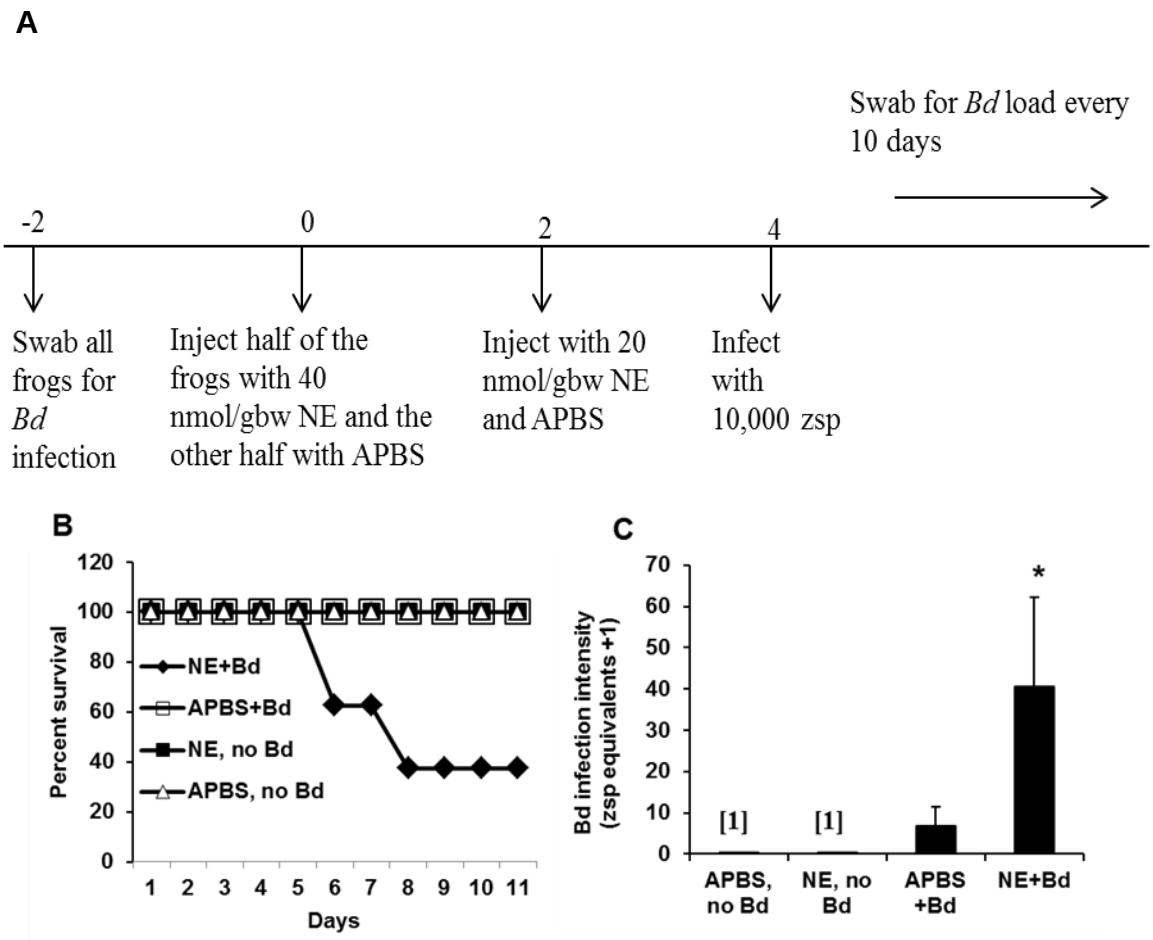

Figure 3-5. Peptide-depletion induced by norepinephrine increases susceptibility of postmetamorphic juveniles to *B. dendrobatidis*. (A) The peptide-depletion and infection protocol is shown above. Animals were housed individually and were infected by putting zoospores directly into their water. (B) Percent survival of postmetamorphic juveniles peptide-depleted and exposed to *B. dendrobatidis* (NE+*Bd*, $N = 8$), APBS-injected and exposed to *B. dendrobatidis* (APBS+*Bd*, $N = 8$), norepinephrine-injected but not exposed to *B. dendrobatidis* (NE, 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*, $P \leq 0.1$. (C) 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*, $N = 8$) or injected with norepinephrine (NE) and not exposed to *Bd* (NE, no *Bd*, $N = 2$) or APBS-injected and exposed to *Bd* (APBS+ *Bd*, $N = 7$) or injected with NE and exposed to *Bd* (NE+*Bd*, $N = 3$). The data represent the mean log zoospore equivalents + 1 ± standard error. *Greater number of zoospore equivalents present on the skin of NE+*Bd* frogs compared to APBS+*Bd* frogs by a one-tailed Student's *t*-test after log transformation, $P = 0.0352$.

