THE ANTIFUNGAL ARSENAL IN AMPHIBIAN SKIN: INNATE IMMUNE DEFENSES AGAINST *BATRACHOCHYTRIUM DENDROBATIDIS* IN SOUTHERN LEOPARD FROGS.

By

Whitney Marie Holden

Dissertation

Submitted to the Faculty of the

Graduate School of Vanderbilt University

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

In

Microbiology and Immunology

May 2014

Nashville, Tennessee

Approved:

H. Earl Ruley, Ph.D., Chair

Patrick Abbot, Ph.D.

Andrew Link, Ph.D.

Luc Van Kaer, Ph.D.

Louise Rollins-Smith, Ph.D., Dissertation Advisor

ACKNOWLEDGEMENTS

First, I would like to thank my mentor, Dr. Louise Rollins-Smith. She has been supportive of all my endeavors both in and out of the lab and has always been willing to discuss new ideas. During my time in lab, she has been there to offer me advice and encouragement and has taught me so much. Likewise, I thank the other members of the Rollins-Smith Lab, including lab manager Laura Reinert, who is always willing to lend a helping hand and listening ear, and fellow graduate students Scott Fites, Jim Pask, and Jeremy Ramsey, who offer insightful comments and are often willing to collaborate on interesting side projects. I would also like to thank my thesis committee for their investments in my graduate education: Dr. Earl Ruley, Chair, Dr. Patrick Abbot, Dr. Andrew Link, and Dr. Luc Van Kaer. Over the course of my graduate career, they have offered many helpful suggestions, constructive criticism, and helped me learn how to think about research and ask the right questions. Thank you all.

Much of this work has been made possible by our collaborators. I would like to thank the following people for their contributions of animals, equipment, or other materials: Dr. Shane Hanlon and Dr. Matt Parris (University of Memphis, Memphis, TN) for raising *R. sphenocephala* metamorphs used in my experiments, the lab of Dr. Eric Skaar (Vanderbilt University, Nashville, TN) for the use of the Olympus microscope, camera, and software for histology and cellular photography, the lab of Dr. Alex Hyatt (Australian Animal Health Laboratory, Victoria, Australia) for providing *B. dendrobatidis* DNA standards for use in real time PCR assays, Dr. John N. Galgiani (Valley Fever Center for Excellence, Tucson, AZ) for providing nikkomycin Z-HCl, and

the laboratory of Dr. Janet Foley (University of California – Davis, CA) for providing the strain of *B. dendrobatidis* used in infection studies.

I would like to thank the following people for their contributions of time, skills, and information: Dr. Douglas Woodhams and Dr. Valerie McKenzie (University of Colorado – Boulder, CO) for help with 16S rDNA sequencing of bacterial isolates; Dr. Laetitia Tatiersky (University of Guelph, Ontario, Canada) for her advice in histology staining methods, Dr. David Friedman (Vanderbilt Proteomics Laboratory, Nashville, TN) for tandem mass spectrometry analysis, Dr. Thomas Kehl-Fie (Vanderbilt University, Nashville, TN) for assistance with Kaplan-Meier statistical analysis, and Laura Reinert (Vanderbilt University, Nashville, TN), Franklin Roman, and Holly Archer (University of Colorado – Boulder, CO) for assistance with bacterial identification.

This work was supported by the National Science Foundation grants IOS-0619536, IOS-0843207, and IOS-1121758 (to Louise Rollins-Smith, Ph.D.), and DEB-1146282 (to Valerie McKenzie, Ph.D. and Rob Knight, Ph.D.) and a National Science Foundation Graduate Research Fellowship (to Whitney Holden). Isolation of the Section Line strain of *B. dendrobatidis* was supported by a grant from California Department of Fish and Wildlife and the United States Fish and Wildlife Service to Jonah Piovia-Scott, Janet Foley, and Karen Pope).

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

Figure Page

LIST OF ABBREVIATIONS

AMP	Antimicrobial peptide
AmB	Amphotericin B
ANOVA	Analysis of variance
APBS	Amphibian phosphate buffered saline
BCA	Bicinchoninic acid
Bd	*Batrachochytrium dendrobatidis*
BLAST	Basic Local Alignment Search Tool
cfu	Colony forming units
CHCA	α-Cyano-4-hydroxycinnamic acid
Chl	Chloramphenicol
dH_2O	Distilled water
Fig	Figure
gbw	Body weight in grams
hr	Hours
H&E	Hematoxylin and eosin
HPLC	High performance liquid chromatography
IUCN	International Union for Conservation of Nature
JEL197	*B. dendrobatidis* strain
LB	Luria-Bertani media
m	Meters
m/z	Mass to charge ratio
MALDI-TOF	Matrix-assisted laser desorption ionization time-of-flight
MBS	Meeman Biological Field Station
MIC	Minimal inhibitory concentration
min	Minutes
MS	Mass spectrometry
MW	Molecular weight
N	Number, sample size
NE	Norepinephrine

NZ	Nikkomycin Z
$OD_{\#}$	Optical density at a given (#) wavelength
OTU	Operational taxonomic unit
PAMP	Pathogen-associated molecular pattern
PAS	Periodic acid-Schiff
QIIME	Quantitative Insights Into Microbial Ecology
qPCR	Quantitative polymerase chain reaction
RDP	Ribosomal Database Project
s	Seconds
SEM	Standard error of the mean
t	Time
T	Tryptone

CHAPTER I

INTRODUCTION AND RESEARCH GOALS

Global amphibian declines

In recent years, the scientific community has observed an alarming number of severe amphibian population declines (Collins 2010), with some resulting in extinctions. These declines are especially striking because the current rate of amphibian species disappearances far exceeds that of birds or mammals (Stuart et al. 2004). It is estimated that one third of all known amphibian species are threatened or extinct (IUCN Red List for Amphibians, http://www.iucnredlist.org/initiatives/amphibians) and the rate of amphibian extinctions since 1980 is over 100 times the background rate (McCallum 2007), marking this the largest extinction event in 10,000 years (Wake and Vredenburg 2008). In this light, severe declines have been documented around the globe with massive die-offs reported on multiple continents (Laurance et al. 1996, Bosch et al. 2001, Ron et al. 2003, Burrowes et al. 2004). Possible causes for declines include climate change, various anthropogenic factors, and emerging infectious diseases (Collins and Storfer 2003, Burrowes et al. 2004, Collins 2010).

Climate change and its effect on amphibians

The largest factors in climate change are temperature and precipitation changes. Because amphibians are ectothermic and breathe through their skin, temperature changes

and dehydration can have serious effects on survival, immunity, and reproduction. There are multiple reports of climate change events correlating with a direct decline or extinctions of local amphibian populations, including frost, severe drought, and rising temperatures linked to global warming (Pounds and Crump 1994, Donnelly and Crump 1998, Pounds et al. 2006, McMenamin et al. 2008, Collins 2010). Despite this, it has proven difficult to firmly establish a link between climate change and amphibian declines on a global scale, most likely because multiple factors may result in compounding stressors for amphibian population health (Carey and Alexander 2003, Lips et al. 2008). One such example is known as the "chytrid thermal optimum hypothesis." Proponents of this hypothesis suggest that rising temperatures due to global warming resulted in increased cloud cover and misting, causing higher nighttime temperatures and lower daytime temperatures, resulting in improved conditions for the fungal pathogen *B. dendrobatidis* (Pounds et al. 2006). Though subsequent studies of other amphibian declines have cast doubt on this hypothesis (Lips et al. 2008, Vredenburg et al. 2010), it is clear that climate change is an important factor irrespective of infectious diseases, especially on local scales.

Anthropogenic causes of amphibian decline

Various human activities have unfortunately resulted in amphibian declines around the globe. These activities include commercial use of amphibians, introduction of non-native species, release of environmental contaminants, and habitat destruction. Amphibians are commonly used commercially both as sources of food and for the international pet trade, both legal and illegal (La Marca and Reinthaler 1991, Collins et

al. 2009). Such stress on wild populations inevitably leads to declines, especially for already endangered species (La Marca et al. 2005). Introduction of non-native (invasive) species into amphibian habitats may result in amphibian declines directly, such as through increased amphibian predation, or indirectly, via a reduction in available resources due to increased competition (Knapp et al. 2001, Collins 2010, Johnson et al. 2011, Leivas et al. 2013). An additional anthropogenic stressor on amphibian populations is environmental chemicals, like pesticides, fertilizers, and pollutants. Amphibians exposed to these chemicals experience a range of effects, like reduced growth, developmental and reproductive deformities, and death (Lefcort et al. 1998, Sparling et al. 2001, Hayes et al. 2002, 2003, Collins 2010). Finally, habitat destruction may play a major role in the decline of many amphibian populations. Reduced habitat area and disrupted breeding sites are both results of urban development, drainage of wetlands, and deforestation for commercial reasons (Blaustein and Wake 1995, Lehtinin et al. 1999, Collins and Storfer 2003, Becker et al. 2007, Johnson et al. 2011).

As with climate change, it is possible that these anthropogenic factors work in conjunction with other factors to cause amphibian declines. Movement of large numbers of amphibians on a global scale inevitably leads to spread of infectious fungal and viral diseases into previously naïve populations, an all too common occurrence (Mazzoni et al. 2003, Picco and Collins 2008, Catenazzi et al. 2010, Schloegel et al. 2012), despite the fact that two amphibian diseases (the fungus *B. dendrobatidis* and members of the ranavirus family) are now internationally notifiable diseases designated by the World Organization for Animal Health (Schloegel et al. 2010). One of the best known examples of this is global movement of *Xenopus laevis*. These frogs have been used for decades in

research, as pets, and for pregnancy tests, and have more recently been discovered as a common carrier species of *B. dendrobatidis* (Weldon et al. 2004, Rachowicz et al. 2005). In another example of collaborating stressors, larval tiger salamanders experienced reduced survival when exposed to the pesticide carbaryl and the *Ambystoma tigrinum* virus, when compared to exposure to either stressor alone. Likewise, carbaryl inhibits the antimicrobial peptide defense of frogs, which is thought to be protective against many pathogens (Davidson et al. 2007). Interestingly, the pesticides thiophanate-methyl, chlorothalonil, and atrazine appeared to reduce the infection levels or completely cure amphibians infected by the lethal fungal pathogen *Batrachochytrium dendrobatidis* (Hanlon et al. 2012, McMahon et al. 2013), most likely due to the ability of the pesticides to inhibit *B. dendrobatidis* growth (Hanlon and Parris 2012).

Emerging infectious diseases of amphibians

Amphibians are known to be hosts to a variety of bacterial, viral, and fungal pathogens. *Aeromonas hydrophila* is an opportunistic bacterium present in healthy frogs (Hird et al. 1981) that is capable of causing "red leg" disease (Hubbard 1981, Carey et al. 1999). Other pathogenic bacteria that infect amphibians are *Mycobacterium* spp. and *Flavobacterium indologenes* (Olson et al. 1992, Carey et al. 1999, Hill et al. 2010). Amphibians are also hosts to iridoviruses in the genus *Ranavirus,* such as the *Ambystoma tigrinum* virus. These are double-stranded DNA viruses that can infect reptiles and fish in addition to amphibians (Daszak et al. 2003) and can cause mortality (Bollinger et al. 1999, Docherty et al. 2003). Fungal pathogens can also infect amphibians, including *B. dendrobatidis* (Berger et al. 1998, Longcore et al. 1999, Pessier et al. 1999),

Batrachochytrium salamandrivorans (Martel et al. 2013), and *Basidiobolus ranarum* (Carey et al. 1999, Taylor et al. 1999). While not common, it is possible for some amphibian species to experience simultaneous infections by bacterial pathogens, ranaviruses, and *B. dendrobatidis* (Miller et al. 2008, Hill et al. 2010). The recently discovered *Batrachochytrium salamandrivorans* may prove to be linked to multiple declines in the future due to its extreme lethality (Martel et al. 2013), but little is known about this newly emerging pathogen. Although bacteria, viruses, and *B. ranarum* can be lethal to amphibians, the only pathogen clearly linked to population declines is the fungus *B. dendrobatidis* (Carey et al. 1999, Daszak et al. 2003). Because many of these pathogens infect the skin, studying skin immune defenses will provide information about protection against *B. dendrobatidis* and other pathogens of this vital organ.

Batrachochytrium dendrobatidis

Batrachochytrium dendrobatidis causes the amphibian skin disease chytridiomycosis (Berger et al. 1998, Longcore et al. 1999, Pessier et al. 1999). This pathogen is in the phylum Chytridiomycota, a basal fungal lineage (James et al. 2006). It has been linked to multiple amphibian population declines around the globe (reviewed in Wake and Vredenburg 2008, Collins 2010, Fisher et al. 2012). It may have played a role in as many as 90 amphibian extinctions since 1980 (Pennisi, 2009), including the disappearance of the Monteverde harlequin frog (La Marca et al. 2005; Pounds et al. 2006), the Eungella gastric-brooding frog (Retallick et al. 2004), and the sharp-snouted day frog (Schloegel et al. 2006). *Batrachochytrium dendrobatidis*-related declines have even been observed in protected habitats (Bosch et al. 2001, Rovito et al. 2009).

This pathogen's life cycle (Fig. 1-1) begins with a motile, flagellated zoospore (Longcore et al. 1999, Pessier et al. 1999, Berger et al. 2005a). Upon reaching a host cell, zoospores encyst, resorb their flagellum, and form a cell wall, a germ tube, and rhizoids as they proceed through the germling and thalli stages (Berger et al. 2005a, Greenspan et al. 2012; Van Rooij et al. 2012). This eventually results in mature, urn-shaped zoosporangia which fill with newly differentiated zoospores. Zoosporangia have discharge papillae through which zoospores are released and go on to infect other hosts (Berger et al. 1998, 2005, Longcore et al. 1999). The duration of this life cycle *in vitro* is 4-5 days at 22ºC (Berger et al. 2005a, Woodhams et al. 2008).

Batrachochytrium dendrobatidis colonizes keratinized epithelial cells of adult amphibians and keratinized mouthparts of tadpoles and causes the skin disease chytridiomycosis (Berger et al. 1998, Longcore et al. 1999, Pessier et al. 1999, Fellers et al. 2001). The pathogen infects cells in the outermost layers of the epidermis, specifically in the stratum granulosum and stratum corneum. The keratinoyctes in these layers are gradually shed during the natural skin turnover process. Interestingly, the life cycle of *B. dendrobatidis* seems almost synchronized with this process, suggesting that *B. dendrobatidis* has adapted specifically as an amphibian skin pathogen (Berger et al. 2005a, Voyles et al. 2011). Symptoms of chytridiomycosis include appetite loss, reduced weight, lethargy, excess skin sloughing, skin reddening, abnormal posture, and loss of righting reflex (Berger et al. 1998, 2005b, Parker et al. 2002). The disease ultimately results in cardiac arrest due to disruption of sodium and potassium ion transport resulting in osmotic imbalance (Voyles et al. 2007, 2009, 2012, Marcum et al. 2010).

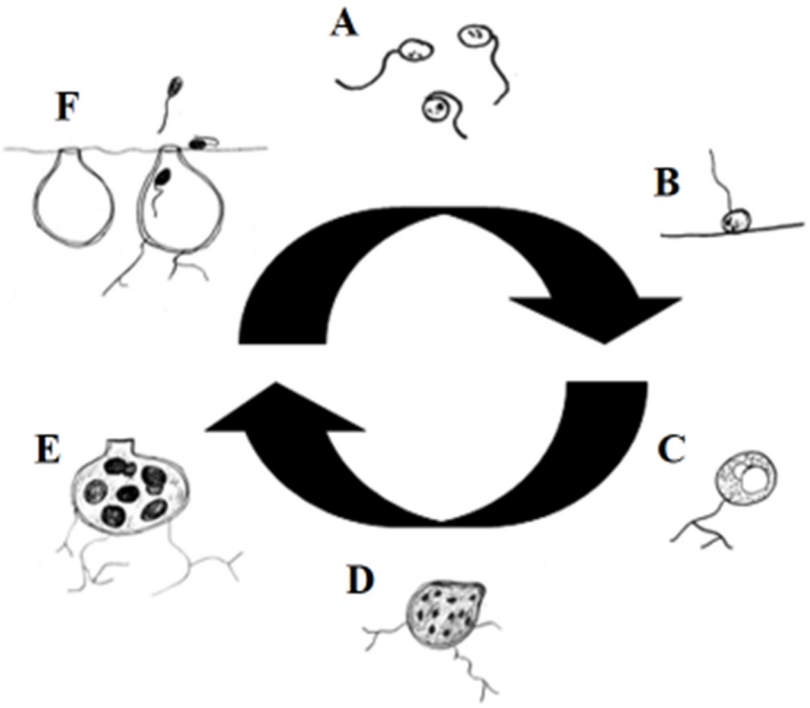


Figure 1-1. ***Batrachochytrium dendrobatidis*** **life cycle.** The life cycle of this pathogen begins with a (A) flagellated zoospore that (B) enters an amphibian host cell, (C) matures into a thallus and (D) becomes multinucleate by mitotic divisions. (E) Zoospores are formed within the zoosporangium by a cleavage process and (F) released into the environment through one or more discharge papillae. (Images of *B. dendrobatidis* life stages were drawn by Dr. Robert Brucker, Vanderbilt University, Nashville, TN)

In order to infect a host cell, zoospores must survive chemical defenses in the host mucus, including bacterial antifungal metabolites, antimicrobial peptides (AMPs), and mucosal antibodies (reviewed in Rollins-Smith et al. 2009; 2011). Development of pathogen-specific IgM, IgX, and IgY antibodies of unknown effectiveness following skin exposure in *Xenopus laevis* indicates a role for adaptive immunity (Ramsey et al. 2010); however, the adaptive and lymphocyte-mediated immune responses to *B. dendrobatidis* are known to be impaired (Berger et al. 1998, 2005b, Rosenblum et al. 2009, Fites et al. 2013). While some amphibian species are more resistant to chytridiomycosis than others (Peterson et al. 2007, Woodhams et al. 2007, Murphy et al. 2009), the reasons for this are not well understood. Thus, a thorough understanding of the antimicrobial peptide defenses and symbiotic skin bacteria in the mucus is critical to understanding effective anti-chytrid defenses.

Symbiotic Bacteria

An often quoted assertion is that the human body contains ten times more bacterial cells than human somatic cells (Luckey 1972). The microbial communities are a subject of much study regarding the physiological and ecological roles that symbionts play for numerous organisms, from insects to humans (reviewed in Fraune and Bosch 2010, Feldhaar 2011, Rosenberg and Zilber-Rosenberg 2011, Goodrich-Blair and Hussa 2013). Benefits for the host organism are diverse, with symbionts contributing to nutrition by synthesizing essential amino acids and vitamins or enabling host digestion of otherwise indigestible dietary components (Hill 1997, Moran 2007, Goodman et al. 2009, Gunduz and Douglas 2009, Chaucheyras-Durand and Durand 2010, Muegge et al. 2011).

Microbes are also important for successful immune system function. Evidence supports a role for microbial symbionts in peripheral and germinal center lymphocyte activity, development of gut immunity, and protection of the skin from pathogens and from overgrowth of already present opportunistic pathogens (Weinstein and Cebra 1991, Dobber et al. 1992, Cebra 1999, Mazmanian et al. 2005, Gallo and Nakatsuji 2011, Royet et al. 2011, Hooper et al. 2012). When the normal symbiotic state is altered by microbial imbalances, a state known as dysbiosis ensues, the result is often disease. For example, inflammatory bowel disease, obesity, non-alcoholic fatty liver disease, and type 1 diabetes all may be related to dysbiotic states (Tamboli et al. 2004, Ley 2006, Kang et al. 2010, Henao-Mejia et al. 2012, Vaarala 2013).

Symbiotic bacteria of amphibian skin may also play an important role in protection against disease for their hosts. Recent studies suggest that antifungal metabolites produced by symbiotic skin bacteria may be a significant defense against *B. dendrobatidis* (Becker et al. 2009, 2010, Becker and Harris 2010, Woodhams et al. 2012a). The most well-known example is *Janthinobacterium lividum*, which produces violacein and indole-3-carboxaldehyde (Brucker et al. 2008a). Another bacterium, *Lysobacter gummosus*, secretes 2,4-diacetylphloroglucinol (Brucker et al. 2008b). These metabolites inhibit *B. dendrobatidis* growth *in vitro* at micromolar concentrations and it has also been demonstrated that *B. dendrobatidis* zoospores move away from these compounds (Lam et al. 2011). Thus, the skin microbiome may constitute an important innate skin defense against chytridiomycosis (Fig. 1-2).

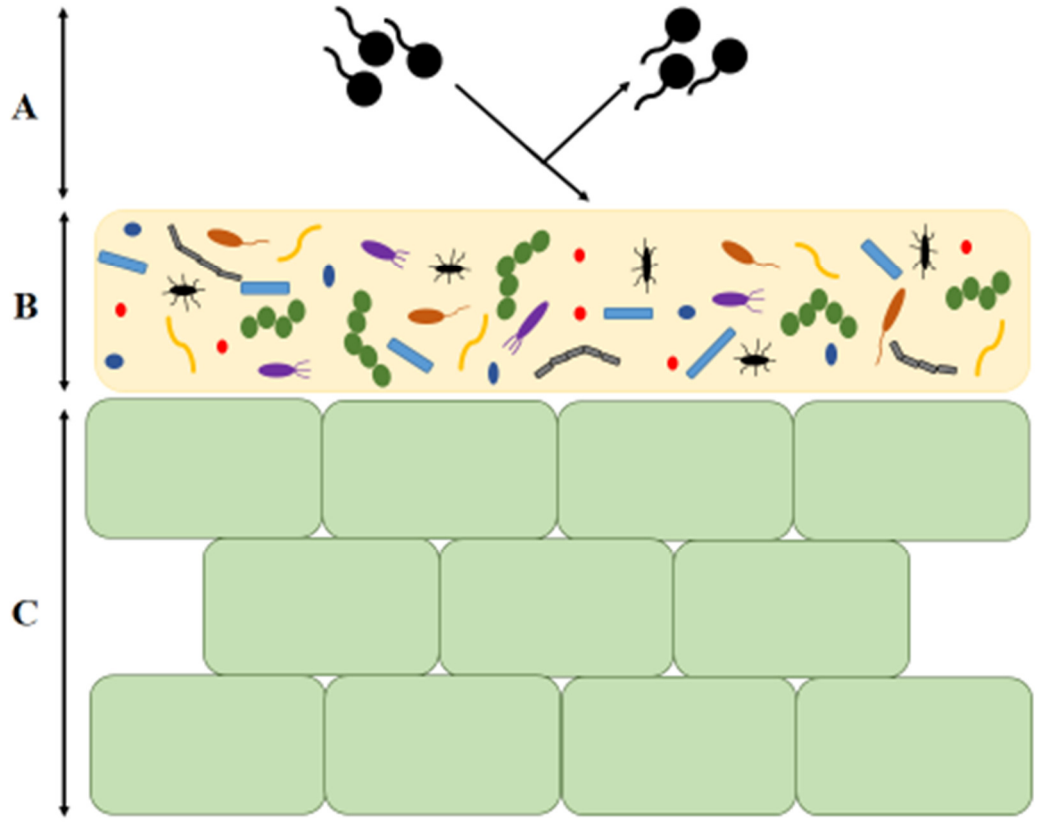


Figure 1-2. Skin bacteria defend against *Batrachochytrium dendrobatidis*. Chytridiomycosis may be prevented or ameliorated when (A) *B. dendrobatidis* zoospores are killed or exhibit chemotaxis away from (B) antifungal metabolites secreted by diverse symbiotic skin bacteria present in the mucosal layer that covers the (C) host epithelium. (Note, diagram is not drawn to scale.)

Bioaugmentation as a conservation strategy

One potential conservation strategy for endangered amphibians involves the addition of bacteria with known antifungal activity to the skin of new hosts in a prophylactic process called bioaugmentation (Becker et al. 2009, Harris et al. 2009a, 2009b). Such bioaugmentation techniques might be improved through the use of antifungal bacteria isolated from closely related species or through the use of antibiotics to reduce native bacteria and create a niche in which beneficial bacteria can establish colonization. Furthermore, whether the skin microbiome offers a significant defense against *B. dendrobatidis* infection *in vivo* independent of the antimicrobial peptide defense has not been satisfactorily established. Answering this question will be critical to a field endeavoring to improve this defense against a deadly pathogen.

Antimicrobial peptides

Antimicrobial peptides (AMPs) have long been known to be an important part of innate immunity in many organisms, including insects, plants, and vertebrates like humans and amphibians (Nicolas and Mor, 1995; Zasloff 2002). In humans, AMPs are primarily produced by skin keratinocytes as a mechanism to limit microbial overgrowth on the skin. However, AMPs are also found in the antimicrobial arsenal of human mast cells, neutrophils, saliva, and sweat as part of the larger immune system (Wiesner and Vilcinskas 2010, Bernard and Gallo 2011).

In amphibians, AMPs are synthesized and stored in dermal granular glands (Fig. 1-3A). Natural secretion from these glands results from local α-adrenergic nerve stimulation (Sjoberg and Flock 1976), which can be induced by subcutaneous injection of norepinephrine into the dorsal lymph sac. This causes contraction of myoepithelial cells, forcing peptide granules onto the surface of the skin where they co-exist in the mucus with symbiotic skin bacteria (Benson and Hadley 1969, Dockray and Hopkins 1975). This AMP secretion process occurs constitutively, but is upregulated upon frog alarm or stress. While most active in the first 15 minutes after release onto the skin, peptides are detectable for up to two hours. Degradation of the peptides over time in this way is likely important to protect the integrity of the skin as well as symbiotic skin bacteria also present in the mucus (Pask et al. 2012).

Amphibian AMP activity against *B. dendrobatidis*

These amphipathic cationic α-helical peptides are typically 10-50 amino acids and inhibit fungi, viruses, and bacteria via membrane disruption (reviewed in Nicolas and Mor 1995, Rinaldi 2002). Specifically, cationic peptides are attracted to negatively-charged membranes, which they easily disrupt by way of their amphipathic structure (Fig. 1-3). Antimicrobial peptides from multiple amphibian species are active against *B. dendrobatidis* at micromolar concentrations (Rollins-Smith et al. 2002a, 2002c, Ramsey et al. 2010, Conlon et al. 2013, reviewed in Rollins-Smith and Conlon 2005, Rollins-Smith 2009). Species that express skin peptides that inhibit *B. dendrobatidis* growth *in vitro* tend to be more resistant to *B. dendrobatidis*. In contrast, declining species are more likely to express peptides with decreased *in vitro* activity (Woodhams et al. 2006).

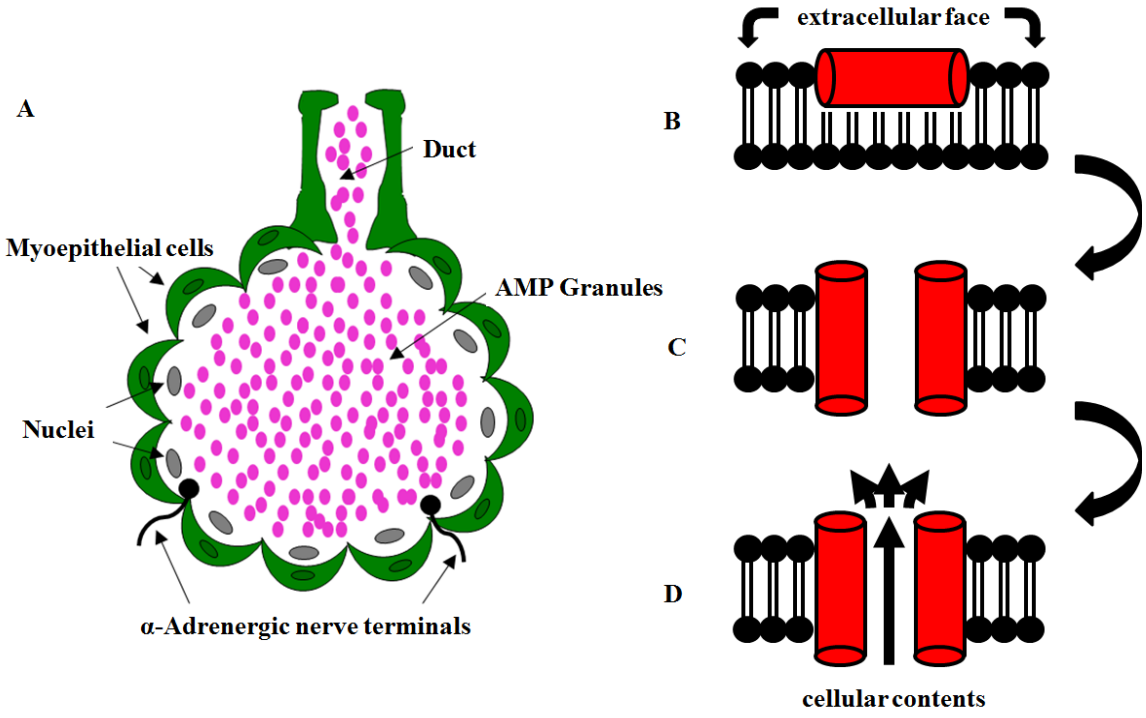


Figure 1-3. Stimulated amphibian granular glands secrete antimicrobial peptides that act via membrane disruption. (A) Myoepithelial cells surrounding granular glands contract due to stimulation of α-adrenergic nerve terminals. This causes secretion of antimicrobial peptides (AMP granules) through a duct onto the epidermal surface. (Figure from Gammill et al. 2012). AMP inhibitory activity is a result of their ability to (B) interact with biological membranes due to their amphipathic, cationic nature. Once associated with the membrane, (C) AMPs aggregate and insert themselves across the bilayer, forming pores that (D) lead to cell lysis and allow the cellular contents to exit the cell.

Antifungal drug treatments for chytridiomycosis

Amphibians brought into captivity are often infected with *B. dendrobatidis* and require quarantine and treatment before introduction into captive colonies. Because some species are now secure only in captivity (Weldon and du Preez 2004, Gagliardo et al. 2008), this is especially important for zoos, wildlife refuges, and conservation centers. The only antifungal drug in widespread use is itraconazole (Nichols and Lamirande 2000). This drug causes membrane permeability by binding fungal cytochrome P-450, which prevents synthesis of ergosterol (Panda 1997, Leyden et al. 1998), an important component of the fungal cell membrane (Fig. 1-4A). However, itraconazole can be toxic to tadpoles and adults (Garner et al. 2009, Woodhams et al. 2012b). Identification and validation of new antifungal drugs to combat chytridiomycosis is needed (Fig. 1-4).

One drug worthy of further study is amphotericin B. It inhibits the growth of this pathogen *in vitro* (Berger et al. 2009, Martel et al. 2011) by binding to ergosterol (Fig. 1-4A) and disrupting cell membrane stability (Gray et al. 2012), but it has yet to be examined *in vivo*. The well-known antibacterial drug chloramphenicol has had some success in treating infected frogs (Bishop et al. 2009, Young et al. 2012), though sample sizes were low and its inhibitory properties have not been quantified *in vitro*, so it is unclear if ideal concentrations were used in these trials. While chloramphenicol is known for inhibiting 70S ribosomes and disrupting bacterial protein synthesis (Gale and Folkes, 1953), the mechanism through which it inhibits *B. dendrobatidis* is unknown. Another potential drug of interest is the chitin synthase inhibitor, Nikkomycin Z (Fig. 1-4A). It is known for its inhibition of *Coccidioides immitis,* which causes coccidioidomycosis (Hector et al. 1990), but it has yet to be tested against *B. dendrobatidis*.

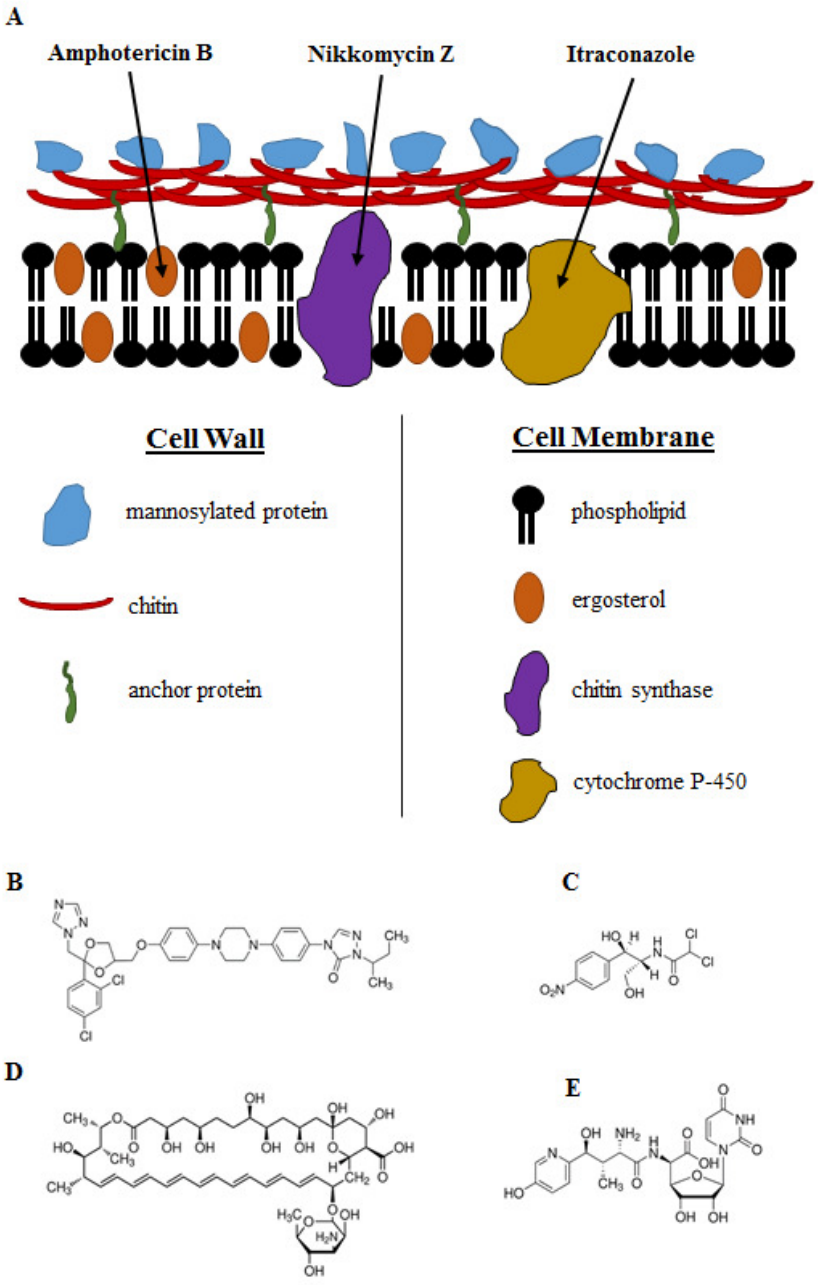


Figure 1-4. Alternative antifungal drugs for chytridiomycosis. (A) Many components of the *B. dendrobatidis* cell wall and cell membrane make excellent antifungal drug targets, including ergosterol and enzymes that synthesize ergosterol and chitin. Unlike other types of fungi, *B. dendrobatidis* lacks genes for synthesis of β-1,3-glucans and β-1,6-glucans, which appear to have entered the fungal lineage by horizontal gene transfer following chytrid divergence (Ruiz-Herrera & Ortiz-Castellanos 2010). (B) The triazole drug itraconazole is currently the only commonly used antifungal drug for *B. dendrobatidis*, though it is not without serious side effects. Alternative antifungal drugs to examine include (C) Amphotericin B, which binds to ergosterol and disrupts fungal cell membranes, (D) the antibacterial agent chloramphenicol, whose antifungal mechanism is not understood, and (E) the chitin synthase inhibitor Nikkomycin Z. Images of compounds in Panels B – E are from Sigma-Aldrich.

Significance

Amphibians have long been considered by the scientific community to be environmental sentinels due to their ectothermic character and the importance their skin plays in many biological processes (Roy 2002). Thus, amphibians are indicators of ecological integrity in addition to being integral members of food chains. Emerging diseases like chytridiomycosis that result in declines or extinctions constitute a serious threat to the health of entire ecosystems (Fisher et al. 2012). Further, amphibian skin has many similarities with human skin and has been helpful as a research model, especially in studies focused on ion transport through tight epithelia. Better understanding of the amphibian integument and how its various components function may improve understanding of similar facets of human skin (Haslam et al. 2013).

Batrachochytrium dendrobatidis-related extinctions may result in lost medicinal potential of amphibian natural products. Important human bacterial, fungal, and viral pathogens, including *Staphylococcus aureus*, *Escherichia coli*, *Candida albicans*, and HIV, are sensitive to amphibians AMPs (Goraya et al. 1998, 2000, VanCompernolle et al. 2005). There is also evidence that amphibian AMPs can target endotoxin (Schadich et al. 2013), human tumors (Koszałka et al. 2011, Wang et al. 2012), and could be used as a spermicide/microbicide to prevent pregnancy while reducing transmission of HIV and other sexually-transmitted infections (Zairi et al. 2009). Many AMPs have comparably low activity against human cells (Conlon et al. 2007, Mechkarska et al. 2010, Asoodeh et al. 2012, Wang et al. 2012) and those with undesirable activity against human cells can be selectively mutated to decrease human cell toxicity without reducing antimicrobial activity (Kamech et al. 2012).

Amphibian AMPs have the potential to contribute to human agricultural and biotechnical endeavors as well. For example, temporin A, an AMP from *Rana temporaria*, has been transfected into potatoes and successfully defended against potato blight in laboratory studies, indicating that it may be a feasible option to protect one of the world's most common food crops against a costly pathogen (Osusky et al. 2004). Thus, there is a significant risk of lost medicinal and biotechnical potential from species declining due to *B. dendrobatidis*. Further study of these compounds and identification of new AMPs may contribute to significant advances in human medicine and important agricultural endeavors.

Antifungal drugs work by targeting differences between host and fungal cells to kill the fungal pathogen while causing little to no harm to the host. Unlike bacteria, both fungi and amphibians are eukaryotes and thus have many molecular similarities, making drug development more challenging. Antifungal drug development for human hosts is challenging for the same reason. Many antibiotics originate as natural products from bacteria, including the antifungal drugs chloramphenicol (Gottlieb 1954), amphotericin B (Trejo and Bennett 1963), and nikkomycin Z (Möhrle et al. 1995). The amphibian skin symbiont *J. lividum* has been studied mostly for its ability to secrete metabolites that inhibit *B. dendrobatidis*, but when cocultured with *Trichophyton rubrum*, the agent of athlete's foot, *T. rubrum* colony growth was impaired (Ramsey et al. 2013). Thus, amphibian skin symbionts may be a rich source of novel antifungal compounds. By isolating and characterizing amphibian symbiotic skin bacteria that naturally produce potent antifungal metabolites, new drugs for both amphibian and human therapies may be identified.

Research Goals

There is ample evidence from field studies that multiple amphibian species are highly susceptible to the pathogen *B. dendrobatidis*, while others are relatively resistant. The factors contributing to this spectrum are uncertain, but may be a result of differing potencies of amphibian innate skin defenses, specifically antimicrobial peptides and symbiotic skin bacteria. The central objectives of this research are to understand which immune defenses in amphibian skin are active against this pathogen and develop novel antifungal drug treatments that cooperate with naturally-present innate skin defenses to treat amphibians with chytridiomycosis.

Objective 1 (Chapter II) was to determine the contribution of the skin microbiome against *B. dendrobatidis* in the absence of AMPs in juvenile (metamorph) *Rana sphenocephala* and identify individual bacterial symbionts that inhibit *B. dendrobatidis in vitro*. Using an antibiotic cocktail to reduce *R. sphenocephala* skin bacteria levels, I examined the effect of the skin microbiome to protect against *B. dendrobatidis* in an infection study. Using MALDI-TOF mass spectrometry to confirm absence of AMPs in juveniles of this species, I conducted this experiment prior to the development of an AMP defense. Finally, I identified culturable skin symbionts of *R. sphenocephala* by 16S sequencing and examined their ability to inhibit *B. dendrobatidis* growth *in vitro.*

Objective 2 (Chapter III) was to quantify the efficacy of known AMPs *in vitro,* characterize new AMPs, and determine the ontogeny of AMP development in *R. sphenocephala*. I determined the minimal inhibitory concentrations (MICs) of four *R. sphenocephala* peptides (Conlon et al. 1999), brevinin-1Sa, brevinin-1Sb, brevinin-1Sc, and Peptide C using highly pure, synthetic peptides. I used norepinephrine to induce

peptide secretion at multiple developmental time points to assess the amounts of peptides produced and the individual peptides present in each frog's secretions. I also determined the impact norepinephrine-induced AMP secretion has on skin bacteria levels and developed a method using two injections of norepinephrine to fully deplete granular glands of AMPs, enabling use of amphibians without AMPs in *in vivo* studies (Appendix A).

Objective 3 (Chapter IV) was to conduct *in vitro* studies with the drug Nikkomycin Z to determine if it is an effective inhibitor of *B. dendrobatidis*. Using a series of *in vitro* assays, I determined this drug's ability to inhibit *B. dendrobatidis* growth, examined its antifungal effects at the cellular level, confirmed its mechanism of action, and determined whether this drug is capable of cooperation with natural mixtures and synthetic purified antimicrobial peptides. This is an important first step in the validation of this drug as a potential inhibitor of *B. dendrobatidis.*

Objective 4 (Chapter V) was to conduct studies with the drugs amphotericin B and chloramphenicol to better quantify their ability to inhibit *B. dendrobatidis in vitro* and determine their effects on known skin innate immune defenses. Amphotericin B and chloramphenicol have previously been reported for their ability to inhibit *B. dendrobatidis* (Berger et al. 2009, Bishop et al. 2009, Martel et al. 2011, Young et al. 2012). However, amphotericin B has not been tested *in vivo* while chloramphenicol trials have had low sample sizes or lacked proper controls. I used *in vitro* growth inhibition assays to identify the MIC for chloramphenicol, to confirm the MIC of amphotericin B, and to assess the ability of these drugs to impact the growth of known *R. sphenocephala* bacterial symbionts. I used my peptide depletion by norepinephrine method, a

microBCA assay, and MALDI-TOF mass spectrometry to assess the effects of these drugs on antimicrobial peptide synthesis. Finally, I conducted *in vivo* treatment trials to determine if these drugs are feasible treatments for chytridiomycosis.

Objective 5 was to develop a method to result in highly pure zoospore cultures or populations of *B. dendrobatidis* cells enriched for a given life stage (Appendix B). Such a method will allow researchers to draw more specific conclusions about specific life stages and the effects of innate immune defenses against them.

CHAPTER II

SKIN BACTERIA DEFEND *RANA SPHENOCEPHALA* JUVENILES AGAINST *BATRACHOCHYTRIUM DENDROBATIDIS*, THE FUNGUS IMPLICATED IN GLOBAL AMPHIBIAN DECLINES

Abstract

A great concern for scientists and naturalists alike is the continuing loss of global biodiversity. This is especially true for amphibians, which are disappearing at an alarming rate. Recent studies suggest that symbiotic bacteria present on amphibian skin may provide some protection against the fungal pathogen *B. dendrobatidis*. Most studies of this "defensive skin microbiome" have focused on the ability of certain bacterial isolates to secrete antifungal metabolites that inhibit *B. dendrobatidis* growth *in vitro*. Few experiments have addressed the effects of reduction of the microbial skin assemblage in combination with assessment of antifungal activity of individual bacterial isolates. Using the southern leopard frog, *Rana sphenocephala*, I demonstrated that the skin of these animals harbors multiple bacterial species capable of inhibiting *B. dendrobatidis* growth *in vitro* and that reduction of bacteria on post-metamorphic juvenile (metamorph) skin using a potent antibiotic cocktail resulted in increased *B. dendrobatidis* infection. Further, I present evidence that this innate bacteria-mediated skin defense precedes the development of an antimicrobial peptide skin defense in juveniles of this species and thus protects at a critical period of development.