Discussion

The current study supports previous work that showed a correlation between the survival of amphibians exposed to *B. dendrobatidis* with beneficial AMPs (195,197,198). It also supports a previous study showing that depletion of skin peptides in *Xenopus laevis* (naturally resistant to chytridiomycosis) resulted in increased pathogen burden (144).

Development of a method to deplete skin peptides

Using adult northern leopard frogs (*Rana pipiens*) as a model, we developed a protocol to deplete skin AMPs by injection of norepinephrine and observed the relatively slow renewal of the same set of peptides over a period of approximately two 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* (144). Although one injection of 40 nmol norepinephrine/g significantly reduced available skin peptides, some peptides were recovered at three days after the first injection. We previously showed in *X. laevis* that two norepinephrine injections separated by two days were necessary to completely deplete skin peptides (62). Thus, for the experimental infection study with juvenile *R. pipiens*, two norepinephrine injections were used.

Kinetics of peptide recovery and characteristics of the recovered peptides

Previous studies of peptide recovery following norepinephrine injection examined relatively mild inductions, and recovery was examined after a very short time. In *Xenopus laevis*, recovery of peptides following a mild norepinephrine injection (0.5 to 1 nmol/g) was detected by fast atom bombardment mass spectrometry. The full complement of expected peptides was detected within two to six days (68) suggesting that recovery is very rapid or peptides were not depleted by this injection. Following injection of a slightly higher concentration of norepinephrine (3 nmol/g), gland morphology was not completely restored for two weeks as determined by histology and electron microscopy (53). This suggested that a higher concentration of norepinephrine is necessary to more completely deplete peptides. We have shown that in *X. laevis*, two injections of 80 nmol/g within two days were necessary to completely deplete peptides (62), and peptide recovery following depletion with 80 nmol/g required seven to nine weeks (144). In the current study, we showed that peptide recovery after nearly complete depletion of *R. pipiens* peptides (injected with 40 nmol/g norepinephrine) required greater than 56 days (Fig. 3-2). 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. 3-3B and 3-3C). 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 three days earlier (Fig. 3-3D).

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 (144). 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 (51,87,88,153,156). Presence of high affinity IgY antibodies in *Rana catesbeiana* juveniles at 3 months post-metamorphosis (132) suggests that the adult B cell population in ranid frogs develops during the postmetamorphic 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, previous studies in *R. pipiens* showed that granular glands are completely developed in the dermal plicae of *R. pipiens* at the conclusion of metamorphosis (19). 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 twelve of these young frogs and found the expected AMPs in the majority of these animals (9/12) as shown in Fig. 3-4A. 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 norepinephrine at the relatively high concentrations used in our experiments is the depletion of skin peptide reserves, the norepinephrine could also have other immunosuppressive effects such as activation of corticosterone release (166). Future studies will determine whether there are other immunosuppressive effects of norepinephrine-treatment. This very strong norepinephrine 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 (131) that the stress to a frog of being chased for ten minutes resulted in the total peptide release of about 6,400 μg of peptides/ml of mucus comparable to that observed following the injection of 2 nmol/g of norepinephrine in the experiments presented here (Fig. 3-1). Thus, the treatment to deplete peptides (40 nmol/g norepinephrine), which induced about 90,000 μg of peptides/ml of 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 (59). In *Xenopus*, tadpoles are fasting after stage 62 but their glucose levels in body fluids do not decrease (77). As suggested by Jaudet and Hatey (95), glycogen, lipids, and proteins are probably converted to glucose (74,77). Elevation of corticosteroids hormones is observed during metamorphosis of all

amphibian species studied (49,97). Therefore, it is likely that a tradeoff 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 (115). 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 tradeoff in decreased total leukocyte counts and diminished cellular immune response (skin swelling in response to a plant lectin) (67). Precocious metamorphosis induced by administration of thyroid hormones limited lymphocyte development and impaired allograft rejection responses in *X. laevis* (153). During metamorphosis the immune system undergoes involution due to elevated corticosteroid hormones (156), and new metamorphs are particularly susceptible to diseases including chytridiomycosis (17,142,143,179,189).

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 (Fig 3-5B). Peptide-depleted juveniles that survived the infection had significantly higher *B. dendrobatidis* zoospore loads compared to the peptide intact animals (Fig 3-5C). 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* (131). The current study demonstrates that those

constitutively released AMPs provide critical protection for the most susceptible life stage against *B. dendrobatidis*.