Introduction

In order for *B. dendrobatidis* to infect amphibian keratinocytes, the fungal cells must overcome chemical defenses that are present in the host mucus. These include antifungal metabolites from symbiotic skin bacteria, antimicrobial peptides secreted from dermal granular glands, and mucosal antibodies (reviewed in Rollins-Smith et al. 2009, 2011). Because the adaptive immune response to *B. dendrobatidis* is impaired (Berger et al. 1998, 2005b, Pessier et al. 1999, Rosenblum et al. 2009, Fites et al. 2013), understanding the symbiotic skin bacteria and AMP innate defenses is essential to gaining a better understanding of effective anti-*B. dendrobatidis* defenses.

The best examples of beneficial amphibian symbionts are *Janthinobacterium lividum* and *Lysobacter gummosus*, which secrete metabolites that impair *B. dendrobatidis* growth *in vitro* (Brucker et al. 2008a, 2008b). Addition of cutaneous bacteria like these to new hosts in a process called bioaugmentation is a possible conservation strategy (Becker et al. 2009, Harris et al. 2009a, 2009b), which will only be successful if the introduced bacteria persist long-term on the skin. This has not occurred in some bioaugmentation trials (Becker et al. 2012). Development of a short-term antibiotic reduction protocol that would reduce native cutaneous bacteria before the addition of probiotic bacteria may increase the feasibility of bioaugmentation as a conservation strategy. Becker and Harris (2010) used an antibiotic reduction protocol and found that it was associated with increased chytridiomycosis symptoms in *Plethodon cinereus*. However, the highest *B. dendrobatidis* infection levels measured by quantitative PCR were less than a single zoospore per salamander and infections did not differ between groups. Using a more virulent *B. dendrobatidis* strain in a cohort of

amphibians that do not yet express AMPs would be both novel and possibly more effective in testing the role of the skin microbiome.

Thus, my aims in this study were to (1) develop a robust protocol for reducing bacteria on the skin of *Rana sphenocephala*, (2) examine the ability of the skin microbiome of *R. sphenocephala*, as well as individual symbionts, to inhibit *B. dendrobatidis* in highly susceptible juvenile frogs, and (3) report any deleterious health effects of short-term reduction of skin bacteria by antibiotic treatment.

Materials and Methods

Organisms

B. dendrobatidis strain JEL197 (Longcore et al. 1999) was used to test the antifungal activity of individual bacteria *in vitro*. This strain has been used in many previous studies (Rollins-Smith et al. 2006, Woodhams et al. 2006, 2010, Ramsey et al. 2010, Pask et al. 2012, 2013, Conlon et al. 2013), but it has been in culture for a number of years, and its virulence had not been recently tested. Thus, I used zoospores of the isolate "Section Line" for exposures in order to establish significant infections. This isolate was collected by Dr. Jonah Piovia-Scott from *Rana cascadae* and isolated by Joy Worth in the laboratory of Dr. Janet Foley (University of California – Davis, School of Veterinary Medicine). Further, it was highly virulent in recent studies (J. Piovia-Scott, unpublished). Cultures of both strains were maintained in 1% tryptone broth and on tryptone agar at 19 – 21°C and passaged weekly.

Rana sphenocephala juveniles were raised from eggs in outdoor mesocosms and were naïve to *B. dendrobatidis*. *Rana sphenocephala* juveniles from the 2012 cohort

were used in the following experiments: (1) characterization of culturable *R. sphenocephala* skin bacteria, (2) pilot experiments optimizing the bacterial reduction by antibiotics protocol, and (3) when frogs reached one year old, as positive controls for AMP expression in mass spectrometry studies determining peptide presence in newly metamorphosed juveniles. *Rana sphenocephala* juveniles from the 2013 cohort were raised under the same conditions and were used in the following experiments: (1) norepinephrine stimulation of granular glands to assess AMP production at early post-metamorphic time points or (2) in a bacterial reduction and infection experiment to assess the contribution of skin bacteria to defense against chytridiomycosis. The Institutional Animal Care and Use Committee at Vanderbilt University Medical Center or the University of Memphis approved all animal procedures.

Rana sphenocephala **care and husbandry**

In 2012 and 2013, metamorphs were reared by Dr. Shane Hanlon and Dr. Matthew Parris (University of Memphis) in polyethylene tank mesocosms (diameter = 1.83 m) at the University of Memphis Edwards J. Meeman Biological Field Station (MBS), Shelby County, TN (35° 22' N / 90° 01' W). Each tank was filled with ~613 L (30.5 cm) of well water and 300 g of dry deciduous (primarily *Quercus* spp.) leaf litter. One 500 ml aliquot of concentrated plankton suspension, originally collected from a nearby pond, was added to each tank. Fiberglass mesh screens (1-mm mesh) were used as lids to prevent tank colonization by possible *B. dendrobatidis*-infected adults, predators, and other amphibian competitors and to provide shading for each tank. A ramp (65.59 x 121.92 cm piece of fiberglass composite) was placed in each tank at a 30°

angle to simulate the margin of a pond. *Rana sphenocephala* egg clutches (N = 8) were collected and evenly distributed between two prepared tanks. After hatching and reaching the free-swimming stage (Gosner stage 25 [Gosner 1960]), ~270 tadpoles were randomly selected from both tanks and distributed across nine rearing tanks at densities of ~30/tank (densities comparable to natural *R. sphenocephala* densities [Morin 1983]). Rearing tanks were undisturbed until the first metamorphs were observed in June and were removed and placed into previously established terrestrial enclosures comprised of 3 polyethylene tanks, each placed at a 25° slant. The lower half of each tank was filled with water taken from the tadpole rearing tanks while the upper half was filled with sand and leaf litter, allowing metamorphs to assume their natural semi-terrestrial life. Metamorphs were removed from the tanks and transported to Vanderbilt University in two batches in mid-summer.

Once in the laboratory, frogs were kept at 21–25°C and fed live vitamin-dusted crickets three times weekly, with water changed four times weekly. Frogs were kept either in dechlorinated tap water or in mesocosm water. Mesocosm water was water containing plant matter, zooplankton, environmental bacteria, etc. removed from mesocosms the same day the metamorphs were collected. Mesocosm water was refrigerated at 4–7ºC until its use in the experiment described, beginning 7 days post-metamorphosis and continuing for a period of five weeks after the metamorphs were collected. Containers were placed at an incline to result in wet and dry areas in each tank. During the *B. dendrobatidis* infection study, metamorphs were housed individually in sterile 280 ml plastic containers. Metamorphs used to assess the antimicrobial peptides

of this species were housed in sterile polystyrene tanks measuring 44 × 24 × 20 cm at a density of 10 frogs per tank.

Quantification and MALDI-TOF mass spectrometry analysis of amphibian AMPs

To prevent unnecessary stress, I collected peptides from a group of metamorphs separate from those used in the infection experiment. I collected peptides at one week (2013 cohort) and one year (2012 cohort) post-metamorphosis by injection of 40 nmol/g body weight norepinephrine-HCl (Sigma, St. Louis, MO) dissolved in amphibian phosphate-buffered saline (APBS, 6.6g NaCl, 0.2g KH_2PO_4, 1.15g anhydrous Na_2HPO_4 in 1L distilled water, pH = 7.4) as previously described (Gammill et al. 2012). This dose of norepinephrine is a pharmacological dose that exceeds concentrations that frogs would experience under natural physiological conditions (Pask et al. 2012, 2013). I chose this dosage to induce a high level of peptide secretion resulting in a large quantity of peptides for use in *in vitro* studies. Metamorphs at one week post-metamorphosis were submerged in 10 ml collection buffer (50 mM sodium chloride, 25 mM sodium acetate, pH = 7.0) (Nutkins and Williams 1989) for 15 min while larger year-old frogs were submerged in 100 ml so the same portion of skin area was covered. The collection buffer was acidified by addition of 1% trifluoroacetic acid following removal of the frog to disable endogenous proteases (Resnick et al. 1991). I enriched collected secretions for antimicrobial peptides by passage over C18 Sep-Pak cartridges (Waters Corporation, Milford, MA) and quantified the resulting product with a microBCA assay (Pierce, Rockford, IL) using bradykinin standards (amino acid sequence: RPPGFSPFR) as previously described (Rollins-Smith et al. 2006). Finally, I calculated peptide

concentrations as μg peptide/ml of mucus on the skin based on the surface area of each frog's skin and the approximate volume of mucus on the skin as previously described (McClanahan and Baldwin 1969, Brucker et al. 2008a, Ramsey et al. 2010).

I determined the presence or absence of individual peptides in skin secretions of each frog by matrix-assisted laser-desorption ionization time-of-flight (MALDI-TOF) mass spectrometry using a previously described protocol (Rollins-Smith et al. 2006, Gammill et al. 2012). Briefly, I spotted 0.6 μl peptides resuspended at 1 mg/ml in HPLC-grade water onto a stainless steel MALDI plate with the same volume of matrix [α-cyano-4-hydroxycinnamic acid (CHCA, Fluka, Sigma, St. Louis, MO), 60% acetonitrile, 39.6% HPLC-grade water, and 0.4% trifluoroacetic acid (v/v/v)] and allowed the mixture to air dry. I used the following peptide standards (Sigma, St. Louis, MO) to calibrate the mass spectrometer: bradykinin fragment 1-7 (*m/z* 757.3997), human angiotensin II (*m/z* 1046.5423), $P_{14}R$ synthetic peptide (*m/z* 1533.8582), adrenocorticotropic hormone fragment 18-39 (*m/z* 2464.1989), and bovine oxidized insulin chain B (*m/z* 3494.6513). I used an Ultraflex III mass spectrometer (Bruker Daltonics, Billerica, MA) in delayed extraction, positive ion, reflector mode to collect 250 laser shots and analyzed the resulting MS spectra with Data Explorer v4.4 software (Applied Biosystems, Foster City, CA).

A previous study examining peptides in juvenile *R. pipiens* observed a phenomenon in which some unknown component within the peptide samples prevented desorption or ionization of known peptides within the samples and standard peptides added to the samples (M. Groner, personal communication). To confirm that a similar effect was not preventing peptide desorption or ionization in one-week post-

metamorphosis samples, I spiked synthetic brevinin-1Sb (>90% pure, MW =2535.22, obtained from Lifetein, South Plainfield, NJ), into the samples (N = 10) at a 1:1 ratio such that the final concentrations of brevinin-1Sb and partially purified one-week post-metamorphosis peptides were each at a concentration of 500 µg/ml in the mixture. A negative control of HPLC-grade water and positive controls (N = 5) containing 500 µg/ml brevinin-1Sb in HPLC-grade water alone were also prepared. I performed MALDI-TOF MS analysis to compare the amount of brevinin-1Sb signal in the positive controls to the amount of brevinin-1Sb signal in samples containing the one-week post-metamorphosis peptides in addition to the brevinin-1Sb. Data Explorer v4.4 software (Applied Biosystems, Foster City, CA) was used to calculate the area under the brevinin-1Sb peaks, including the monoisotopic mass and the sodium adduct of that peptide.

Antibiotic reduction and quantification of skin bacteria

In order to reduce the levels of skin bacteria on *Rana sphenocephala*, I modified a previously reported method (Becker and Harris 2010) by adding penicillin, streptomycin and enrofloxacin to the cocktail. The final solution to which frogs were exposed was 24 mg/L cephalexin (Teva Pharmaceuticals, North Wales, PA), 14.5 mg/L sulfamethazine and 2.9 mg/L trimethoprim (Mardel Maracyn Plus, Sergeant's Pet Care Products, Inc., Omaha, NE), 60 mg/L enrofloxacin (Fluka, Sigma, St. Louis, MO), 100 mg/L streptomycin, and 100,000 I.U./L penicillin (Mediatech, Inc., Manassas, VA). At 7–10 days post-metamorphosis, I placed metamorphs in 20 ml cocktail for 48 hours and refreshed the cocktail at 24 hr to ensure continued effectiveness. Control metamorphs were in the same volume of mesocosm water that was changed at the same time. Prior to

and immediately after this treatment, metamorphs (N = 80) were rinsed with 50 ml sterile water to remove transitory environmental bacteria and swabbed with sterile swabs (Medical Wire & Equipment Co., Advantage Bundling, Durham, NC), which were added to 1 ml sterile dechlorinated water and vortexed gently (t = 5s) to dislodge bacteria from the cotton swab. Then, I conducted plate counts on Difco R2A agar (Becton, Dickinson and Co., Sparks, MD) with incubation at 19-21°C for 7 days prior to counting colony-forming units (cfu) per swab. Finally, I conducted an additional plate count to assess the number of cfu per ml mesocosm water by plating multiple dilutions on Difco R2A agar and incubating in the same conditions.

***Batrachochytrium dendrobatidis* (*Bd*) exposure**

I harvested zoospores of the *B. dendrobatidis* "Section Line" isolate as previously described (Rollins-Smith *et al.* 2002a, 2002b, 2002c, Pask et al. 2012) by flooding agar culture plates with 1% tryptone broth containing 100 µg/ml streptomycin and 100 I.U./ml penicillin (Mediatech, Inc., Manassas, VA) for 10 min and filtering broth through 20 µm pore mesh filters (Spectrum Laboratories Inc., Rancho Dominguez, CA). I counted zoospores in the filtrate on a hemocytometer (Hausser Scientific, Horsham, PA,) and resuspended them in 1% tryptone broth at 10^6 cells/ml. I exposed metamorphs with or without skin bacteria to zoospores on days 4, 11, and 18. Metamorphs (Bd+ groups) in individual sterile containers were exposed to 10^6 zoospores/ml by adding approximately 250 µl of concentrated zoospores in tryptone broth to metamorphs in 20 ml of either dechlorinated tap water with 100 µg/ml streptomycin and 100 I.U./ml penicillin (Bacteria– = Bacteria-reduced groups) or mesocosm water (Bacteria+ = Bacteria-intact

groups). Control frogs (Bd– groups) were exposed to a vehicle control containing the same volume of tryptone broth without zoospores. Each exposure lasted 24 hr, when I refreshed the water to remove zoospores. I examined metamorphs for signs of chytridiomycosis and death each day after setting up the experiment.

Quantification of *B. dendrobatidis* infection levels

To quantify the level of *B. dendrobatidis* infection, I swabbed the skin of each metamorph (Kriger et al. 2006) on days 6, 13, 20, 27, and 34 with sterile cotton swabs (Medical Wire & Equipment Co., Advantage Bundling, Durham, NC). I extracted DNA from each swab per manufacturer's instructions (DNeasy Blood and Tissue DNA Extraction Kit, Qiagen, Valencia, CA). A swab containing a known amount of *B. dendrobatidis* zoospores served as a positive control in each extraction. A sterile swab without zoospores provided a negative control. I used a real time quantitative PCR (qPCR) to quantify *B. dendrobatidis* DNA from each swab. Briefly, 5 µL DNA was added to each well of a 96-well PCR plate (Fisher Scientific, Pittsburgh, PA) with 12.5 µL 2X TaqMan Buffer (Applied Biosystems, Carlsbad, CA), 5.75 µL sterile water, 0.625 µL of chyt3 and 5.8S primers (Boyle et al. 2004) (Eurofins MWG Operon, Huntsville, AL), and 0.5 µL TaqMan MBG probe (Applied Biosystems, Carlsbad, CA). Reagents were assembled in a 'master mix' prior to their combination with the DNA in order to ensure identical amplification conditions for each DNA sample. Finally, a no template control containing reagents without DNA was included in each qPCR. Zoospore equivalents were quantified by comparing fluorescence intensity to a standard curve of known zoospore DNA dilutions equivalent to 1000, 100, 10, and 1 *B. dendrobatidis*

zoospores using an Mx3000P Real-Time PCR machine (Stratagene, La Jolla, CA) and default Stratagene conditions (10 min at 95°C; 40 cycles of 30 s at 95° C, 1 min at 55°C, and 1 min at 72° C). All controls, standards, and experimental samples were run in triplicate and values were averaged to quantify a final infection level for each sample. DNA standard for quantification of *B. dendrobatidis* zoospore equivalents were kindly provided by Dr. Alex Hyatt, CSIRO, Geelong, Victoria, Australia.

Isolation, identification, and characterization of bacterial isolates

I rinsed newly metamorphosed *R. sphenocephala* with 50 ml sterile water to remove transient environmental bacteria, and swabbed each frog with sterile cotton swabs at approximately 18 hr after arrival in the laboratory. During this time, metamorphs were housed in sterile polystyrene containers to assure that they did not acquire laboratory bacteria before swabbing. After swabbing, the frogs were placed in tanks containing mesocosm water to simulate their natural environment and reduce the chance of acquiring non-native skin bacteria. I struck swabs across R2A plates to isolate individual colonies of diverse morphologies in collaboration with Tim Chappell (Vanderbilt University), an undergraduate student whom I helped to mentor in the lab. We recorded phenotypic characteristics of the individual colonies, including size and color, and froze stock cultures in Luria-Bertani broth and glycerol for storage at -80°C.

Individual isolates of interest were identified by 16S rDNA sequencing by Dr. Douglas Woodhams with assistance from Franklin Roman and Holly Archer in the laboratory of Dr. Valerie McKenzie (University of Colorado, Boulder). Isolates were grown in 96-well plates with R2A media. DNA was extracted using an UltraClean 96-

Well Microbial DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA) with the centrifugation protocol. The PCR reaction contained: 12 µL PCR water, 10 µL 5 Prime Master Mix, 1 µL of the forward and reverse primers at 10 µM concentration, 1 µL $MgCl_2$, and 1 µL genomic DNA. PCR universal primers consisted of 515F (5'-GTG CCA GCM GCC GCG GTA A-3') and 1391R (5'-GAC GGG CRG TGW GTR CA-3'). The PCR profile included an initial denaturation step of 94°C for 2 min, followed by 29 cycles of 94°C for 30 s, 55.5°C for 60 s, and 72°C for 90 s and final extension at 72°C for 12 min. Amplicons were sent to Beckman Coulter Genomics for Sanger sequencing. Geneious v.6.1 was used to create contigs by combining forward and reverse reads. The Quantitative Insights into Microbial Ecology (QIIME) v.1.7 was used to identify isolates based on RDP (Ribosomal Database Project) classification (Caporaso et al. 2010). To confirm each isolate's identity with BLAST computational analysis, I searched for each FASTA sequence using a standard nucleotide BLAST within the nucleotide collection database (nr/nt) optimized for highly similar sequences (megablast). Accession numbers of closest sequence matches are reported. All BLAST matches reported here matched isolate sequences with identities of ≥99%.

Growth inhibition of *B. dendrobatidis* by bacterial supernatants

Following inoculation of sterile 1% tryptone broth with frozen stock cultures, pure cultures of bacterial isolates were grown to optical densities of 0.20 ± 0.02 at λ = 630 nanometers (OD_{630}) then pelleted by centrifugation for one minute at room temperature at 15,000×g in a Beckman Microfuge E™ centrifuge (Beckman Coulter, Inc., Brea, CA). Supernatants were harvested following centrifugation and filtered

through 0.2 μm pore filters (VWR International, LLC, Radnor, PA). Zoospores were harvested from agar plate cultures of JEL197 (Longcore et al. 1999), counted, and resuspended in 1% tryptone broth at 10^6 cells/ml as described above then cultured 1:1 with bacterial supernatants in replicates of five for 7 days at 19-21°C in 96-well flat-bottom microtiter plates (BD Falcon, Franklin Lakes, NJ) using a previously published protocol (Rollins-Smith et al. 2002a, 2002b, 2002c). Five replicate wells each of positive and negative controls of either living or heat-killed (10 min at 60°C) zoospores in tryptone broth without bacterial supernatant were included on each plate along with five replicate wells of 100 μl tryptone broth to serve as a blank. During the incubation, plates were wrapped in parafilm to prevent evaporation. Optical densities (OD_{490}) were measured by an MRX Microplate Reader (Dynex Technologies, Inc., Chantilly, Virginia) at day 0 and day 7 with a change in optical density indicating *B. dendrobatidis* growth. Each supernatant's effect on *B. dendrobatidis* growth was expressed as percent inhibition or percent enhancement. This measure was calculated as % inhibition/enhancement = [(growth of experimental group – positive control growth) ÷ (positive control growth)] × 100. These assays were primarily conducted by undergraduate students Heather Wells and Samantha Glisson (Vanderbilt University) using methods that I previously optimized.

Statistical comparisons

Statistical tests used are described in the figure legends. Kaplan-Meier analysis was accomplished using GraphPad Prism® software (GraphPad Prism Inc. San Diego, CA) with the Log-rank (Mantel-Cox) Test that weights all deaths equally and the Gehan-Breslow-Wilcoxon Test that assigns higher weights to earlier deaths (Machin et al. 2006).

I was assisted by Dr. Thomas Kehl-Fie (Vanderbilt University) in all Kaplan-Meier analyses. Whenever data were compared by Student's t tests or ANOVA, I log-transformed data to meet the hypothesis of normal distribution for parametric statistics (Bland and Altman 1996, Manikandan 2010). Rarely, outliers detected by Dixon's Q test were removed. Whenever more than one Student's t-test was performed on the same data set, I adjusted p-values to satisfy the Bonferroni correction (Bland and Altman, 1995).