CHAPTER IV

SWAB VS SOAK: THE BEST WAY TO DETECT *B. DENDROBATIDIS*

Abstract

The chytrid fungus, *Batrachochytrium dendrobatidis*, has been linked to amphibian declines around the world. Motile zoospores colonize the keratinized epithelium of adult frog and the mouth parts of tadpoles. To further understand the magnitude and scope of this problem and determine pathogen burdens, effective testing for the pathogen is imperative. The standard method for quantifying zoospore loads on infected amphibians is to swab animals on the most infected areas including the ventral abdomen, legs, and footpads. Although this method is optimal for the field, the main drawback is that it does not take into account the zoospore load on the whole animal. In this study a new method for testing amphibians was developed and compared to standard methods. The new method includes a protocol in which frogs were submerged in sterile water for an hour, removed, and the water was filtered. DNA was isolated from laboratory samples containing a known number of zoospores and live animals using the methods of filtration, swabbing, and centrifugation. It was found that filtration was the most accurate method of detection.

Introduction

Amphibians are facing serious declines from a fungal pathogen, *B. dendrobatidis* (10,134). This pathogen, which causes the disease chytridiomycosis, has been identified in Africa, South America, Central America, North America, Europe, Australia, and Asia (10,17,111,122,189,190). *B. dendrobatidis* has been detected in wild and captive populations including the amphibian orders of Anura, Caudata, and Gymophiona (133).

The early testing for *B. dendrobatidis* involved highly invasive methods such as collecting toe clips (whole toes) or skins sections and performing histology using haemotoxylin and eosin staining (46). Histological techniques have their own pitfalls. The problems with histological diagnosis are: 1) the investigator must develop the skills for accurate identification of the fungal cells within the skin; 2) the invasive nature of collecting toe clips from live animals; 3) the method is time consuming; 4) low sensitivity; and 5) there is variability in infection levels between different toes or skin sections (89).

In *B. dendrobatidis* infection, early detection is imperative to allow for treatment and to prevent further spread within a captive colony. Infected amphibians are frequently transported to new locations such as zoos, for the international pet trade, food trade, and the laboratory animal trade (118,122,130,134). If detected early, amphibians can be treated and possibly cured. It has been shown that simply by elevating body temperature *B. dendrobatidis* infection can be cleared (193). Drug treatments have also shown to be effective (125).

A PCR technique has been developed to more rapidly identify *B. dendrobatidis* infection. This technique is quick, non-invasive, specific, and sensitive. In fungal

ribosomal DNA, there are about 100-400 copies per haploid genome (113). Fungal ribosomal DNA contains conserved regions of 5.8, 18, and 28S DNA interspersed with quickly evolving internal transcribed spacers and an intergenic spacer (113). These characteristics were optimal for developing a real-time Taqman assay of *B. dendrobatidis* (20).

The standard technique for testing a frog for *B. dendrobatidis* is to swab the abdomen, legs, and footpads 10 times each. The DNA is isolated from the swab, and real-time PCR is performed. This sampling method is optimized for the field. Researchers can catch the amphibians, swab them, and release the animal back into the wild in a matter of minutes. The swabs are then able to be transported to the lab to be analyzed. The method is quick and easy, but it does have its drawbacks. First, swabbing methods are highly variable from person to person. Second, it only measures the heavily infected areas of the frog and not the whole organism.

One sampling method that does take into account *B. dendrobatidis* infection from the whole organism is the centrifuge method. Here, frogs are placed in sterile water for an hour and removed. The water is centrifuged and DNA isolation is performed on the pellet (89). The pitfall to this technique is that *B. dendrobatidis* does not pellet well. As a result, infection levels can be drastically underestimated compared to what is actually present on the frog.

To solve the problems of both sampling methods, a new method was developed in our lab. In the new sampling method, frogs were placed in sterile water, the water was then filter sterilized, and DNA was isolated from the filter. This sampling method has the benefit of sampling from the entire frog, removing the variability from the swabbing

technique, and eliminating the pelleting problem. In addition, the technique developed is able to be standardized and easily repeated in the laboratory setting.

In this chapter the newly developed technique was tested against the centrifuge and swabbing method. Factors considered included accuracy against known standards and zoospore load detected. It was hypothesized that the filtration method is a better measure of quantifying infection load compared to the standard methods of *B. dendrobatidis* sampling.

Materials and Methods

Frogs

Adult *Rana pipiens* measuring between 2 and 2.5 inches were purchased from Connecticut Valley Biological (Southampton, MA). Frogs were housed in groups of 5 to 6 in covered polystyrene containers in dechlorinated tap water at a temperature of 20-24°C. Containers were placed at an angle allowing the animals to choose between a wet or dry environment. They were fed live crickets, and water was changed three times a week. All protocols used were approved by the Vanderbilt University Medical Center Institutional Animal Care and Use Committee.