Results

Assessment of AMP defenses in newly metamorphosed frogs

I consistently observed peaks of *m/z* values corresponding to known AMPs previously reported in *R. sphenocephala* (Conlon et al. 1999) in the mass spectra (2012 cohort) in frogs examined at one year post-metamorphosis (Fig. 2-1A) and in multiple adult *R. sphenocephala* collected in Wilson County, TN (Charles Sullivan Co., Nashville, TN). However, in pilot experiments conducted in 2012, I did not detect AMP signals in secretions from new metamorphs. Therefore, I carefully examined AMP expression in skin secretions from the 2013 cohort for expression of known peptides at early time points. Mass spectrometry indicated an absence of expected AMPs at one week post-metamorphosis (Fig. 2-1B). At this time point, the total amount of hydrophobic skin peptides (μg) recoverable per frog (110 ± 16) was <10% of the amount recovered from mature frogs in the same experiment (1760 ± 290). Peptides recovered from metamorphs at one week post-metamorphosis were significantly reduced compared to older frogs, but still increased compared to APBS-injected metamorphs (two-tailed Student's t tests, *$p < 0.05$, **$p < 1 \times 10^{-5}$) (Fig. 2-1C).

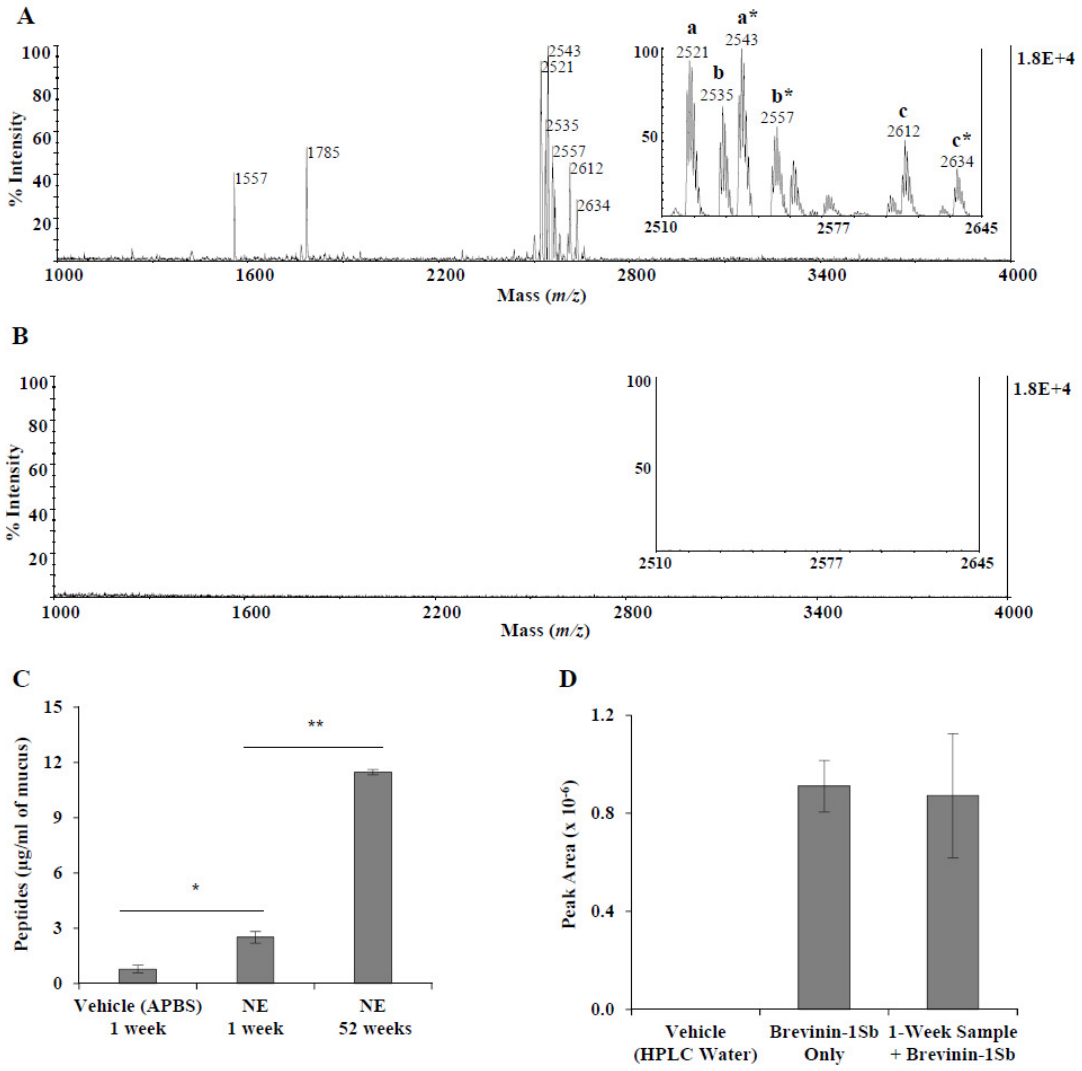


Figure 2-1. AMPs were not detected by MALDI-TOF MS in newly metamorphosed *R. sphenocephala*, but were present in older frogs. (A) MALDI-TOF profile of skin secretions from frogs at one year post-metamorphosis with known peptides including brevinin-1Sa (inset, a), brevinin-1Sb (inset, b), and brevinin-1Sc (inset, c) shown. Asterisks (e.g. a*) denote signals for sodium adducts of brevinins-1Sa, 1Sb, and 1Sc. (B) MALDI-TOF profile from newly-metamorphosed juveniles showing complete absence of expected AMP mass signals. (C) Peptides recovered from skin secretions following injection of norepinephrine (NE) or APBS at one week or one year post-metamorphosis (significant differences by two-tailed Student's t tests, *$p < 0.05$, **$p < 1 \times 10^{-5}$). (D) Peak area for brevinin-1Sb spiked into HPLC-grade water ("Brevinin-1Sb Only", N = 10) or into the same volume of one-year post-metamorphosis samples in HPLC-grade water ("One-Week Samples + Brevinin-1Sb", N = 10) (two-tailed Student's t test, $p > 0.05$). Panels A and B show representative spectra from *R. sphenocephala* sampled at one week (N = 10) and one year (N = 7) post-metamorphosis. In panel C, p-values were multiplied by two to correct for two t-tests in one experiment. In panels A and B, the insets represent the right end of the spectra at *m/z* range 2510 to 2645.

In order to confirm that the one-week samples did not contain an unknown component preventing peptide desorption or subsequent ionization, I spiked synthetic brevinin-1Sb into each one-week sample and examined them by MALDI-TOF mass spectrometry. Upon finding the signal for brevinin-1Sb, I quantified its peak area in each sample relative to the positive control samples of brevinin-1Sb alone and found that it did not differ significantly (Fig. 2-1D, $p > 0.05$), indicating that no components in the one-week samples prevented peptide identification by MALDI-TOF mass spectrometry. I concluded that newly metamorphosed *R. sphenocephala* do not express AMPs at the detection limit of the MS techniques employed, an important finding as it allows analysis of skin bacteria to proceed in a model without confounding antifungal activity by AMPs.

Optimization of bacterial reduction protocol

In several pilot experiments, I observed a reduction in cfu of non-antibiotic-treated (control) frogs compared to when first brought into captivity (Fig 2-2A). For this reason, I used mesocosm water for the 2013 study to maintain the natural microbial environment during the month-long experiment. Mesocosm water resulted in the retention of equal numbers of bacteria as detected prior to the treatments (Fig 2-2B). The cfu detected on frogs following antibiotic exposure were 0.3 ± 0.1 with zero cfu detected on 85% of frogs. The frog weights among groups did not differ before or after treatment or between control and bacterially-reduced groups (Fig 2-2C), demonstrating that groups were not significantly different in mass at the beginning of the experiment or following the antibiotic treatment regimen. Culturable bacterial numbers in the mesocosm water by plate counts indicated that the number of cfu per milliliter was $12{,}180 \pm 2{,}710$.

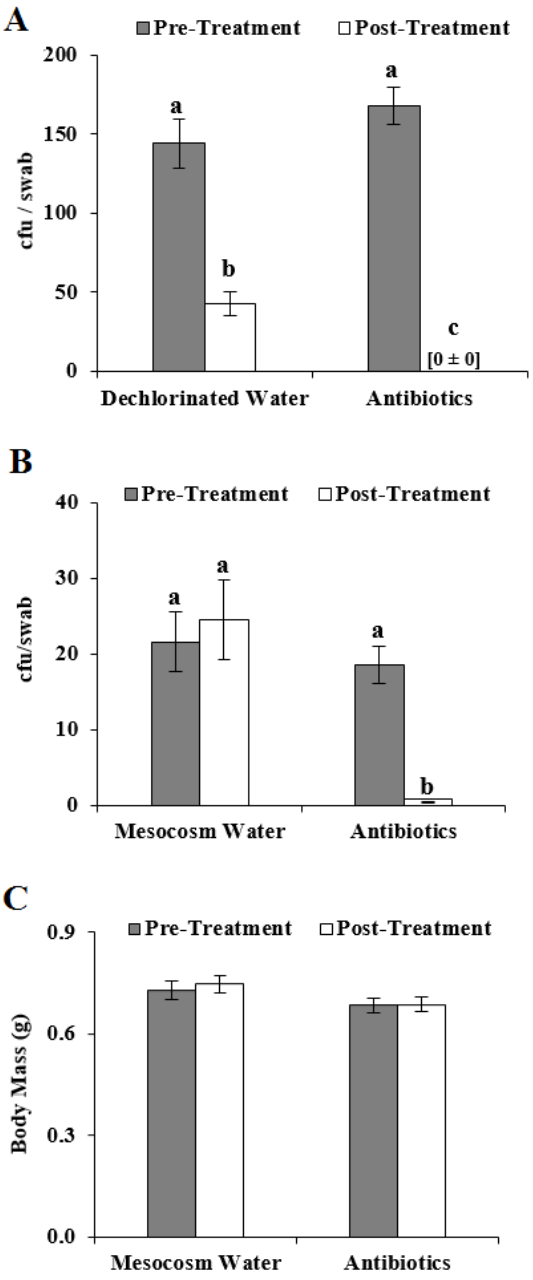


Figure 2-2. An antibiotic drug cocktail significantly reduced bacteria on *R. sphenocephala* skin. (A) In a pilot experiment, a reduction in skin bacteria was observed in control frogs (untreated) maintained in dechlorinated tap water in the laboratory (N = 5) as well as those treated with antibiotics (antibiotic-treated) (one-way ANOVA with Tukey post hoc test, $p < 0.01$). (B) Bacterial counts after swabbing frogs treated with an antibiotic cocktail (N= 39) or mesocosm water (N= 39) (significantly different by one-way ANOVA with Tukey post hoc test, $p < 1 \times 10^{-4}$). In Panels A and B, different lower case letters (a, b, or c) represent significant differences among groups. (C) Weights of frogs before and after bacterial reduction treatment (no significant differences by two-way ANOVA with Tukey post hoc test, $p > 0.05$).

Effects of bacterial reduction on *B. dendrobatidis* infection and survival of frogs

All *R. sphenocephala* metamorphs from the mesocosm-bred cohorts tested negative for *B. dendrobatidis* by qPCR. Frogs not exposed to *B. dendrobatidis,* with or without bacteria (Bacteria+, Bd– and Bacteria–, Bd–), tested negative for *B. dendrobatidis* at the beginning and end of the experiment by qPCR, and all non-exposed metamorphs that died during the experiment tested negative as well.

All metamorphs exposed to *B. dendrobatidis* in this experiment tested positive by qPCR at every time point examined. On days 6 and 13, a significantly increased infection level was observed in the Bacteria–, Bd+ frogs in comparison with the Bacteria+, Bd+ frogs (Fig. 2-3A). Approximately 10^5 additional zoospores were detected on metamorphs without their native skin bacteria when compared to those with intact skin bacteria at time of death (Fig. 2-3B). Despite the significantly lower zoospore loads observed in bacteria-intact groups, survival of these post-metamorphic juveniles was not significantly improved (Fig. 2-4, Kaplan-Meier analysis, $p = 0.578$). In addition to being swabbed for *B. dendrobatidis*, metamorphs were weighed on days 1, 3, 6, 13, 20, 27, and 34 and each individual's slope of weight change was calculated. There were no significant differences in weight change between treatment groups (Kruskal-Wallis test, $p = 0.491$). However, initial weight was found to predict metamorph survival. That is, metamorphs surviving to the end of the experiment had significantly greater weight on day 1 (mean = 0.79 g) than metamorphs that died (0.64 g; T-test, $t = 4.329$, $df = 76$, $p < 0.001$). This factor may explain the lack of significance in survival between groups as each group included metamorphs from a range of weights, including small metamorphs less likely to survive.

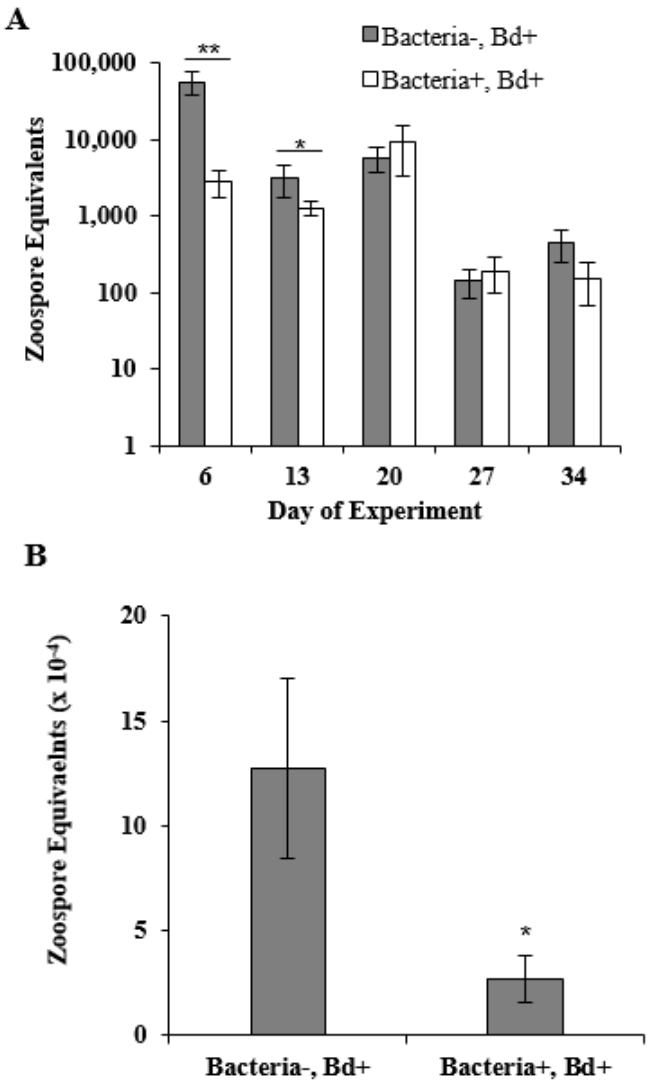


Figure 2-3. Depletion of skin bacteria on *R. sphenocephala* resulted in increased *B. dendrobatidis* detectable on the skin. The infection experiment described here involved four groups of metamorphs: (1) Bacteria-depleted, non-exposed (Bacteria–, Bd–; N = 20); (2) Bacteria-intact, non-exposed (Bacteria+, Bd–, N = 19); (3) Bacteria-depleted, *B. dendrobatidis*-exposed (Bacteria–, Bd+; N = 19); and (4) Bacteria-intact, *B. dendrobatidis*-exposed (Bacteria+, Bd+; N = 20). (A) *B. dendrobatidis* levels on skin of metamorphs with or without skin bacteria (significant differences at days 6 and 13 by two-way ANOVA with Tukey post hoc test, *$p < 0.05$, **$p < 0.001$). (B) *B. dendrobatidis* infection load at time of death in bacteria-depleted or bacteria-intact juveniles (significantly different by two-tailed Student's t test,*$p < 0.001$).

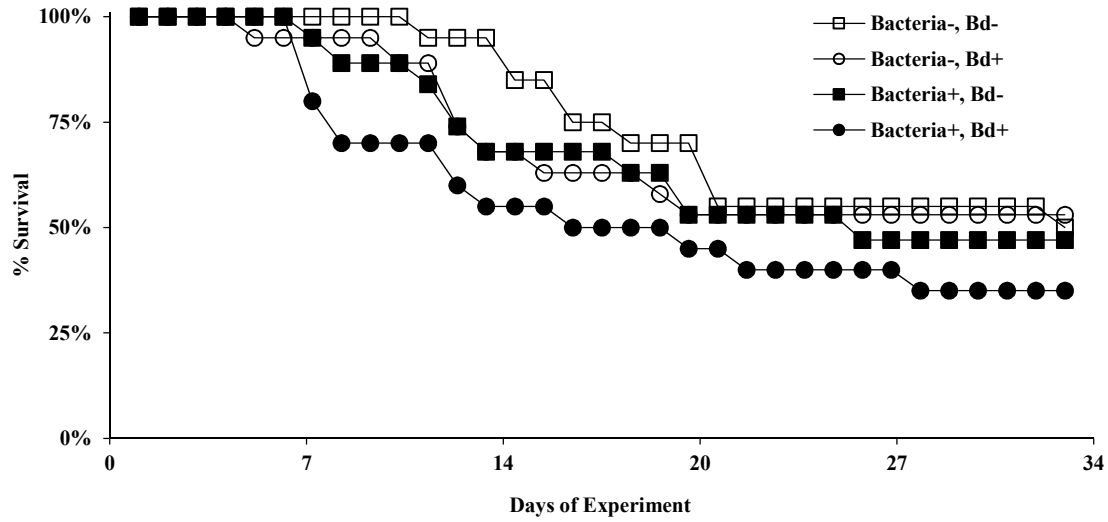


Figure 2-4. Percent survival of post-metamorphic juveniles depleted of bacteria or not and exposed or not to *B. dendrobatidis*. Treatment groups were (1) Bacteria-depleted, non-exposed (Bacteria–, Bd–; N = 20); (2) Bacteria-intact, non-exposed (Bacteria+, Bd–; N = 19); (3) Bacteria-depleted, *B. dendrobatidis*-exposed (Bacteria–, Bd+; N = 19); and (4) Bacteria-intact, *B. dendrobatidis*-exposed (Bacteria+, Bd+; N = 20). Difference between groups was assessed by GraphPad Prism® software using Kaplan-Meier analysis with the Log-rank (Mantel-Cox) test and Gehan-Breslow-Wilcoxon tests. Default conditions were used for each test and $p < 0.05$ was considered significant.

Characterization of the culturable microbiome of juvenile *R. sphenocephala*

Multiple isolates of skin bacteria collected from 15 *R. sphenocephala* metamorphs were identified by sequencing of the 16S rDNA region, and classified into 39 operational taxonomic units (OTUs) (Table 2-1). Each isolate's supernatant was harvested and tested for its effects on *B. dendrobatidis* growth. I designated isolates as belonging to one of three categories: inhibitors, enhancers, and isolates whose supernatants had no significant effect on *B. dendrobatidis* growth (Fig. 2-5A, summarized in Table 2-1). In total, the most prominent phyla observed were *Proteobacteria*, *Actinobacteria*, and *Bacteriodetes* (Fig. 2-5B), with greater than 80% of isolates in these phyla. Those isolates characterized as inhibitory to *B. dendrobatidis* also fell into these phyla (Fig. 2-5C). Two OTU's, including one inhibitory OTU, were also identified from *Deinococcus-Thermus*, an unexpected occurrence as this phylum is home to several extremophiles. Analysis of the bacterial classes also points toward increasing taxonomic diversity, especially among *Proteobacteria*, with members identified from α-, β-, and γ-*Proteobacteria* (Fig. 2-5D). Interestingly, every γ-*Proteobacteria* isolate identified was a significant inhibitor of *B. dendrobatidis* growth, making this class the largest contributor to *B. dendrobatidis* inhibition identified in this study (Fig. 2-5E).