Culturing B. dendrobatidis

B. dendrobatidis isolate JEL 197 was cultured on tryptone agar plates. Zoospores were isolated > 95% purity by filtration over sterile nylon spectramesh filters as previously described (Chapter II). Zoospores were stained with Lugol's solution and

counted using a hemocytometer. Zoospores were diluted to a concentration of 10^2, 10^3, 10^4, 10^5 and 10^6 zoospores total in a volume of 50 ml.

Swabbing of B. dendrobatidis

B. dendrobatidis standards with a known number of zoospores (3 µl) were loaded onto swabs. Frogs were rinsed with deionized sterile water to remove any dead zoospore DNA. Animals were then swabbed on the ventral abdomen 10 times, legs 10 times, and footpads 10 times. DNA was isolated from the swabs according to the method of Boyle et al., (20) by putting the swabs in 60 µl of PrepMan Ultra (Applied Biosystems, Foster City, CA) and 30 to 35 mg of zirconium/silica beads (0.5 mm in diameter)(Biospec Products, Bartlesville, OK). Swabs were homogenized in a Mini Beadbeater (MP Bio, Solon, OH) for 45 seconds followed by a 30 second centrifugation at 15,000 x g. Homogenization and centrifugation were repeated once more. Samples were then boiled for 10 minutes, and cooled for 2 minutes room temperature, and centrifuged for 3 minutes at 15,000 x g. Supernatants containing *B. dendrobatidis* DNA were removed for real-time PCR.

Centrifugation of B. dendrobatidis

B. dendrobatidis standards with a known number of purified zoospores were centrifuged for 20 minutes at 3,000 rpm. Supernatants were removed and the pellet was resuspended with 200 µl or PrepMan Ultra (Applied Biosystems, Foster City, CA), and DNA was isolated as described above.

Filtration of B. dendrobatidis

B. dendrobatidis standards with a known number of purified zoospores were filtered through a 0.22 micron filter. Following filtration, the filters were submerged into a solution containing 200 µl of PrepMan Ultra (Applied Biosystems, Foster City, CA) and 50 mg of zirconium/silica beads (0.5 mm in diameter)(Biospec Products, Bartlesville, OK). After this step the protocol for DNA isolation was identical to the swabbing protocol. For live animals, frogs were rinsed with sterile deionized water to remove any dead *B. dendrobatidis* DNA. Animals were then placed in 100 ml of sterile dechlorinated water for 1 hour, the water was filtered through a 0.22 micron filter, and DNA was isolated as described above.

B. dendrobatidis real-time PCR

Probe-based real-time PCR was performed on the Mx3000P Real-Time PCR system (Stratagene, La Jolla, CA) according to the protocol of Boyle et al. (20). The standard PCR cycles were as follows: 95°C for 10 minutes, then 95°C for 30 seconds, 55°C for 1 minute and 72°C for 1 minute for 40 cycles according to previous protocol (20,89). The number of zoospore equivalents was calculated based on a standard curve of threshold values from control zoospore DNA.

Statistical comparisons

All parameters compared were averaged, and plotted with standard error bars. The means were compared by one-tailed or two tailed Student's t test. A *P* value of less than 0.05 was considered statistically significant.

Results

Filtration vs centrifugation

Known standards containing 10^2, 10^3, 10^4, and 10^6 zoospores were made in sterile deionized water. DNA was isolated, and real-time PCR was performed to assess accuracy and detection limits and centrifuged or filtered. The filtration method was much more accurate and sensitive than the centrifuge method. For all standards the filtration method detected more zoospore equivalents, and the numbers detected were within 1 log of the input zoospore numbers. The centrifugation method did detect *B. dendrobatidis* DNA, but numbers of zoospores detected was often much lower than the input number of zoospores (Fig 4-1).