Discussion

Skin bacteria protect *R. sphenocephala* metamorphs in the absence of AMPs

My analysis of the skin peptides showed convincingly that the AMPs previously identified for *R. sphenocephala* (Conlon et al. 1999) were not detectable by mass

Taxonomy (GenBank accession number of closest match in parentheses)	Activity	% Change	p-value
Actinobacteria			
Microbacteriaceae			
1. *Curtobacterium flaccumfaciens* (KF818635)	Inhibitor	-39.1%	6.1E-06
2. *Microbacterium* lacus (KF057952)	Enhancer	16.2%	4.5E-07
3. *Microbacterium* sp. KBKU19 (KF751800)	Inhibitor	-26.7%	1.8E-13
Micrococcaceae			
4. *Arthrobacter* sp. strain SD41 (KC415036)	No Effect	0.9%	8.6E-01
5. *Arthrobacter globiformis* strain B2S5 (EU221365)	Enhancer	18.0%	9.6E-06
6. *Arthrobacter globiformis* strain cp45 (JN082276)	No Effect	4.1%	9.3E-01
7. *Arthrobacter aurescens* (FM213390)	Inhibitor	-15.3%	1.0E-05
8. *Kocuria kristinae* (HF548363)	No Effect	0.1%	9.5E-01
Nocardiaceae			
9. *Rhodococcus fascians* (HG796188)	Enhancer	11.3%	9.1E-04
Bacteroidetes			
Flavobacteriaceae			
10. *Chryseobacterium defluvii* (JX500178)	Inhibitor	-22.2%	1.9E-10
11. *Chryseobacterium hispalense* (EU336941)	No Effect	3.1%	6.4E-01
12. *Chryseobacterium chaponense* (JX287903)	Inhibitor	-18.6%	5.4E-08
13. *Chryseobacterium* sp. strain L7-15 (AB819816)	No Effect	-5.9%	5.7E-02
14. *Chryseobacterium indologenes* (AB517708)	No Effect	-10.8%	9.5E-02
15. *Chryseobacterium* sp. strain PanRB004 (AB581570)	Inhibitor	-19.0%	5.6E-09
Firmicutes			
Bacillaceae			
16. *Bacillus* sp. strain SG2 (HM057848)	No Effect	6.7%	7.5E-02
Enterococcaceae			
17. *Enterococcus* sp. CD23 (AB673465)	No Effect	0.1%	8.9E-01
18. Uncultured *Enterococcus* sp. clone SL26 (HQ264086)	N/A	N/A	N/A
Proteobacteria			
Caulobacteraceae			
19. *Brevundimonas nasdae* (KF006808)	Inhibitor	-79.9%	9.3E-26
20. *Brevundimonas* sp. strain 266XY5 (KF818659)	No Effect	-18.4%	5.9E-02
21. *Brevundimonas vesicularis* (KF818658)	Inhibitor	-27.2%	4.4E-06
Methylobacteriaceae			
22. *Methylobacterium aquaticum* (EU977594)	N/A	N/A	N/A
23. *Methylobacterium extorquens* (KF572999)	N/A	N/A	N/A
Rhizobiaceae			
24. *Agrobacterium* sp. strain CRRI-74_SB13A (KF840387)	No Effect	-21.5%	3.1E-02
25. *Rhizobium* sp. strain M37C698A00 (JX292614)	N/A	N/A	N/A
Sphingomonadaceae			
26. Uncultured clone 300CC03, related to *Novosphingobium* spp. (AY662023)	Inhibitor	-21.0%	5.0E-06
27. Uncultured clone 2.31, related to *Novosphingobium* spp. (JN256101)	No Effect	-13.7%	9.5E-03
28. Uncultured clone ncd09h10c1, related to *Sphingomonas* spp. (HM251127)	Inhibitor	-32.9%	5.0E-11
29. Uncultured clone 16slp96-1f07, related to *Sphingomonas* spp. (GQ158667)	No Effect	10.2%	6.3E-03
Comamonadaceae			
30. *Comamonas* sp. ZYM5 (AB847926)	No Effect	13.8%	7.8E-02
Enterobacteriaceae			
31. *Citrobacter freundii* (FN997616)	Inhibitor	-42.8%	7.2E-05
32. *Enterobacter aerogenes* (AB844449)	Inhibitor	-90.4%	1.8E-25
Moraxellaceae			
33. *Acinetobacter calcoaceticus* (KF843714)	Inhibitor	-22.6%	1.0E-19
34. *Acinetobacter rhizosphaerae* (JX133182)	Inhibitor	-15.8%	8.1E-06
Xanthomonadaceae			
35. *Pseudomonas geniculata* (KF254513)	Inhibitor	-93.6%	4.7E-24
36. *Pseudomonas hibiscicola* (KC172017)	Inhibitor	-90.3%	5.3E-46
37. *Stenotrophomonas maltophilia* (KC581677)	Inhibitor	-89.6%	8.5E-38
Deinococcus-Thermus			
Deinococcaceae			
38. *Deinococcus* sp. SA1 (KF790633)	Inhibitor	-17.8%	1.8E-08
39. *Deinococcus aquaticus* (NR_043472)	No Effect	-4.0%	9.3E-02

Table 2-1. *R. sphenocephala* skin hosts a diverse bacterial community. Each p-value was calculated by comparing fungal growth (% Change) in the presence and absence of supernatant by two-tailed Student's t-test and was classified as an enhancer, inhibitor, or having no effect. In order to determine significance, the Bonferroni correction was used to establish a conservative p-value cut-off to reduce the chance of false positives: $p < 1.4 \times 10\text{-}3$. Isolates labeled "N/A" did not grow in tryptone broth, precluding analysis.

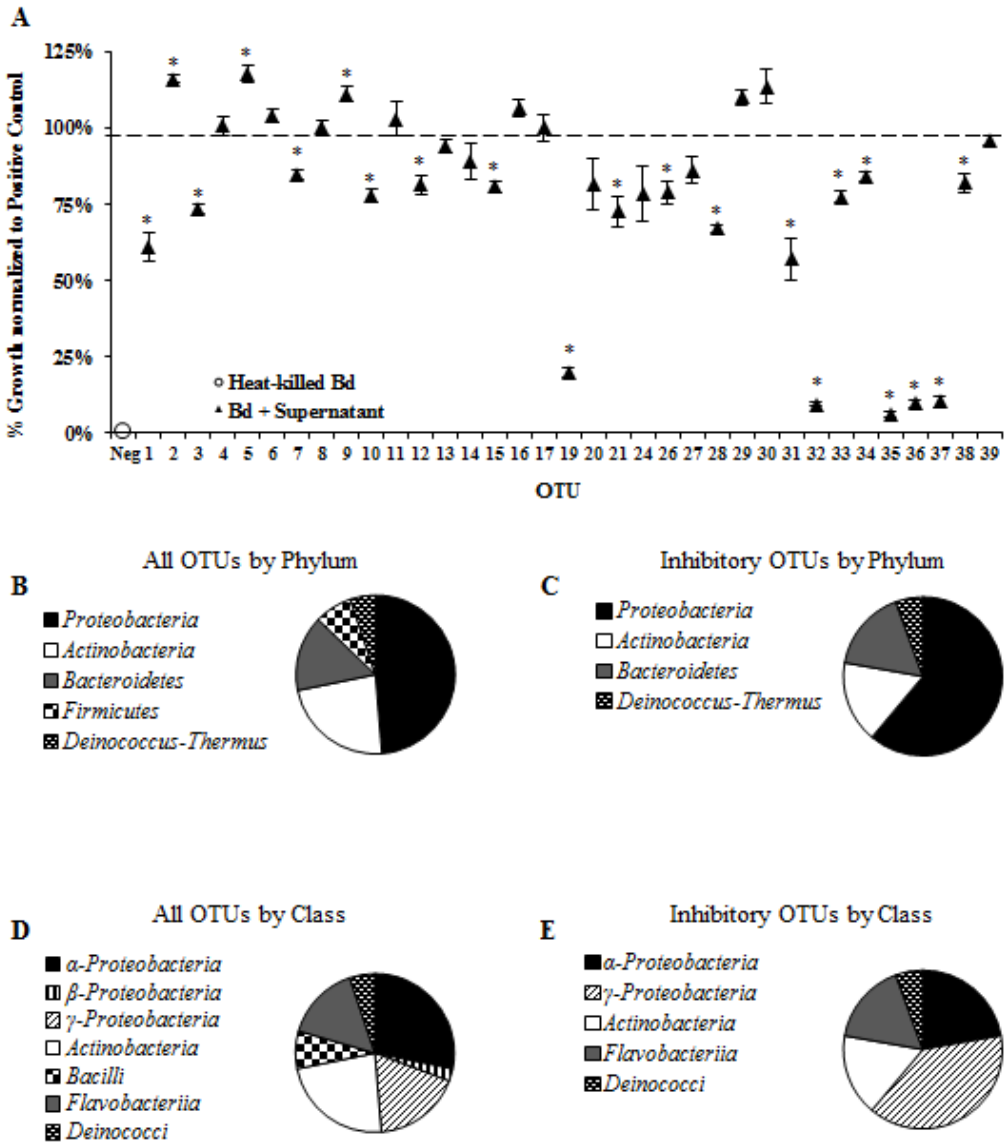


Figure 2-5. Skin bacteria isolates from *R. sphenocephala* have diverse impacts on *B. dendrobatidis* growth *in vitro*. (A) Growth inhibition or enhancement by individual isolates in comparison with *B. dendrobatidis* only positive control. The mean ± standard error (SEM) of five replicate optical densities at 490 nanometers were graphed. If no SEM is shown, it was smaller than the data symbol. Significant differences between each supernatant's effect on *B. dendrobatidis* and the positive control were determined by two-tailed Student's t-tests where $p < 1.28 \times 10^{-3}$ (post-Bonferroni correction) was considered significant. Isolates whose supernatants stimulated significant growth above that of the positive control were classified as enhancers. Isolates whose supernatants significantly inhibited growth compared to the positive control are classified as inhibitors. Both inhibitors and enhancers are designated by asterisks (*) in Panel A. Those isolates without asterisks did not significantly impact growth of *B. dendrobatidis*. Pie charts show the phylum diversity of bacteria isolated from *R. sphenocephala* skin for (B) all OTUs and (C) inhibitory OTUs and the class diversity among these isolates for (D) all OTUs and (E) inhibitory OTUs.

spectrometry at one week post-metamorphosis. This observation demonstrates that the AMP defense at this early time point is immature, and thus, any significant defense against *B. dendrobatidis* at this point in development would be a result of other factors, including the skin microbiome. Because the *B. dendrobatidis* exposure experiment was begun at one-week post-metamorphosis, I interpret the increase in infection intensity in the bacteria-depleted frogs as being due to the loss of their protective skin bacteria.

Here, I present evidence for the importance of the skin bacterial defense in providing some protection of juvenile *R. sphenocephala* against chytridiomycosis. My results support previous studies in other amphibian species (Woodhams et al. 2007, Becker and Harris 2010, Lam et al. 2010), with the added benefit of assessing the bacterial contribution to defense prior to the development of skin peptide or adaptive immune defenses and the use of a more robust bacterial depletion protocol. Because this skin bacteria defense is present at metamorphosis prior to the development of other known immune defenses, it may represent an important defense again *B. dendrobatidis* in species who lack peptide defenses altogether or, like *R. sphenocephala*, do not produce AMPs until later in development.

Rana sphenocephala **do not secrete peptides immediately after metamorphosis**

Previous studies have observed metamorphosing frogs to be at a particularly susceptible life stage to *B. dendrobatidis* infection in multiple species and populations (Rachowicz and Vredenburg 2004; Walker et al. 2010), which may be explained by lack of AMPs at early time points. This conclusion is further supported by a study reporting that *B. dendrobatidis* infection of *Anaxyrus americanus* was more severe for newly

metamorphosed juveniles than for those first exposed at 28 days post-metamorphosis (Ortiz-Santaliestra et al. 2013). This disparity could be due to development of a more effective peptide defense in older individuals, leading me to conclude that the additional time necessary for the peptide defense to mature may apply to several amphibian species. It is also possible that this peptide defense would develop more rapidly under natural environmental conditions in which the post-metamorphic juveniles might encounter more diverse bacteria in the environment. Finally, in one-week old metamorphs in this study, I observed a difference in the amount of total hydrophobic peptides recovered from metamorphs receiving a norepinephrine or APBS (vehicle) injection. This is most likely due to the fact that norepinephrine stimulates additional mucus gland secretion (Gammill et al. 2012) as no known AMPs were present according to mass spectrometry analysis.

Uses and potential modifications of the bacterial reduction protocol

In these experiments, one limitation in maintaining bacteria-reduced metamorphs quickly became apparent. I used penicillin and streptomycin in sterile water to maintain reduced skin bacteria levels and prevent colonization of the skin by environmental bacteria present in the lab, introduced food crickets, and metamorph excrement. However, long-term exposure to these drugs appeared to be detrimental to survival, possibly because of reduction of non-target microbiota such as those in the gut that are essential for proper digestion and nutrient absorption (Kupferberg 1997, Stevens and Hume 1998). Another possible outcome of long-term maintenance of frogs in water containing these drugs is the potential overgrowth of rare resistant bacteria. In frogs swabbed at the end of one month, I observed bacterial growth on R2A plates, suggesting

that re-colonization of the skin occurs despite the presence of penicillin and streptomycin. All frogs swabbed at this point appeared healthy and re-colonization of the skin by bacteria may explain the lack of difference in survival between groups and the lack of significant difference in infection intensity observed at later time points. Further, smaller metamorphs were statistically less likely to survive, which could also explain reduced survival in all groups as each group contained a mixture of small, medium, and large metamorphs. Finally, the difficulty of rearing newly metamorphosed juveniles cannot be overstated, as this is a period in which immune system development is still incomplete (reviewed in Rollins-Smith 1998). As a result, close monitoring of amphibians is important during studies using long-term reduction of skin bacteria in metamorphs, including future bioaugmentation trials that require this protocol to open niches on the skin to increase the likelihood of persistence of introduced antifungal skin bacteria.

Analysis of *R. sphenocephala* bacterial isolates

The larger number of cfu observed in mesocosm water compared to cfu detected by skin swabs of control metamorphs was not surprising, as the swabbing technique collects a smaller volume of liquid than 1 milliliter. It has also been reported that while co-habitation of two species within the same pond is not a significant predictor of skin symbionts, host species is (McKenzie et al. 2012, Kueneman et al. 2013). Thus, many bacteria common in the mesocosm water and in the environment of this species are unlikely to be skin symbionts.

I identified inhibitory isolates on 87% (13/15) of metamorphs sampled and found isolates with enhancing supernatant activity on 33% (5/15) of metamorphs. Most *R.*

sphenocephala isolates characterized as inhibitory fell into the phyla *Proteobacteria*, *Actinobacteria*, and *Bacteriodetes*, suggesting that these are the most critical phyla on which future studies of amphibian skin microbiota should focus. Diverse bacteria are known to produce a variety of chitinases and small antifungal compounds (reviewed in Verschuere et al. 2000, Garbeva et al. 2004) that would be of use in any anti-*B. dendrobatidis* arsenal. Interestingly, OTUs from *Deinococcus-Thermus* were also identified among my isolates. To my knowledge, this is the first such identification among amphibian skin isolates, representing a novel contribution to the known biogeography of bacteria typically considered to be extremophiles.

These results confirm the existence of rare isolates capable of enhancing *B. dendrobatidis* growth *in vitro*, a finding suggested by one previous study (Bell et al. 2013). We identified three such enhancers among *R. sphenocephala* isolates, all *Actinobacteria*, although they are members of three different families: *Microbacteriaceae*, *Micrococcaceae*, and *Nocardiaceae*. Interestingly, we identified other isolates in two of these families (*Microbacteriaceae* and *Micrococcaceae*) capable of significantly inhibiting *B. dendrobatidis* growth. Further, the genera *Microbacterium* and *Arthrobacter* were each home to one inhibitor and one enhancer. Such diversity within a genus suggests that identification of antifungal isolates at the species level will be important in future studies, especially those with the aim of bacterial identification for bioaugmentation. Finally, one enhancing isolate, *Rhodococcus fascians*, is a well-known plant pathogen whose primary infection can result in additional opportunistic fungal infections (reviewed in Putnam and Miller 2007), although it is unclear whether the same mechanisms are responsible for its enhancement of *B. dendrobatidis* growth.

CHAPTER III

ANTIMICROBIAL PEPTIDE DEFENSES OF SOUTHERN LEOPARD FROGS, *RANA SPHENOCEPHALA*, AGAINST THE PATHOGENIC CHYTRID FUNGUS, *BATRACHOCHYTRIUM DENDROBATIDIS*

Abstract

Antimicrobial peptides produced in dermal granular glands by many amphibian species may be an important defense against the pathogen *B. dendrobatidis*. However, little is known about the ontogeny of this innate immune component or its impact on symbiotic skin bacteria, which may be another important antifungal defense in their own right. Here, I conducted a series of studies to show that *R. sphenocephala* produces skin peptides active against *B. dendrobatidis* and I report the MIC of each peptide using synthetic pure peptides. Using mass spectrometry and protein quantification assays, I observed that *R. sphenocephala* does not express a mature suite of peptides until approximately twelve weeks post-metamorphosis and examined expression between two different populations. To evaluate the effects of an AMP-depletion protocol, I examined the impact that concentrations of induced AMPs from norepinephrine stimulation have on symbiotic skin bacteria and found that culturable bacteria levels on the skin are significantly reduced after this process.

Introduction

Despite the large number of population declines caused by *B. dendrobatidis*, there are species that appear to be more resistant than others to the effects of chytridiomycosis,

both in wild populations and in laboratory infection studies (Woodhams et al. 2006, 2007, Peterson et al. 2007, Murphy et al. 2009). Evidence suggests that adaptive and lymphocyte-mediated immune responses to this pathogen are significantly impaired (Berger et al. 1998, Pessier et al. 1999, Berger et al. 2005b, Fites et al. 2013). My own experiments (Chapter II) as well as others (Brucker et al. 2008a, 2008b, Becker et al. 2009, 2010, Lam et al. 2011) have also identified symbiotic skin bacteria that may be protective. However, secretion of AMPs from dermal granular glands into the mucus is another important potential defense against this pathogen that cannot be ignored.

Each species has its own distinct suite of peptides (Conlon et al. 2004), suggesting that peptide repertoires evolve to combat specific pathogens a given species encounters. Different populations of the same species can also exhibit variability in the specific peptides expressed (Tennessen et al. 2009, Song et al. 2013). This evidence of positive selection (Tennessen and Blouin 2007; 2010) suggests that AMPs play an essential role in survival. However, no one has undertaken a thorough study of peptide ontogeny, even though energy trade-offs during metamorphosis are known to be detrimental to other immune defenses (reviewed in Rollins-Smith 1998, Rollins-Smith and Woodhams 2012). As metamorphosing frogs are a particularly susceptible life stage to *B. dendrobatidis* infection in multiple species (Rachowicz and Vredenburg 2004, Walker et al. 2010), this could be due to a weakened peptide defense following metamorphosis, a possibility that requires further study. Finally, it is essential that the scientific community more fully understand the interplay that exists between *B. dendrobatidis* and host antimicrobial peptides, especially in the context of a species like *R. sphenocephala*, which lives in regions where *B. dendrobatidis* is endemic without suffering massive declines.

The aims of this study were to: (1) quantify the effects of *R. sphenocephala* AMPs against *B. dendrobatidis* growth *in vitro*, (2) elucidate the dynamics of AMP ontogeny in *R. sphenocephala* from metamorphosis to maturity, and (3) determine the effect of norepinephrine-induced depletion of AMPs on *R. sphenocephala* skin bacteria.

Materials and Methods

Organisms

Rana sphenocephala described here as adults were collected in middle Tennessee (Wilson County) in March 2011 or 2013 (Charles Sullivan Co., Nashville, TN) or in western Tennessee (Shelby County) in March 2013 and were infected with *B. dendrobatidis* (determined by qPCR, for method details, see Chapter II). *Rana sphenocephala* described as post-metamorphic juveniles (metamorphs) were reared from eggs in western Tennessee (Shelby County) in outdoor mesocosms from February through June in 2012 or 2013. All were *B. dendrobatidis*-negative (determined by qPCR) and were kept in *B. dendrobatidis*-free conditions in captivity. For further details on care and husbandry of *R. sphenocephala*, refer to Chapter II. *In vitro* culture experiments were conducted using *B. dendrobatidis* isolate JEL197 (Longcore et al. 1999). For culture conditions, refer to Chapter II.

Synthetic antimicrobial peptides

Brevinin-1Sa (>81% pure, molecular weight (MW) = 2521.15), brevinin-1Sb (>90% pure, MW =2535.22), brevinin-1Sc (>95% pure, MW = 2612.26), and Peptide C (>93% pure, MW = 1443.80) were synthesized (Lifetein, South Plainfield, NJ) using

previously reported sequences (Conlon et al. 1999). I weighed lyophilized peptides on an analytical balance and dissolved them at known concentrations in HPLC-grade water.

Growth inhibition assays

Batrachochytrium dendrobatidis zoospores were cultured with *R. sphenocephala* AMPs in growth inhibition assays. These are similar to the assays with bacterial supernatants described in Chapter II, except for the following changes: AMPs dissolved in HPLC-grade water replaced bacterial supernatants and multiple dilutions of AMPs were tested (500 μg/ml to 3 μg/ml). Optical density at $\lambda = 490$ nanometers (OD_{490}) was calculated at Day 0 and Day 7 of each assay. Similar assays were conducted with AMPs and bacterial isolates with the following changes: Pure cultures of each isolate were grown in Luria-Bertani broth (10g tryptone/L, 5g yeast extract/L, 5g NaCl/L) to an optical density at 630 nanometers (OD_{630}) of 0.1 (~10^8 cells/ml). Optical densities were assessed on days 0 and 2. All growth inhibition assays were repeated a minimum of three times to confirm results.

Computational analysis of putative antimicrobial peptides

I analyzed previously reported amino acid sequences of Peptide A, Peptide B, and Peptide C (Conlon et al. 1999) for AMP characteristics using a variety of computational tools. These included the Antimicrobial Peptide Database's Calculator and Predictor (Dept. of Pathology & Microbiology, University of Nebraska Medical Center, Omaha, NE, http://aps.unmc.edu/AP/prediction/prediction_main.php) and a helical wheel prediction program (Armstrong and Zidovetzki, University of California – Riverside,

Riverside, CA, http://rzlab.ucr.edu/scripts/wheel/wheel.cgi). I used the AMP prediction database (Wang et al. 2009) to construct an alignment of Peptide C with other AMPs.

Skin peptide collection and enrichment

Mixtures of skin peptides were collected from *R. sphenocephala* by norepinephrine injection as previously described (Ramsey et al. 2010, Pask et al. 2012). The volume of collection buffer varied with frog size (adults = 100 ml, metamorphs = 10 ml), and frogs received only one injection of either APBS or 40 nmol norepinephrine per gbw. For additional details on this procedure, refer to Chapter II.

Mass spectrometry

MALDI-TOF mass spectrometry was used to identify individual peptides present in individuals in the *R. sphenocephala* ontogeny study, and to confirm the molecular weight of synthetic peptides used to determine MICs. For details about the methodology for MALDI-TOF mass spectrometry, refer to Chapter II. MALDI-TOF-TOF mass spectrometry was performed by Dr. David Friedman (Vanderbilt Proteomics Laboratory, Nashville, TN) to confirm amino acid sequences of peptide signals identified in MALDI-TOF mass spectrometry analysis.

Plate counts to assess levels of culturable skin bacteria

Plate counts were conducted to assess levels of skin bacteria before and after injection with either 40 nmol norepinephrine per gram body weight (gbw) or APBS (vehicle). For details on the methodology, refer to Chapter II.

Statistical comparisons

Two-tailed Student's t tests were used to compare optical densities and one-way ANOVA with Tukey post hoc tests was used to compare cfu/ml in plate counts following log-transformation of data (Bland and Altman 1996, Manikandan 2010). In rare cases, outliers identified by Dixon's Q test were removed. To determine whether significant ($p < 0.05$) differences existed in peptide expression, Chi-square tests were performed to compare numbers of frogs expressing AMPs at specific ontogeny time points.

Results

Rana sphenocephala antimicrobial peptides are active against *B. dendrobatidis*

Rana sphenocephala regularly express three known antimicrobial peptides: brevinin-1Sa, brevinin-1Sb, and brevinin-1Sc (Conlon et al. 1999). Their activity against *Escherichia coli* has been reported, but no studies have focused on their ability to inhibit *B. dendrobatidis*, one of the most significant global threats to amphibian diversity.

Following my use of norepinephrine injection to collect peptide mixtures from *R. sphenocephala* skin secretion, Dr. David Friedman (Vanderbilt Proteomics Laboratory) conducted tandem mass spectrometry analysis to confirm the published sequences of these peptides (Appendix C). Next, I obtained purified synthetic versions of each brevinin peptide to examine their individual abilities to inhibit *B. dendrobatidis*. Using *in vitro* growth inhibition assays, I observed dose-dependent inhibition by each peptide, with MICs ranging from 10 to 40 μM (Fig. 3-1), defined as the concentration of peptide that resulted in optical density changes not significantly different from the heat-killed *B. dendrobatidis* negative control.

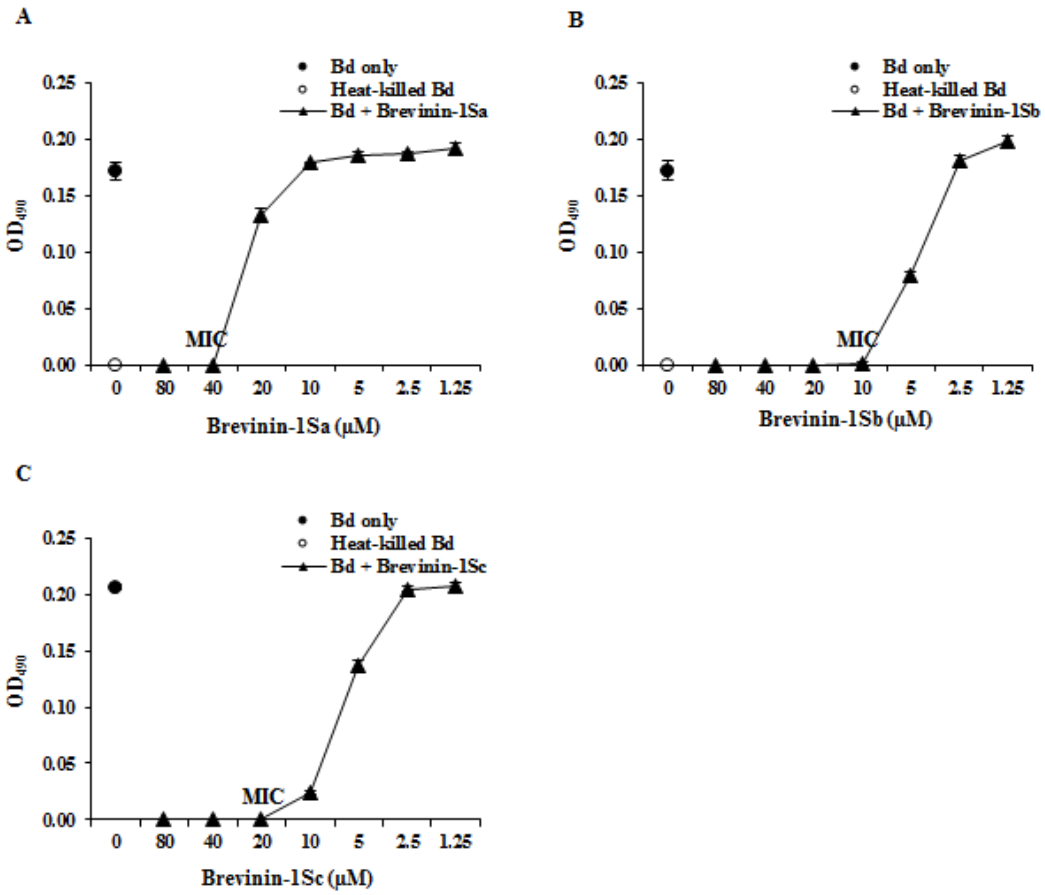


Figure 3-1. *R. sphenocephala* produces three brevinin family antimicrobial peptides with activity against *B. dendrobatidis*. (A) Brevinin-1Sa, (B) brevinin-1Sb, and (C) brevinin-1Sc were cultured with *B. dendrobatidis* and their minimal inhibitory concentrations were 40, 10, and 20 μM, respectively (defined as no significant difference, $p > 0.05$, between experimental sample and negative control by two-tailed, Student's t test). Optical densities from growth inhibition assays were averaged and are presented as the mean ± standard error (SEM), where SEM = standard deviation / $\sqrt{n}$, where n = number of replicates. Some standard error values are not observable because they are within the limits of the data symbol. Panels are representative of at least three assays conducted per peptide.

Peptide C is an antimicrobial peptide active against *B. dendrobatidis*

Three other skin peptides have been identified in this species: Peptide A, Peptide B, and Peptide C (Conlon et al. 1999), although nothing beyond their amino acid sequences has been reported. Using a variety of computational tools, I assessed the percent hydrophobicity, total charge, and predicted helical topology of these three peptides to identify which, if any, exhibit typical hallmarks of antimicrobial peptides (Table 3-1), which include cationic, amphipathic, and helical characteristics (Nicolas and Mor 1995, Rinaldi 2002, Zasloff 2002). My results suggest that Peptide C is the only one of these three with sufficient antimicrobial peptide properties to warrant additional study. Similar to the three brevinin peptides, it is cationic (net charge = +2) with a percent hydrophobicity of 53% and predicted to be helical.

To confirm the amphipathic nature of Peptide C, I used a helical wheel projection program (Don Armstrong and Raphael Zidovetzki, University of California – Riverside, Riverside, CA) (Fig. 3-2A). Specifically, the only two potentially charged residues, lysine-5 and arginine-9, were positioned close to one another on the same side of the peptide as the hydrophilic residues serine-8 and asparagine-13. On the other side of the peptide were the hydrophobic residues isoleucine-6 and -7, leucine-10 and -11, and phenylalanine-3. This qualitatively indicated that Peptide C has amphipathic character expected of classical antimicrobial peptides.

Peptide[1]	Amino Acid Sequence[1]	Hydrophobicity[2]	Net Charge[2]	Helical Topology[2]
Brevinin-1Sa	FLPAIVGAAGQFLPKIFCAISKKC	62%	+3	Yes
Brevinin-1Sb	FLPAIVGAAAKFLPKIFCAISKKC	66%	+4	Yes
Brevinin-1Sc	FFPIVAGVAGQVLKKIYCTISKKC	54%	+4	Yes
Peptide A	SLVSDIQDRQGPIA	35%	-1	No
Peptide B	SLVSDISDRWGPIALN	43%	-1	No
Peptide C	LLFGKIISRLLGN	53%	+2	Yes

[1]Reported by Conlon et al. (1999).
[2]Antimicrobial Peptide Predictor (University of Nebraska Medical Center, Omaha, NE).

Table 3-1. Computational analysis of *R. sphenocephala* skin peptides. Each peptide is listed along with its percent hydrophobicity, its net charge, and whether its topology is predicted to be helical by the Antimicrobial Peptide Predictor.

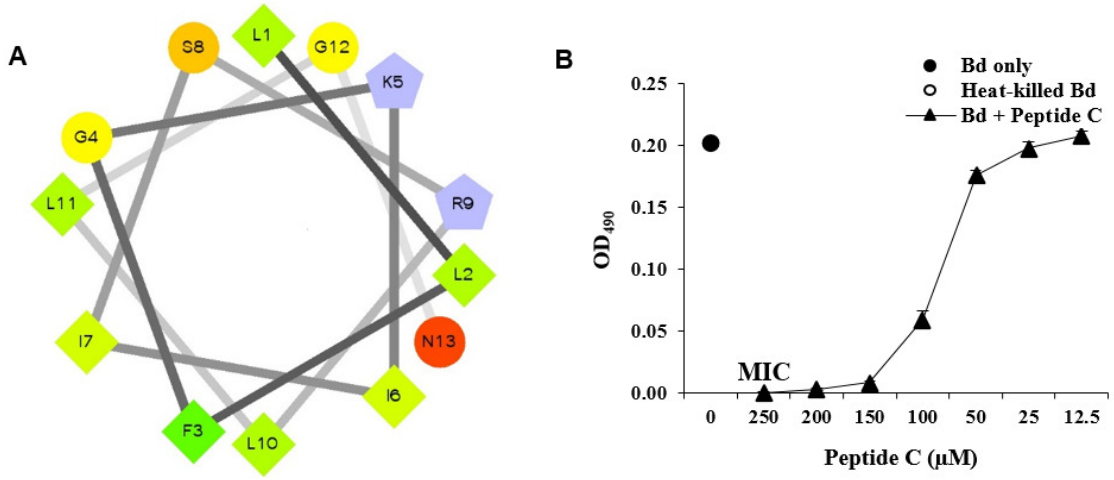


Figure 3-2. Peptide C is an amphipathic peptide with antimicrobial activity against *B. dendrobatidis*. (A) A helical wheel projection of Peptide C: Hydrophilic residues are represented as circles, hydrophobic residues as diamonds, and positively charged residues as pentagons. Peptide C does not have any negatively charged residues. Hydrophobicity is color coded: hydrophobic residues are green, with the amount of green decreasing proportionally to the hydrophobicity. Zero hydrophobicity is coded as yellow. Hydrophilic residues are red, with the amount of red decreasing proportionally to the hydrophilicity. Charged residues are blue. (B) Synthetic purified Peptide C was cultured with *B. dendrobatidis* and its minimal inhibitory concentrations was 250 µM (defined as no significant difference, $p > 0.05$, between experimental sample and negative control by two-tailed, Student's t test). Optical densities from growth inhibition assays were averaged and are presented as the mean ± standard error (SEM), where SEM = standard deviation / $\sqrt{n}$, where n = number of replicates. Some standard error values are not observable because they are within the data symbol. This panel is representative of three assays.

A sequence alignment of Peptide C with a comprehensive database of other known antimicrobial peptides indicated that it aligns most closely with members of the temporin family of peptides, specifically those expressed by other species of *Rana* and *Hylarana* amphibians (Antimicrobial Peptide Database ID numbers: AP00586, AP00611, AP00874, and AP00658). Due to its similarity to other temporins, I suggest Peptide C be designated "Temporin-1S." Finally, this peptide also aligns closely with a wasp venom that is active against fungi (Baek et al. 2011, Antimicrobial Peptide Database ID number: AP01680), further supporting the likelihood of Temporin-1S to be active against fungi like *B. dendrobatidis*. Using purified synthetic Temporin-1S, I determined its activity against *B. dendrobatidis* to be dose-dependent with an MIC of 250 μM (Fig. 3-2B). As with the three brevinins, I also collaborated with Dr. David Friedman (Vanderbilt Proteomics Laboratory) to confirm its sequence in *R. sphenocephala* skin peptide mixtures using tandem mass spectrometry (Appendix C).

Rana sphenocephala **skin peptide mixtures are active against** ***B. dendrobatidis***

In MALDI-TOF MS analysis, I commonly observed all four peptides, brevinin-1Sa, brevinin-1Sb, brevinin-1Sc, and Temporin-1S (Peptide C), in multiple individuals of this species (N = 92, Fig. 3-3A). I examined these peptide mixtures for their *in vitro* inhibition of *B. dendrobatidis* and found that peptides mixtures from all frogs tested exhibited MIC's from 250 to 500 μg/ml. For natural peptide mixtures from adult *R. sphenocephala* (Fig. 3-3B, N = 10), the average MIC was 375 ± 42. Similarly, peptide mixtures from post-metamorphic juveniles at 12 to 20 weeks post-metamorphosis (Fig. 3-3C, N = 15) exhibited an average MIC of 383 ± 33. There was no significant difference

in the MICs between these developmental stages ($p > 0.05$, unpaired, two-tailed Student's t test).

I did not find evidence that less potent peptide mixtures (i.e. MIC = 500 μg/ml) correlated with expression of fewer peptides. That is, some crude peptide mixtures with only two or three of the four known antimicrobial peptides observed by MALDI-TOF mass spectrometry had MIC's measured in the 250 to 500 μg/ml range. Similarly, crude peptide mixtures expressing all four known antimicrobial peptides still had MIC's measured in the 250 to 500 μg/ml range, rather than a more potent MIC.

Signals for Peptide A and Peptide B were not observed during MALDI-TOF mass spectrometry analysis of peptide samples following enrichment for hydrophobic peptides (Fig. 3-3A). This further supports the likelihood that any biological function of Peptide A and Peptide B in *R. sphenocephala* skin is not traditional antimicrobial membrane disruption. Thus, I did not pursue further work with these two peptides.

Rana sphenocephala peptide defenses mature by 12 weeks post-metamorphosis

My previous work (see Chapter II) indicated that *R. sphenocephala* at one-week post-metamorphosis did not secrete known antimicrobial peptides upon norepinephrine injection (Fig. 2-1A), suggesting that the development of the skin peptide immune defense may be delayed in this and other species. An immature peptide defense at early developmental life stages could explain why many studies have observed metamorphosing frogs to be a more susceptible life stage to *B. dendrobatidis* infection (Rachowicz and Vredenburg 2004, Walker et al. 2010).

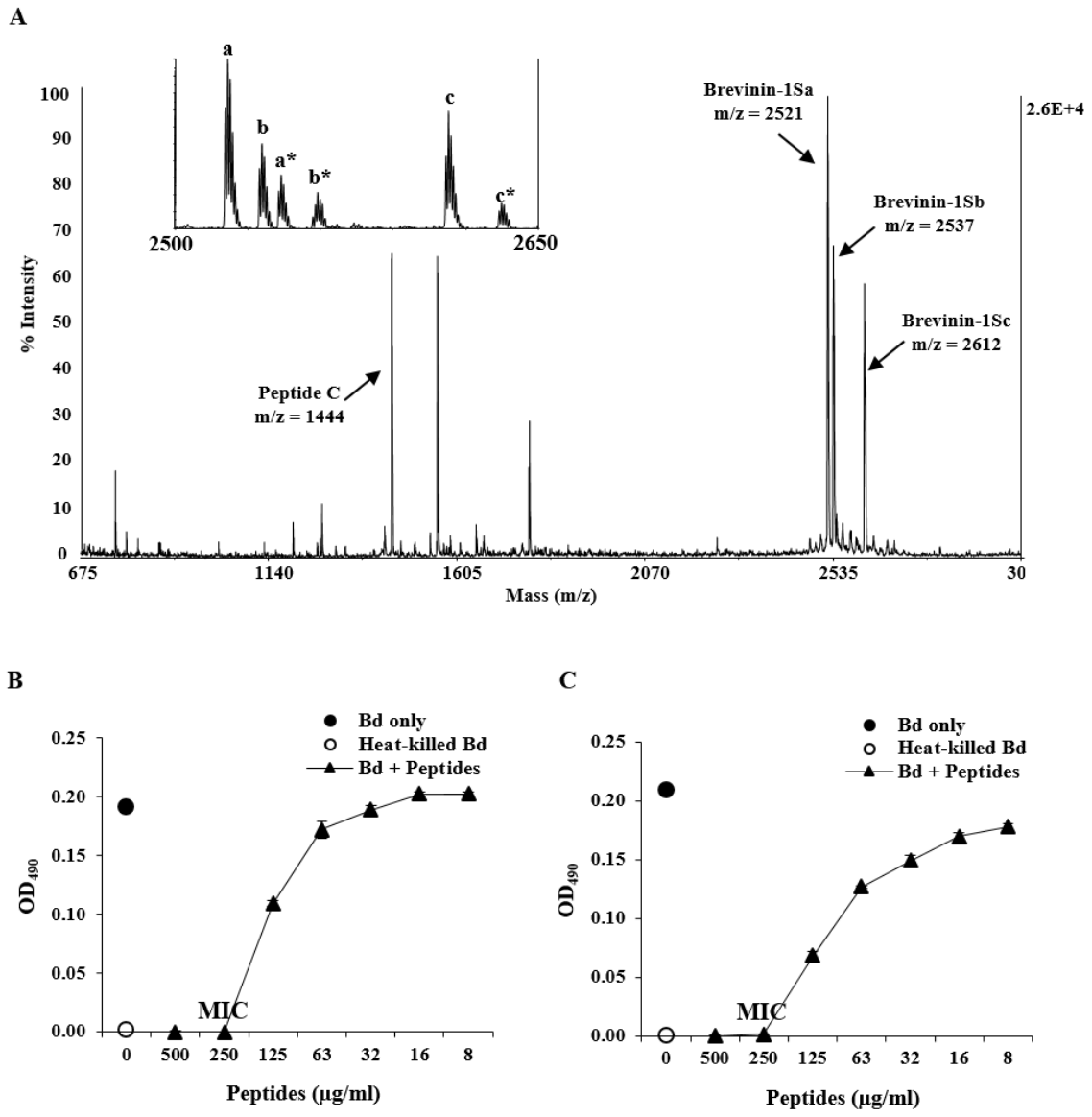


Figure 3-3. Natural peptide mixtures from *R. sphenocephala* inhibit *B. dendrobatidis* growth *in vitro*. (A) Representative spectrum of *R. sphenocephala* skins secretions shows the presence of antimicrobial peptides brevinin-1Sa, brevinin-1Sb, brevinin-1Sc, and Temporin-1S (Peptide C). Inset shows brevinin peptides in more detail. Asterisks ('a*' for brevinin-1Sa) represent sodium adducts of each peptide. Natural skin peptide mixtures from (B) adults and (C) juveniles at 12-20 weeks post-metamorphosis indicate dose-dependent inhibitory activity against *B. dendrobatidis*. Optical densities are presented as the mean ± SE. Some standard error values are not observable because they are within the data symbol. Panels B and C represent results from 10 to 15 individuals.

In order to pinpoint when the peptide defense matures in this species, I collected norepinephrine-induced secretions from individuals of a *R. sphenocephala* cohort at 4, 12, 20, 30, 40, or ≥52 weeks post-metamorphosis. At 4 weeks (N = 18), I observed that peptides were beginning to be expressed with 28% of individuals expressing brevinin-1Sa and one individual expressing brevinin-1Sa, brevinin-1Sb, and brevinin-1Sc (Fig. 3-4A). By 12 weeks (N = 10), expression increased to 90% of individuals expressing brevinin-1Sa, 30% expressing brevinin-1Sb, 20% expressing brevinin-1Sc, and 20% expressing Temporin-1S (Peptide C). This trend continued with brevinin-1Sa in at least 90% of individuals at remaining time points (N = 9 to 10 per time point). In comparison, I observed brevinin-1Sb in 10% to 78% of individuals and brevinin-1Sc in 20% to 40% of individuals at later time points. Temporin-1S (Peptide C) had more variable expression, appearing in 20% of 12-week frogs and 22% of frogs at one year or older.

A Chi-square test comparing peptide expression of each time point with that observed at ≥52 weeks post-metamorphosis indicated that only peptide levels at 4 weeks post-metamorphosis were significantly different from adult frogs ($X^2 = 21.99$; df = 3; $p < 1 \times 10^{-4}$). In contrast, peptides expressed at 12, 20, 30, and 40 weeks were not significantly different from adults at ≥52 weeks (X^2 values = 2.99, 7.71, 3.12, and 2.55, respectively; df = 3, $p > 0.05$ for each time point). I also compared the amounts of peptides secreted at 12 weeks and beyond, and there was no significant difference between time points (one-way ANOVA, $p > 0.05$), confirming my observation that peptide defenses mature in this species by 12 weeks post-metamorphosis (Fig. 3-4B). In contrast, at the previous time point (4 weeks), the amount of peptides was approximately 50% of later levels, a significantly lower amount (one-way ANOVA, $p < 0.01$).