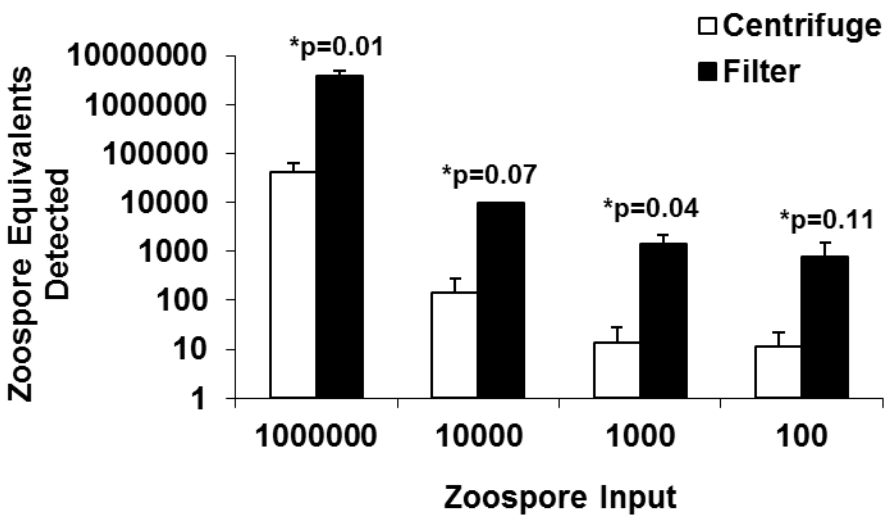

Figure 4-1. Comparison of the filtration and centrifugation methods with known zoospore inputs. Zoospore inputs of 10^2, 10^3,10^4, and 10^6 were compared between the two methods. The number of zoospores detected was log transformed and tested for significance using a Student's two-tailed *t*-test.

Filtration vs swabbing

Next the filtration method was compare to the swabbing method in terms of ability to recover and quantify zoospore DNA. Standards with a known number of zoospores were applied to filters or swabs which were subjected to DNA isolation. In this comparison, the swabs and filters were roughly equivalent with less than a 1 log difference between them in numbers of zoospores detected (Fig 4-2).

Filtration vs swabbing using naturally infected animals

The filtration and swabbing methods were tested on naturally infected live animals. Although there was no difference when known numbers of zoospores were applied to filters or swabs, there was a profound difference in zoospores detected from live animals. Animals were grouped according to their infection status based on the standard swabbing method. Frogs with less than 100 zoospores were considered to have mild infections. Frogs with 100-10,000 zoospores were considered moderately infected, and frogs with more than 10,000 zoospores were considered severely infected. At the lower infection levels the filtration method detected more zoospore DNA than the swab method (Fig 4-3).

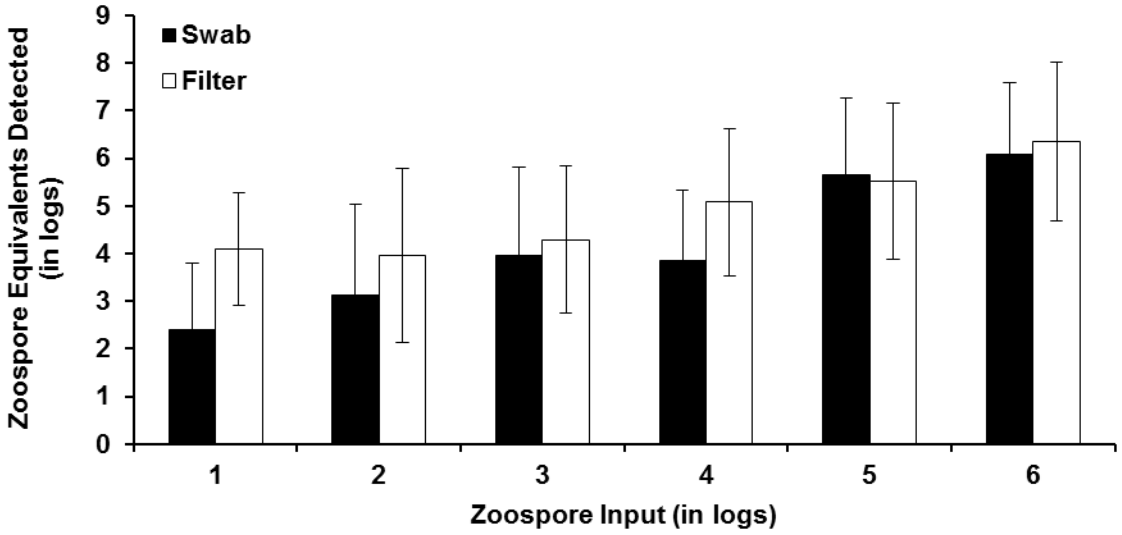

Figure 4-2. Comparison of the swabbing and filtration methods with known zoospore inputs. Zoospore inputs of 10^1, 10^2,10^3,10^4, 10^5 and 10^6 were compared between the two methods. The number of zoospores detected was log transformed and tested for significance using a Student's two tailed *t*-test.