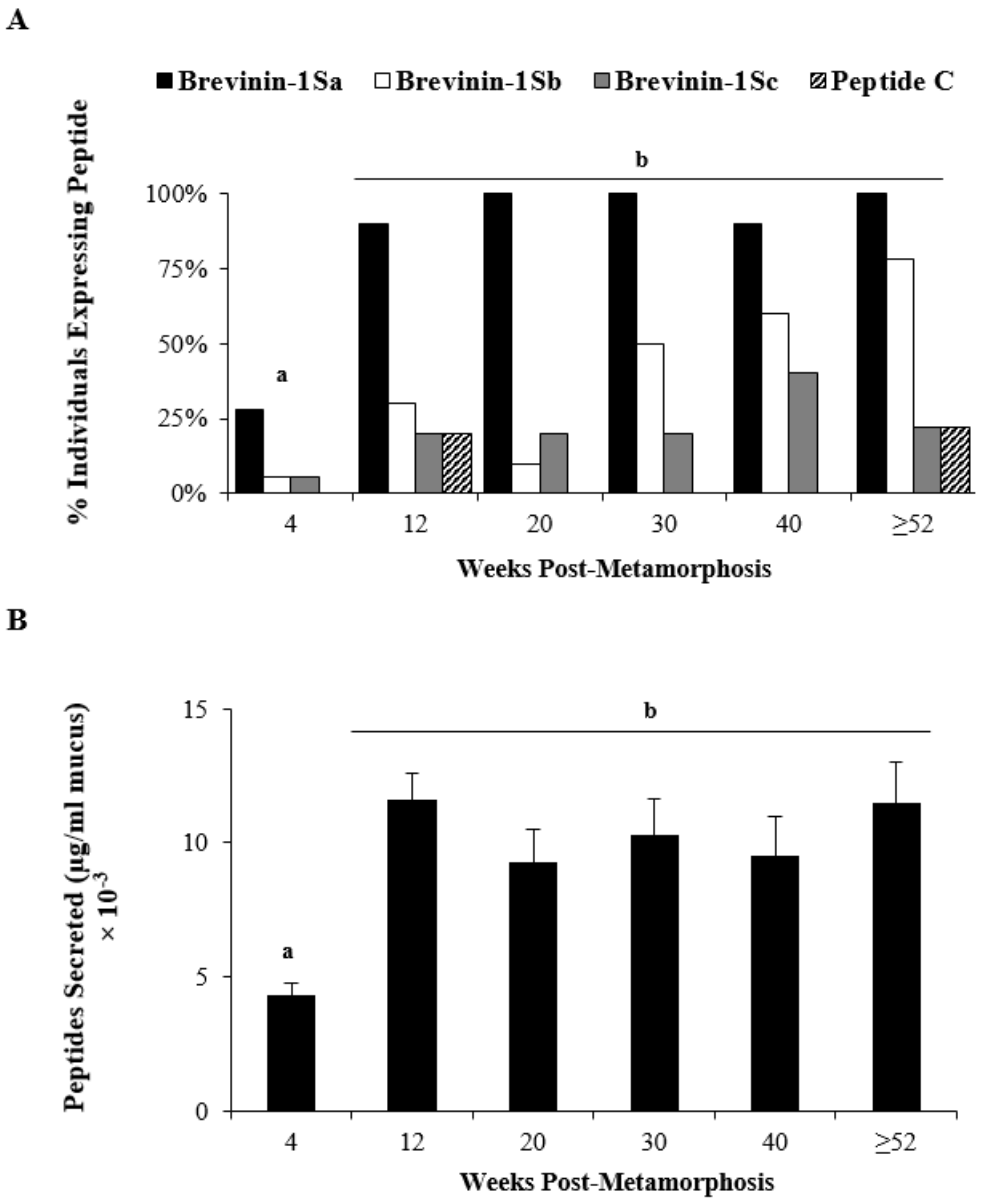


Figure 3-4. *Rana sphenocephala* antimicrobial peptide defenses mature by 12 weeks post-metamorphosis. (A) The percentage of individuals expressing known antimicrobial peptides at multiple time points post-metamorphosis is shown. Peptides expressed at earlier time points were compared to peptides expressed at 52 weeks post-metamorphosis or older by Chi-square test. (B) The amount of peptides secreted at multiple time points post-metamorphosis is shown. Values were compared by one-way ANOVA. In both panels, different letters indicate significant differences among groups, $p < 0.05$. Skin secretions were examined from 9 to 18 individuals per time point.

A year later, I collected skin secretions of individuals from a second cohort of *R. sphenocephala* at either 1, 4, or 12 weeks post-metamorphosis to confirm the trend I observed with the first cohort. As expected, in this second cohort, at 1 week, no AMPs were observed in any individual (N = 10, see Chapter II). Specific peptides expressed at 4 and 12 weeks post-metamorphosis were not significantly different from those expressed in the first cohort when frogs of similar weights were studied (X^2 values = 3.61 and 7.45, respectively; df = 3, $p > 0.05$ for each time point), confirming that peptide expression matures in *R. sphenocephala* by 12 weeks post-metamorphosis.

Frequency of peptide expression differs between *R. sphenocephala* populations

Over the course of many experiments here and in Chapters IV and V, I examined the peptides expressed by numerous *R. sphenocephala* at ≥12 weeks post-metamorphosis from two different Tennessee populations using MALDI-TOF MS. One population included frogs either raised from eggs or collected as adults in Shelby County (western Tennessee) by my collaborator Shane Hanlon (University of Memphis, Memphis, TN). The second population was made up of frogs collected from the wild in Wilson County (middle Tennessee, Charles Sullivan Co., Nashville, TN). Thus, these two populations were separated by a distance of approximately 250 miles. In both populations, I observed brevinin-1Sa in 96 and 98% of frogs (Table 3-2). However, a Chi-square test comparing the expression frequency of all four peptides between the two populations indicated significant difference ($X^2 = 37.20$; df = 3; $p < 1 \times 10^{-7}$). The biggest contributors to the difference between the Wilson and Shelby County frogs were brevinin-1Sc (in 27% and 74%, respectively) and Temporin-1S (Peptide C, in 8% and 61%, respectively).

Population	Brevinin-1Sa	Brevinin-1Sb	Brevinin-1Sc	Temporin-1S (Peptide C)
Shelby County	96%	45%	27%	8%
Wilson County	98%	91%	74%	61%
Total	97%	66%	49%	33%

Table 3-2. Percentage of *R. sphenocephala* expressing antimicrobial peptides from two populations. All *R. sphenocephala* examined (N = 92) were at least 12 weeks-post metamorphosis and many (N = 52/92) were one year or older. Frequencies are expressed as percentages of individuals from each population where I observed the given peptide by MALDI-TOF mass spectrometry. The two populations examined were collected in Shelby County (N = 49) and Wilson County (N = 43). When frequencies of all four peptides were considered, populations were significantly different by Chi-square test ($p < 1 \times 10^{-7}$).

Norepinephrine induction of antimicrobial peptides reduces culturable skin bacteria

It is well-established that both AMPs and symbiotic bacteria are present on the skin of many amphibian species and may contribute to defense against *B. dendrobatidis*. Because AMPs are relatively non-specific and are effective against fungi, bacteria, and viruses, it is attractive to hypothesize that *R. sphenocephala* skin symbionts would be sensitive to *R. sphenocephala* AMPs. It has even been hypothesized that endogenous proteases on the skin are present to degrade peptides within minutes of their original secretion in order to protect natural symbionts while still deterring pathogens (Pask et al. 2012). If true, this may have repercussions for methods that cause high levels of peptides to be secreted onto the skin, including the norepinephrine-stimulated peptide induction necessary to deplete granular glands for experimental purposes (Appendix A).

To determine whether norepinephrine injection affects skin bacteria, I first examined the ability of natural skin peptide mixtures to inhibit *R. sphenocephala* skin bacteria growth (N = 22) *in vitro*. I observed no MIC or dose-dependent response with three isolates in replicated assays, indicating that some symbionts are resistant to peptide concentrations as high as 500 μg/ml. However, for the remaining isolates, I observed the average MIC of peptide activity to be 414 ± 30 μg/ml. Thus, I hypothesized that peptide secretion resulting from one norepinephrine injection (40 nmol/gbw) would significantly reduce skin bacteria levels. In fact, this treatment reduced the level of culturable skin bacteria detected by swab by approximately 75% (Fig. 3-5). The number of cfu/swab detected following norepinephrine was significantly different from the levels detected before norepinephrine injection and before and after APBS (vehicle) injection (one-way ANOVA, $p < 0.05$).

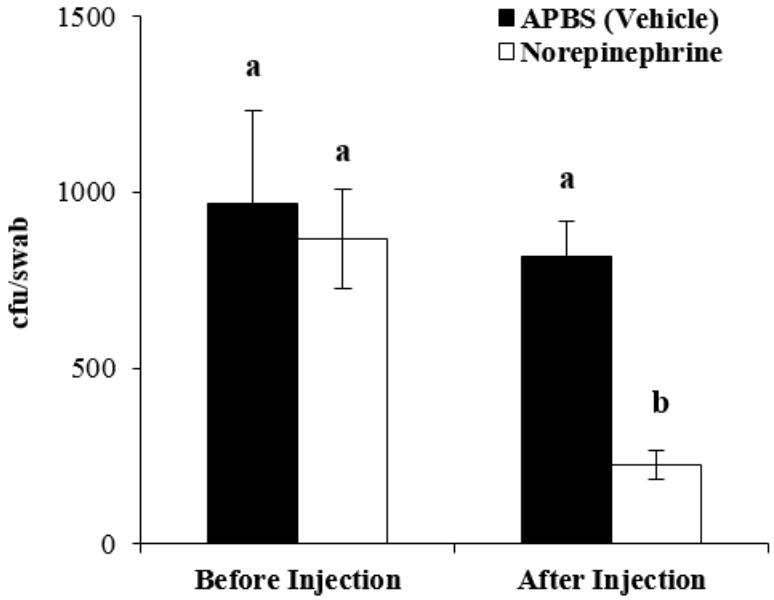


Figure 3-5. Peptide induction by norepinephrine reduces culturable skin bacteria levels. *R. sphenocephala* were swabbed for skin bacteria ("Before Injection"), then injected with either norepinephrine at 40 nmol/gbw (N = 20) or APBS (N = 10). Following 15 minutes of peptide secretion in collection buffer, frogs were swabbed again ("After Injection"). A plate count was performed and cfu counts compared by one-way ANOVA. Different letters represent a significant difference in cfu detected per swab ($p < 0.05$).

Discussion

Rana sphenocephala express skin peptides active against *B. dendrobatidis*.

The MICs I observed for brevinin-1Sa, brevinin-1Sb, and brevinin-1Sb are similar to those reported for other brevinin peptides in different species of *Rana* (Rollins-Smith et al. 2002a, 2002c, Tennessen et al. 2009). And while I observed individual variation in the potency of skin peptide mixtures, all completely inhibited *B. dendrobatidis* growth *in vitro* at 250 to 500 μg/ml. In fact, I observed this range even when the peptide mixtures had only some of the known AMPs. I interpret this to mean that the MIC of any given mixture is a function of the amounts of each peptide expressed, rather than the different types of peptides expressed. Because I frequently observe additional unidentified signals in MALDI-TOF spectra that have the approximate mass and hydrophobicity values associated with known antimicrobial peptides, there may be additional unknown peptides contributing to fungal inhibition by natural peptide mixtures.

Here, I report that Peptide C is an antimicrobial peptide with activity against *B. dendrobatidis*. Based on its amino acid sequence, Peptide C is a temporin, a family of peptides characterized by higher MICs than brevinin peptides against the same targets (Rollins-Smith et al. 2002a, 2002b, 2002c, 2003, Conlon et al. 2013), and may be henceforth known as Temporin-1S. The reduced potency of temporins may be best explained by their smaller size and lesser cationic character. That is, Temporin-1S has a molecular weight of approximately half that of the brevinins. Because the AMP mechanism of action is membrane disruption causing target cell osmotic lysis, smaller AMPs will have greater difficulty disrupting the target cell surface. Further, the three brevinins expressed by *R. sphenocephala* have net charges of +3 and +4, while

Temporin-1S has a net charge of +2. Because these peptides bind to negatively charged membranes, stronger cationic character is important for effective binding.

Antimicrobial peptide expression in *R. sphenocephala*

Though present in some individuals as early as 4 weeks post-metamorphosis, I observed maturation of the antimicrobial peptide defense in *R. sphenocephala* at about 12 weeks post-metamorphosis. I reached this conclusion by examining amounts of peptides secreted and presence of known antimicrobial peptides in skin secretions. To my knowledge, this represents the first detailed study of peptide ontogeny in a ranid species.

Greater susceptibility of juveniles to *B. dendrobatidis* compared to more mature life stages is a well-known phenomenon (Rachowicz and Vredenburg 2004; Walker et al. 2010), and lack of a peptide defense at the early post-metamorphic time points may be an explanation for this increased vulnerability. Potential reasons for this delay include the possibility that mRNA transcripts and the resulting precursor peptides are produced (reviewed in Amiche et al. 1999), but post-translational processing into mature peptides may not occur at early time points. It is also possible that peptides may collect in skin granular glands early in development, but may not be secreted if the adrenergic nerve terminals controlling gland secretion are still undeveloped. Finally, metamorphosis is characterized by a period of fasting as the body, the gastrointestinal tract, and the immune system all undergo massive rearrangements, which are all energetically costly during a period of restricted nutrient intake (reviewed in Fox 1981). Further, adult frog skin peptide secretion is a constitutive process (Pask et al. 2012). Thus, if tadpoles of this species do produce antimicrobial peptides and are capable of constitutive secretion, this

may deplete peptide stores at a point in development where the energy trade-offs described above favor organ and tissue remodeling over new peptide production. It is also possible that under natural conditions in the wild, this peptide response might develop more quickly. Additionally, I found that the frequency at which *R. sphenocephala* individuals express different AMPs differed significantly between two Tennessee populations. This has been reported before in other species (Tennessen et al. 2009, Song et al. 2013). In the case of *R. sphenocephala*, genes for brevinin-1Sb, brevinin-1Sc, and Temporin-1S may not be fixed in either population, though brevinin-1Sa was present in 96% to 98% of individuals in each population, suggesting that it may be the largest contributor to anti-*B dendrobatidis* defenses.

Norepinephrine induction of antimicrobial peptides reduces culturable skin bacteria

My studies and others have indicated that both AMPs and symbiotic bacteria may protect amphibians from *B. dendrobatidis*. One key to understanding their relative contributions is to study them separately with proper experimental controls. In collaboration with J. Scott Fites, I developed a method to experimentally deplete AMPs from granular glands (Gammill et al. 2012, Appendix A), which has proven to be a useful method to assess the protective effects of AMPs during an infection experiment (Pask et al. 2013). However, my new findings that this treatment at least temporarily reduces skin bacteria levels indicates that any experiment using norepinephrine injections to deplete AMPs will result in frogs that have reduced skin bacteria as well. Thus, AMP-intact, bacteria-reduced control frogs should also be part of these experiments. This may be possible using the short term antibiotic cocktail treatment I have developed (Chapter II).

CHAPTER IV

NIKKOMYCIN Z IS AN EFFECTIVE INHIBITOR OF THE CHYTRID FUNGUS LINKED TO GLOBAL AMPHIBIAN DECLINES[1]

Abstract

Recently, many amphibian populations have declined due to chytridiomycosis caused by the fungal pathogen *Batrachochytrium dendrobatidis*. For some endangered species, captive colonies are the best solution towards eventual reintroduction, and effective antifungal treatments are needed to treat chytridiomycosis and limit the spread of this pathogen in such survival assurance colonies. Here, I show that nikkomycin Z, a chitin synthase inhibitor, dramatically alters the cell wall stability of *B. dendrobatidis* cells and completely inhibits growth of *B. dendrobatidis* at 250 μM. Low doses of nikkomycin Z enhanced the effectiveness of natural antimicrobial skin peptide mixtures tested *in vitro*. These studies suggest that nikkomycin Z would be an effective treatment to significantly reduce the fungal burden in frogs infected by *B. dendrobatidis*.

Introduction

The most widely recommended antifungal treatment regimen for amphibians infected with *B. dendrobatidis* uses itraconazole (Nichols and Lamirande 2000). While

[1]This chapter is adapted from: Holden WM, Fites JS, Reinert LK, Rollins-Smith LA (2014) Nikkomycin Z is an effective inhibitor of the chytrid fungus linked to global amphibian declines. *Fungal Biology*, 118:48-60.

this treatment is well tolerated by some amphibians, there are also reports of itraconazole toxicity (Garner et al. 2009, Woodhams et al. 2012b). Therefore, it is essential that new antifungal drugs be explored as potential treatments. One such drug is the chitin synthase inhibitor nikkomycin Z. Studies have confirmed chitin in *B. dendrobatidis* by histological inspection of infected skin (Briggs and Burgin 2004) and by microarray studies that indicate the expression of genes for chitin synthases and chitin binding proteins (Rosenblum et al. 2008). Thus, chitin most likely plays a critical role in chytrid cell wall structure and stability, and drugs that target proteins essential for chitin synthesis may be successful in treating *B. dendrobatidis*-infected amphibians. Nikkomycin Z is a competitive inhibitor of chitin synthases (Hector 1993) that is cell permeable because of naturally-occurring mechanisms for dipeptide uptake (McCarthy et al. 1985). Additionally, previous studies have demonstrated synergism between nikkomycin Z and several other classes of antifungal drugs, including echinocandins and triazoles (Hector and Schaller 1992, Li and Rinaldi 1999, Ganesan et al. 2004). Such activity suggests that it may be useful in combination therapies against *B. dendrobatidis*.

To be an effective treatment, any antifungal drug used on amphibian skin must inhibit *B. dendrobatidis* growth within the context of the skin microenvironment. In many amphibian species, a major component of this microenvironment is the set of AMPs that are produced in dermal granular glands (Dockray and Hopkins 1975) and constitutively secreted onto the surface of the skin (Pask et al. 2012). These peptides alone are effective against *B. dendrobatidis* (Rollins-Smith et al. 2002a, 2002b, 2002c, Ramsey et al. 2010, Pask et al. 2012). Assessing the potential interactions of new drugs with AMPs is an important step in the journey towards amphibian clinical trials.

Here, I report the effects of nikkomycin Z on the cellular morphology and physiology of the chytrid fungus *B. dendrobatidis*, the range of effective concentrations at which it inhibits *B. dendrobatidis* growth, its fungicidal mechanism at 250 μM, and the inhibitory effects of nikkomycin Z in combination with naturally-produced AMPs from leopard frogs. This is the first examination of nikkomycin Z activity against a fungus from the phylum Chytridiomycota and the first to recommend this drug as a disease control agent for the ecologically-important chytrid fungus *B. dendrobatidis*.

Materials and Methods

Materials and Organisms

Nikkomycin Z-HCl (HPLC purity level = 94.6%) was a gift from Dr. John N. Galgiani (Valley Fever Center for Excellence, Tucson, AZ). Chemically synthesized *R. sphenocephala* AMPs brevinin-1Sb (>90% pure) and brevinin-1Sc (>95% pure) were prepared (Lifetein, South Plainfield, NJ) from previously reported sequences (Conlon et al. 1999). I dissolved dried peptides at a known concentration in HPLC water. All experiments with *B. dendrobatidis* were conducted using isolate JEL197 (Longcore et al. 1999). All protocols involving frogs were approved by the Vanderbilt University Medical Center Institutional Animal Care and Use Committee. For more details on fungal culture conditions or animal husbandry, refer to Chapter II.

Collection of amphibian antimicrobial skin peptides

I collected skin peptides from *R. sphenocephala* adults (Charles Sullivan Co., Nashville, TN) and used peptides collected from *R. pipiens* adults (Connecticut Valley

Biological, Southampton, MA) by Dr. James Pask (Vanderbilt University, Nashville, TN). In both cases, peptides were collected as previously described (Chapter II) following one injection of 40 nmol/g norepinephrine (Sigma, St. Louis, MO).

Growth inhibition of *B. dendrobatidis*

I conducted *in vitro* growth inhibition assays to study the effect of nikkomycin Z alone and in combination with amphibian AMPs. This is similar to the assays described in Chapter II, but with these modifications:

Zoospore culture with nikkomycin Z: Enriched zoospores were cultured with serial dilutions of nikkomycin Z in HPLC-grade water (Fisher Scientific, Pittsburgh, PA) to achieve final concentrations of 0.02 to 2000 μM.

Zoospore culture with nikkomycin Z and AMPs: Enriched zoospores were cultured with nikkomycin Z and skin AMPs in HPLC-grade water in parallel with cultures measuring the individual effects of either nikkomycin Z or peptides alone. Similar assays tested the ability of purified synthetic *R. sphenocephala* AMPs to cooperate with nikkomycin Z to inhibit *B. dendrobatidis* growth.

Use of calcofluor white to stain *B. dendrobatidis* cells

Calcofluor white is a non-specific fluorochrome that binds cellulose and chitin (Monheit et al. 1984). I collaborated with J. Scott Fites (Vanderbilt University, Nashville, TN) to stain *B. dendrobatidis* cells with Calcofluor white stain (Sigma, St. Louis, MO) according to manufacturer's instructions. Photographs were obtained using an excitation wavelength of 365 nm with an Olympus BX41 microscope and an Olympus

DP71 camera with DP Controller software, v.3.1.1.267 (Olympus Corporation). Cell diameters were measured to the nearest μm in images using a computer-calibrated scale bar (Gammill et al. 2012).

Effects of nikkomycin Z on multiple life stages of *B. dendrobatidis*

To examine the effect of nikkomycin Z on multiple life stages of *B. dendrobatidis* (zoospores, germlings, intermediate thalli, and mature zoosporangia) (Berger et al. 2005a), whole cultures of *B. dendrobatidis* were resuspended at 10^6 mature cells/ml in either tryptone broth alone or tryptone broth containing 20 μM nikkomycin Z and incubated for 3 days at 19-21°C. The number of mature cells was counted with a hemocytometer on Day 0 and Day 3. In a separate experiment designed to examine the effect of nikkomycin Z on *B. dendrobatidis* zoospore maturation, zoospores were resuspended at 10^7 zoospores/ml in either tryptone broth alone or with 20 μM nikkomycin Z and incubated for 3 days at 19-21°C in cell culture flasks. Cells were counted and zoospores were distinguished from more mature cells (germlings, thalli, or zoosporangia) by visual inspection under a microscope. The total number of each cell type was divided by the original number of zoospores to assess the ability of zoospores to mature in the presence of nikkomycin Z. The experiments in this section were conducted in collaboration with J. Scott Fites (Vanderbilt University, Nashville, TN).

***B. dendrobatidis* recovery after nikkomycin Z exposure**

To determine whether the antifungal activity of nikkomycin Z is fungicidal or fungistatic, I exposed *B. dendrobatidis* zoospores to multiple concentrations of the drug

for 7 days in 96-well flat-bottom microtiter plates. Then I combined the contents of each set of five replicate wells in one microcentrifuge tube and pelleted at 140 × g for 12 min at 4ºC in a Beckman J-6B centrifuge (Beckman Coulter Inc., Brea, CA). I aspirated the supernatants (containing nikkomycin Z) and replaced them with an equal volume of sterile tryptone broth without nikkomycin Z to wash the cells. Following another 12 min centrifugation at 140 × g, I aspirated the second supernatant to ensure removal of nikkomycin Z from each sample. Finally, I resuspended the pelleted cells in 1 ml tryptone broth, plated on tryptone agar, and incubated for 8-12 days. I photographed plates and counted colony forming units (cfu). An agar plate with 1 ml sterile tryptone broth acted as a negative control. Positive control cells (with no drug exposure) were centrifuged with experimental samples and plated as 1:100 dilutions to facilitate counting. If no *B. dendrobatidis* growth was observed following plate incubation, I considered those concentrations of nikkomycin Z to be fungicidal.