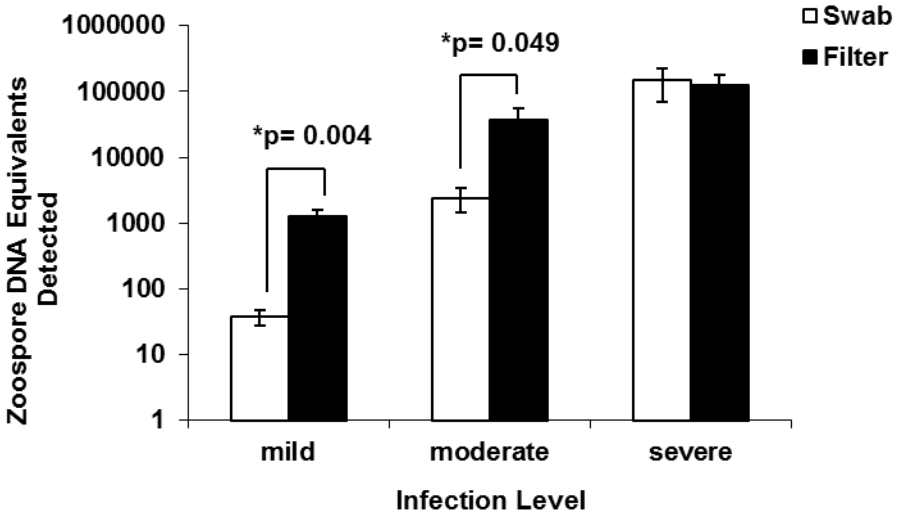

Figure 4-3. Comparison of swabbing and filtration methods in naturally infected animals. Animals were grouped into mild (< 100 zsp), moderate (100< 10,000 zsp), and severe (> 10,000 zsp) by the zoospore equivalents from the swabs. *N* = 3-9 frogs per group. The amount of zoospores detected was log transformed and tested for significance using a two-tailed Student's *t*-test.

Discussion

As *B. dendrobatidis* continues to spread globally, it is important to monitor its prevalence in wild and captive populations. Although the creation of a *B. dendrobatidis* probe-based real-time PCR assay played a critical role in identifying infected individuals, swabbing may not be giving a complete picture of infection status (20). The new method proposed based on sampling *B. dendrobatidis* from frogs by soaking them and filtering their water improves upon the two traditional methods of centrifugation of soak water and swabbing.

In Figure 4-1, known input numbers of *B. dendrobatidis* zoospores were put through either the filter sampling method or the centrifugation sampling method. Both of these techniques sample the entire frog by isolating zoospores washed off from the skin and concentrated by either filtration or centrifugation. The filtration sample method outperformed the centrifugation sample method be detecting more zoospore DNA and being closer to the original input of zoospores. It is suspected that the filtration method performed better because *B. dendrobatidis* zoospores do not pellet well and some of the input may have been lost while removing the supernatant.

Next the filtration sampling method was tested against the swabbing method *in vitro* (Fig 4-2). Here, the swabbing method and the filtration method tested similarly and were within 1 log of each other in terms of zoospore equivalents. Although there was no real difference between the sampling methods, it was thought that there would be a difference when sampling live animals because swabbing only samples particular parts of the frog while filtration would sample the whole frog.

In Fig 4-3, the swab sample method and filtration sample method were tested on

naturally infected adult *R. pipiens.* For each animal the amount of zoospore DNA detected by the filter was higher compared to that of the swab. When grouped by infection loads, there were significant differences between the filtration and swab collection methods. It is thought that sampling the whole frog compared to the select areas of swabbing led to these differences.

The swab method of sampling will remain the main method of sampling *B. dendrobatidis* in the wild. It offers the advantages of being quick, easy, and transportable. We suggest that for laboratory studies and for other animals in captivity the filter method may lead to a more accurate picture of infection status by sampling the entire animal. Swabbing is highly variable by each person performing the sampling method. Filtration removes this variable by soaking the frog in a specific volume of sterile water. Another benefit is that it can be standardized and the data generated by this sampling method could be compared to data generated by other groups and in different species. As a more accurate test for infection, filtration could be performed and the necessary steps could be taken to cure infected animals.

CHAPTER V

DISCUSSION AND FUTURE DIRECTIONS

My dissertation is the first work to specifically examine the dynamics of antimicrobial peptide (AMP) defenses and their role in the immune defense against *Batrachoychytrium dendrobatidis*. It has long been known that AMPs produced in amphibian skin have *in vitro* activity against a whole range of pathogens, but it was unclear if they played a role in the protection of the frog. This work suggests that AMPs play a critical role in protecting frogs against the often lethal skin pathogen, *B. dendrobatidis,* at several life stages. A better understanding of the innate defenses of wild amphibians will aid conservation efforts.

The ebb and flow of antimicrobial peptides on the skin of *Rana pipiens*

R. pipiens populates much of North America and has been shown to have a suite of peptides active against many pathogens. It was hypothesized that if these AMPs were expressed, they would be quickly degraded by proteases and would not be able to prevent pathogen infection. Using a very sensitive technique, direct MALDI-TOF MS, individual AMPs were detected on resting and chased frogs without inducing AMP secretion by norepinephrine injection. When the secretions were tested for anti-*B. dendrobatidis* activity using growth inhibition assays, the peptides recovered from resting and chased frogs were able to inhibit the growth of *B. dendrobatidis,* albeit less effectively than those from norepinephrine-induced animals, suggesting that the AMPs do play a role in immunity against skin pathogens.

Another major question that was answered by my studies is what happens to AMPs following secretion. To induce peptide secretion, frogs were injected with norepinephrine. The secretions were allowed to remain on the skin for various periods of time before being washed off the skin. The total amount of peptides recovered decreased in a time-dependent manner. Secretions also had decreased anti-*B. dendrobatidis* activity over time. To examine the relative quantification of individual AMPs over time, I developed a method in which an alkylated form of one of the native AMPs was added to the peptide mixtures at a known concentration. The relative intensities were compared to the external standard and revealed that individual AMP signal intensities also decreased in a time-dependent fashion.

It was hypothesized that the AMPs were being degraded by a protease. In another frog species, *X. laevis*, an endoprotease was found that cleaved at an amino acid position preceding lysine residues. In secretions from resting and chased animals at the later time points, AMP fragments were found which matched such predicted cleavage products. Taken together this work suggests that AMPs are expressed constitutively at low levels and have anti-*B. dendrobatidis* activity at these concentrations. After secretion onto on the skin, they are broken down by an endopeptidase present in the same skin secretions.

Skin peptides protect juvenile leopard frogs from chytridiomycosis

During metamorphosis, the immune system is suppressed and much of the frog's energy is put into the process of tissue remodeling. *R. pipiens* is thought to be a species that is relatively resistant to chytridiomycosis. In the studies described in chapter III, the goal was to determine the role of AMPs in defense against *B. dendrobatidis* of recently

metamorphosed juvenile frogs. To do so, a skin peptide depletion protocol was established using increasing doses of norepinephrine. It was determined that peptide secretion was maximal at 40 nmol/g.

To determine how long skin peptides would remain at low levels, frogs were then injected with a depleting dose and injected again at various later time points. AMPs had not yet recovered to initial levels at 56 days after depletion with 40 nmol/g, but were able to recover in about 50 days after injection with a 20 nmol/g dose. This demonstrates that the peptide-depletion protocol resulted in a long-term depletion of this important skin defense. The suite of recovered AMPs was identical to the initial peptides, and their activity in anti-*B. dendrobatidis* growth inhibition assays was the same.

Lastly, *B. dendrobatidis* naïve *R. pipiens* at 3-6 months after metamorphosis were depleted of their peptides and exposed to infectious *B. dendrobatidis* zoospores to truly test the role AMPs as a deterrent to *B. dendrobatidis* infection. Individuals that were infected and peptide-depleted died rapidly compared the three other control groups. In addition, they had higher *B. dendrobatidis* loads compared to the controls. This experiment suggests that AMPs provide some degree of protection against chytridiomycosis.

Swab vs soak: The best way to detect *B. dendrobatidis*

In the field, frogs are caught and sampled by swabbing the most infected areas of the frog, the abdomen, legs, and footpads. While sampling these areas can give a quick yes or no determination of infection and such sampling has been used to quantify intensity of infection, it can be misleading because the method does not sample the entire

animal. Although it is acknowledged that this method is optimal for the field, there may be better methods for use in the laboratory setting. In chapter IV, a method was described in which frogs were submerged in sterile water, removed, and water was filtered. *B. dendrobatidis* DNA was then isolated from the filter and *B. dendrobatidis* load was quantified using real-time PCR.

The method developed in this chapter is the first to accurately test zoospore load by sampling the entire organism and removing variability due to differences in the swabbing technique of different investigators. The filtration method also helps to standardize *B. dendrobatidis* sampling by using defined volumes and soak times. This method is proposed for use in laboratory studies but would be cumbersome in the field studies because of the need to haul more equipment and delay the release of the animals into their habitat.

Future Studies

My work showed that antimicrobial skin peptides may be very important for protection of newly metamorphosed juvenile frogs from *B. dendrobatidis* infection. However, little is known about the ontogeny of expression of AMPs at the time of metamorphosis. Future studies will demonstrate whether the granular glands are fully differentiated at the time of metamorphosis or require several weeks or month to develop. MALDI-TOF analysis of skin secretions will demonstrate whether the full repertoire of expected AMPs is present at metamorphosis or takes weeks or months to develop. Answers to these questions will more fully define how vulnerable metamorphosing frogs are to chytridiomycosis.

My work showed that individual AMPs can be detected in the mucus of resting and chased frogs by direct MALDI-TOF. However, we do not yet have a good measure of the actual concentrations of individual peptides in the mucus of these minimally manipulated frogs. Future studies are planned to use direct skin sampling coupled with addition of a known concentration of an external peptide to more rigorously quantify the relative concentration of individual AMPs using MALDI-TOF MS and related methods.

AMPs have been shown to have activity against many bacterial species. In this thesis, it was shown that AMPs are expressed constitutively at low levels and when left on the skin, the AMPs are degraded in a time-dependent fashion. It is thought that the AMPs are degraded to protect the microbiota present on the skin or to protect the integrity of the skin itself. It has been shown that amphibians harbor bacterial species that secrete metabolites capable of inhibiting *B. dendrobatidis* growth (26,78,199). The interplay between these two innate defense mechanisms should be further examined.

Work currently being done in the Rollins-Smith lab is to identify bacterial isolates present on the skin of ranid frogs. Bacterial isolates have been grown up and supernatants were tested for anti-*B. dendrobatidis* activity. Current experiments are being performed to see if there is synergy between the AMPs and bacterial supernatants. It is thought that the presence of both may provide a more effective defense against *B. dendrobatidis* infection.

AMPs from amphibians have been shown to have activity against a wide range of pathogens *in vitro* (203). Further studies of amphibian AMPs such as membrane binding, mechanism of action, intracellular effects may lead to the use of these peptides as therapeutic agents (64,102). For example, the AMP buforin IIb has been shown to have

antitumor activity in P53-deficient mice (34). Furthermore, amphibian AMPs have been shown to have anti-parasite properties showing activity against the causative agents of malaria and Chagas' disease (147). Lastly amphibian AMPs have activity against HIV and the transfer of the virus from dendritic cells to T cells (182).

There is considerable controversy about the use of AMPs for prevention or cure of infections (169). Many AMPs have toxic properties when administered in high doses because of their non-specific binding and membrane disruption properties. Efforts have been made to synthetically modify AMPs to maintain antimicrobial activity but reduce protease, and hemolytic activity.

Specific modifications to improve AMP function and limit toxicity are using truncated forms of the peptides and the use of peptides containing only D-amino acids to limit protease activity. Another modification shown to improve AMPs is using poly-N-substituted glycines (peptoids) that are attached to the amide nitrogen instead of the α-carbon. This change has been shown to have good antibacterial activity but low hemolytic activity (35). Other modifications, such as using β-peptides, arylamide oligomers, and phenylene ethynylenes, have shown similar results (158).

APPENDIX A

LIST OF PUBLICATIONS

I. **Pask, J.D.,** Woodhams, D.C., and Rollins-Smith, L.A. The ebb and flow of antimicrobial skin peptides defends northern leopard frogs (*Rana pipiens*) against chytridiomycosis. *Global Change Biology*. 2012. 18(4):1231-1238.

II. Woodhams, D.C., Kilburn, V.L., Reinert, L.K., Voyles, J., Medina, D., Ibanez, R., Hyatt, A.D., Boyle, D.G., **Pask, J.D.,** Green, D.M., and Rollins-Smith, L.A. Chytridiomycosis and amphibian population declines continue to spread eastward in Panama. *EcoHealth*. 2008. 5:268-274.

III. Rollins-Smith, L.A., Ramsey, J.P., **Pask, J.D**., Reinert, L.K., and Woodhams, D.C. Amphibian immune defenses against chytridiomycosis: Impacts of changing environments. *Integrative Comparative Biology*. 2011. 51(4):552-62.

IV. **Pask, J.D.,** Cary, T.L., and Rollins-Smith, L.A. Resistance of leopard frog (*Rana pipiens)* juveniles to chytridiomycosis is dependent on antimicrobial peptides in the skin secretions. (Submitted, revised version under review)

V. Woodhams, D.C., Chaurand, P., Caprioli, R.M., Bell, S.C., Reinert, L.K., Vazquez, V., Lam, B., Bigler, L., Stalder, U., **Pask, J.D.**, and Rollins-Smith, L.A. 2011. Immune trade-offs in life-history evolution: metamorphosis and amphibian skin peptide defences. Submitted (under revision).

VI. **Pask, J.D.,** Pharr, Z., and Rollins-Smith, L.A. Swab vs soak: the best way to detect *B. dendrobatidis* (In preparation).

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