Following removal of the contents of each replicate well at day 7 for a plate count, I observed an adherent layer of *B. dendrobatidis* cells at the bottom of each well. Due to their ability to adhere to the bottom of the plate, these cells are most likely more mature life stages of the fungus (Berger et al. 2005a). Rather than attempting to scrape these cells out of the 96-well plate for inclusion in the plate count, I added 100 µl sterile tryptone broth to each well. Optical densities were measured on this day and after seven additional days of incubation. My goal was to determine if these cells were capable of additional growth following removal of nikkomycin Z, thus confirming the measured MIC determined in the previously described plate count.

Analysis of *B. dendrobatidis* osmotic lysis following nikkomycin Z treatment

In order to determine if nikkomycin Z treatment increases susceptibility to osmotic lysis, I enriched for zoospores as described in Chapter II and resuspended them in cell culture flasks at 5×10^5 zoospores/ml in a 1:1 mixture of tryptone broth and HPLC water (positive control) or tryptone broth and 50 µM nikkomycin Z in HPLC water. Each assay also included a negative control with heat-killed zoospores. Zoospores were incubated for 5 days at 19-21°C as they matured into zoosporangia. I divided cells into two equal volumes, pelleted them by centrifugation for 12 min at 140 × g at 4ºC, and resuspended them in an equal volume of either distilled water (dH_2O) or APBS in dH_2O. I transferred these cells to a 96-well flat-bottom microtiter plate (BD Falcon, Franklin Lakes, NJ) in five 200-µl replicates per sample and measured the OD_{490} of each well to compare the effects of isotonic (APBS) and hypotonic (dH_2O) environments on *B. dendrobatidis* with and without previous nikkomycin Z exposure. I confirmed these results by counting the number of cells on a hemocytometer immediately before and at multiple time points after resuspension in either APBS or dH_2O.

Statistical comparisons

The statistical tests used are reported in figure legends. When necessary, I multiplied p values to correct for multiple t tests in the same experiment (Bland and Altman 1995). In some cases, the effect of nikkomycin Z is expressed in terms of percent inhibition. This measure was calculated as % inhibition = [(positive control growth – growth of experimental group) / (positive control growth)] × 100.

Results

Effects of nikkomycin Z on *B. dendrobatidis* growth *in vitro*

Nikkomycin Z inhibited growth of *B. dendrobatidis* in a concentration-dependent manner (Fig. 4-1). I observed significant inhibition at concentrations at or greater than 0.3 μM. The percent inhibition of *B. dendrobatidis* growth when cultured with 200 μM nikkomycin Z was 94.0% ± 0.46% in replicated trials, but was still significantly higher ($p < 2 \times 10^{-5}$) than the heat-killed *B. dendrobatidis* negative control (Fig. 4-1A). However, by using a higher concentration of 2000 μM nikkomycin Z, I was able to completely inhibit *B. dendrobatidis* growth (Fig. 4-1B), determined as no change in optical density compared to the negative control ($p > 0.2$).

Effects of nikkomycin Z on *B. dendrobatidis* cellular morphology

To assess whether nikkomycin Z would alter cell wall development and the morphology of *B. dendrobatidis* cells, I exposed zoospores to 0 to 200 μM nikkomycin Z. Over 5 days, zoospores matured into mixed cultures containing zoospores, thalli, and zoosporangia, which were stained with calcofluor white to examine cellular morphology.

A typical mature zoosporangium (Fig. 4-2A) has thread-like rhizoids and a discharge papilla through which zoospores are released, giving it an urn-shaped structure (Longcore et al. 1999, Pessier et al. 1999, Berger et al. 2005a). Zoosporangia typically have a maximum diameter of approximately 15 μm, while thalli and germlings are smaller (Berger et al. 1998, 1999, Rachowicz and Vredenburg 2004). *B. dendrobatidis* zoospores cultured with nikkomycin Z developed into mature life stages and exhibited changes in cellular diameter with increasing concentrations of nikkomycin Z (Fig. 4-2F).

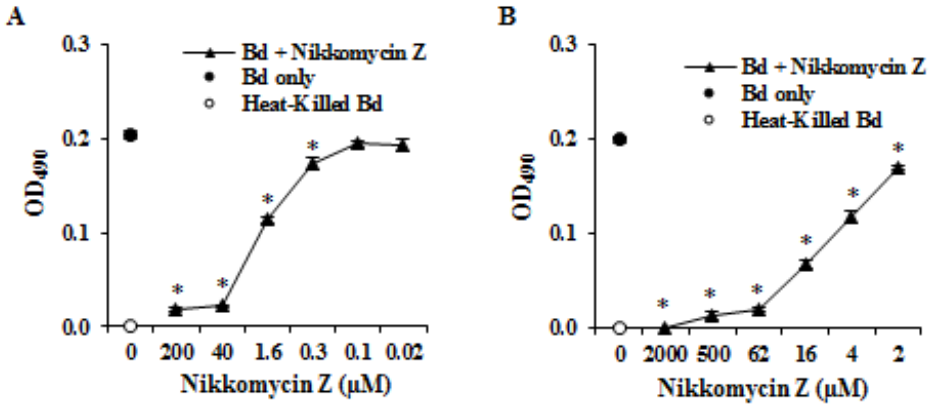


Figure 4-1. Nikkomycin Z impaired *B. dendrobatidis* growth *in vitro*. (A) *B. dendrobatidis* was cultured in equal parts tryptone broth and HPLC water ("Bd only") or in equal parts tryptone broth with various concentrations of nikkomycin Z in HPLC water ranging from 0.02 to 200 µM ("Bd + Nikkomycin Z"). Concentrations ≥ 0.3 µM nikkomycin Z showed significantly lower growth than the positive control (*$p < 0.01$). (B) At 2000 µM nikkomycin Z, *B. dendrobatidis* growth is not significantly different ($p > 0.2$) from the heat-killed *B. dendrobatidis* negative control. Data shown are representative of at least three similar experiments. Optical densities are the mean values ± standard errors for five replicates. In some cases, standard errors are not observable because they are within the limits of the data symbol.

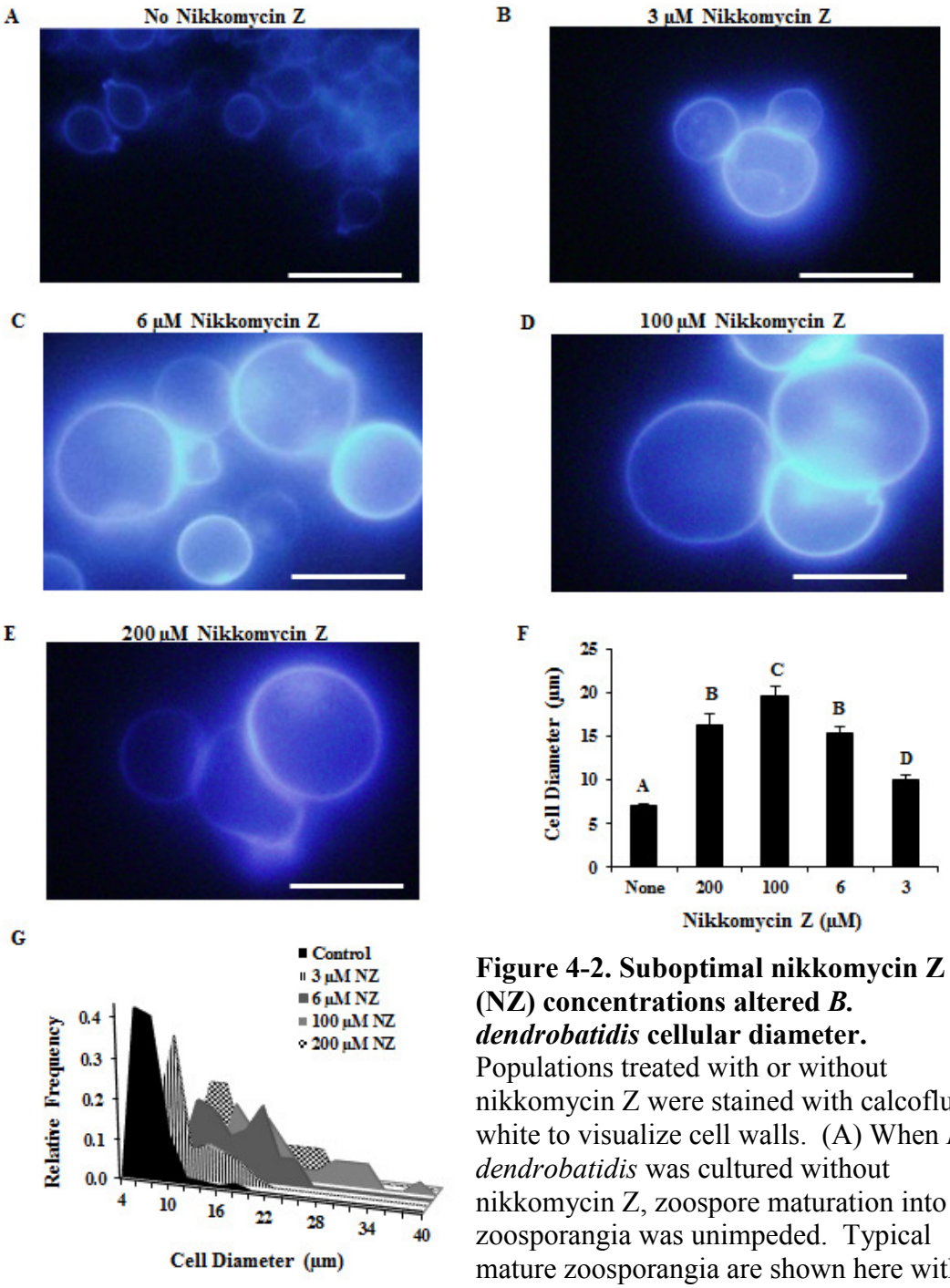


Figure 4-2. Suboptimal nikkomycin Z (NZ) concentrations altered *B. dendrobatidis* cellular diameter. Populations treated with or without nikkomycin Z were stained with calcofluor white to visualize cell walls. (A) When *B. dendrobatidis* was cultured without nikkomycin Z, zoospore maturation into zoosporangia was unimpeded. Typical mature zoosporangia are shown here with a maximum diameter of approximately 15 µm. (B – E) Increasing concentrations of nikkomycin Z resulted in increasing cell diameters in the zoosporangia at all concentrations tested. (F) The largest cell diameters were observed with zoosporangia that developed in culture with 100 µM nikkomycin Z. Letters within the panel indicate groups that differ significantly by one-way ANOVA. (G) Increasing nikkomycin Z concentrations resulted in larger ranges of observed diameters and more heterogeneous cell populations. Scale bars (A – E) represent 20 µm.

Nikkomycin Z exposure led to increased cell size, even at low concentrations (Fig. 4-2B,C). The largest diameters were observed with 100 μM (Fig. 4-2D,F). Increased size suggests that nikkomycin Z's inhibition of chitin synthesis weakens the cell wall and increases chance of osmotic lysis. Exposure to the highest concentration, 200 μM nikkomycin Z, also resulted in increased cell size (Fig. 4-2E), but not to the degree observed with 100 μM nikkomycin Z (Fig. 4-2F). This may occur because larger cells are so destabilized that they are not viable and because at this concentration, cell viability was greatly reduced (Fig. 4-1A). In addition to significantly increased cell diameter, the range of observed cell diameters broadened with increasing nikkomycin Z (Fig. 4-2G), resulting in a mature cell population more heterogeneous in cell diameter.

Fungicidal effects of nikkomycin Z on *B. dendrobatidis* growth.

To determine whether this drug's mechanism of action is fungistatic or fungicidal, I pre-cultured *B. dendrobatidis* zoospores with or without various concentrations of nikkomycin Z for 7 days, washed out the drug, replaced it with sterile broth, and plated cells on agar plates for a plate count. I observed 250 μM nikkomycin Z to be the lowest concentration necessary to completely inhibit *B. dendrobatidis* growth, as no colonies were observed (Fig. 4-3A). Therefore, 250 μM is the MIC and the drug acts in a fungicidal mechanism at this concentration (Fig. 4-3). I observed a dose-dependent response in which samples exposed to decreasing nikkomycin Z concentrations exhibited an increasing number of colonies following drug removal. *B. dendrobatidis* cells exposed to the lowest concentration tested, 0.5 μM nikkomycin Z, showed cfu not significantly different from positive control cell cfu counts ($p > 0.5$).

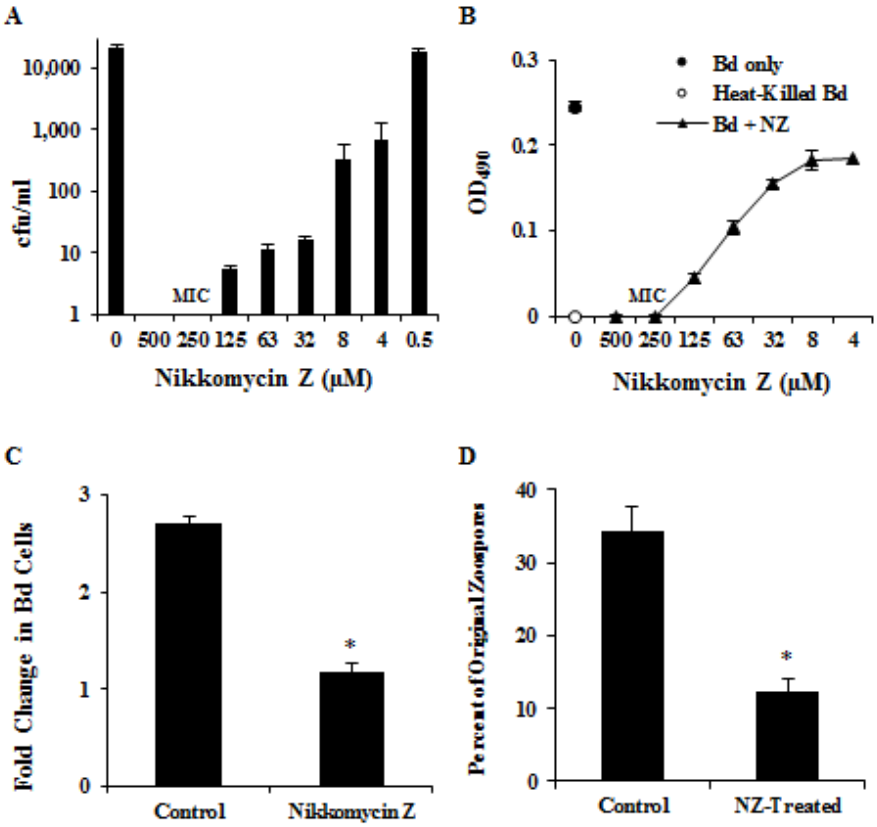


Figure 4-3. Characterization of nikkomycin Z effects on *B. dendrobatidis* growth. (A) Following plating on agar, cells that had been exposed to ≥ 250 μM were not viable. Thus, 250 μM is the minimal inhibitory concentration (MIC). (B) When cells adhering to microtiter plate wells following culture with nikkomycin Z were cultured an additional 7 days without nikkomycin Z, no significant growth ($p > 0.2$) was observed for cells previously exposed to ≥ 250 μM nikkomycin Z, confirming the MIC. (C) When cultured with 20 μM nikkomycin Z, cells exhibited significantly reduced cell replication (*$p < 0.001$). (D) The percent of original zoospores cultured that matured past the zoospore stage was reduced after incubation for 3 days with 20 μM nikkomycin Z (*$p < 0.02$). In panels A, C, and D, the mean of three experiments is graphed. The data shown in panel B is a single experiment representative of at least three similar assays. In some cases, standard errors are not observable because they are within the limits of the data symbol.

In each growth inhibition assay, I observed a layer of *B. dendrobatidis* cells adhering to the bottom of each well. To determine whether these cells were viable following their exposure to nikkomycin Z, I washed the drug out and added fresh tryptone broth to the adherent layer. In replicated trials, *B. dendrobatidis* cells previously exposed to 250 μM nikkomycin Z or greater were not viable following removal of the nikkomycin Z ($p > 0.05$ compared to negative control), whereas *B. dendrobatidis* cells exposed to lower concentrations exhibited an increase in optical density in a dose-dependent manner ($p < 0.01$ compared to negative control) (Fig. 4-3B).

Nikkomycin Z (20 μM) significantly reduced the replication capacity of the fungal cells, as shown by the reduced fold change of cells relative to the number of viable cells present at day 0 (Fig. 4-3C). *B. dendrobatidis* cells also exhibited a significant reduction in the number of matured cells (germling, thallus, or zoosporangium) following zoospore culture with 20 μM nikkomycin Z for 3 days (Fig. 4-3D). This is slightly less than the time it typically takes *B. dendrobatidis* to complete one life cycle (Berger et al. 2005a), suggesting that this drug inhibits the maturation of zoospores, which involves building a cell wall (Berger at al. 1999, Berger et al. 2005a). The number of each cell type following incubation with nikkomycin Z was divided by the original number of zoospores cultured to assess the ability of zoospores to mature in the presence of 20 μM nikkomycin Z. We observed a lower number of mature cells and a decreased number of viable cells surviving the 3 day incubation period (Fig. 4-3D). Experiments in this paragraph were conducted in collaboration with J. Scott Fites (Vanderbilt University, Nashville, TN).

Effects of nikkomycin Z on *B. dendrobatidis* susceptibility to osmotic lysis

I compared effects on zoospores pre-exposed or not to 50 μM nikkomycin Z and exposed or not to hypotonic shock. With cells resuspended in isosmotic APBS, I observed little change in optical density indicating cell stability. When I resuspended cells in dH_2O, I observed an increase in optical density followed by a decrease, indicating increased swelling and subsequent lysis in the hypotonic environment. I observed this in both positive control and nikkomycin Z-treated samples (Fig. 4-4A, 4-4B), but the ratio of optical densities measured in dH_2O and APBS was significantly lower in nikkomycin Z-treated cells, indicating reduced survival in hypotonic conditions due to greater osmotic lysis (Fig. 4-4C). Cell number also significantly decreased in dH_2O, but not in APBS, confirming the decreases observed in optical density were due to cell lysis (Fig. 4-4D).

Combined nikkomycin Z and amphibian AMP effects on *B. dendrobatidis* growth

I hypothesized that nikkomycin Z inhibition of cell wall synthesis might make the plasma membrane more susceptible to AMP activity. To assess possible interactions of AMPs and nikkomycin Z on *B. dendrobatidis* growth, I compared growth inhibition by each agent alone and both agents together and I observed a cooperative effect (Fig. 4-5). The combination of nikkomycin Z and AMP mixtures inhibited significantly more *B. dendrobatidis* growth than either alone (Fig. 4-5A). I observed the same effect when I replaced natural skin peptide mixtures with pure synthetic peptides: brevinin-1Sb (Fig. 4-5B) or brevinin-1Sc (Fig. 4-5C). In both cases, the combination of nikkomycin Z and peptide was significantly more inhibitory than either component alone. This was true at all peptide concentrations tested.

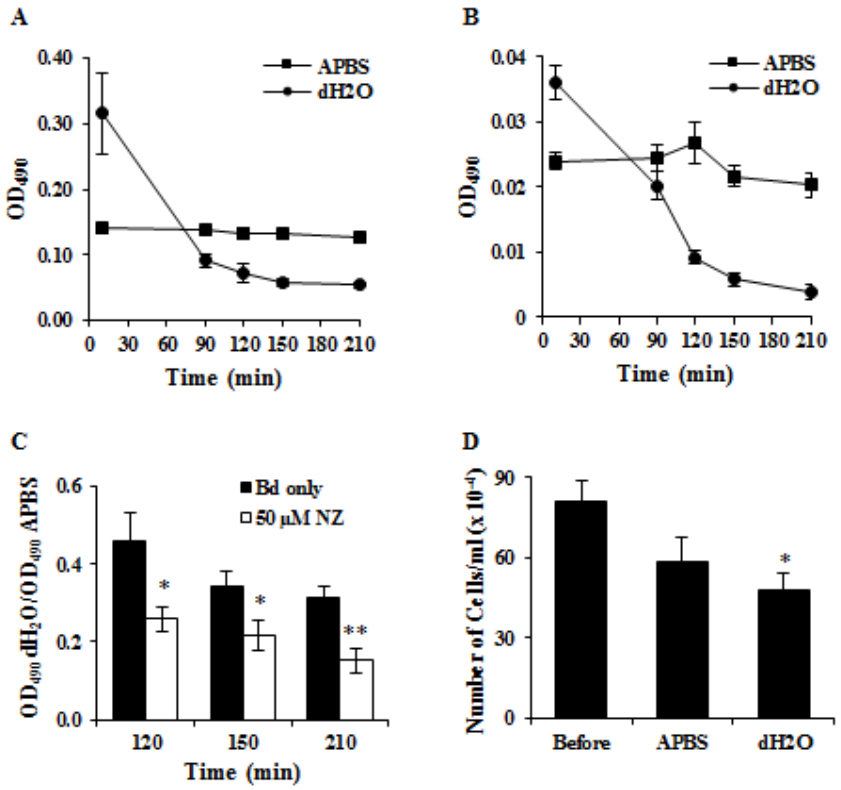


Figure 4-4. Nikkomycin Z increases *B. dendrobatidis* sensitivity to osmotic lysis. *B. dendrobatidis* alone (A) or with 50 µM NZ (B) are stable in isotonic conditions (APBS), but experienced cellular swelling followed by osmotic lysis in hypotonic conditions (dH_2O). Swelling and subsequent lysis are indicated by an initial increase followed by a decrease in optical density (OD_{490}) over a 210 minute time course. In Panels A and B, graphs are representative of three identical experiments for each condition. (C) The ratio of optical densities in dH_2O compared to APBS indicates that osmotic lysis is significantly higher in NZ-treated cells (*$p < 0.05$, **$p < 0.001$ by unpaired, two-tailed Student's t tests). (D) Cell counts immediately prior to resuspension and 120 minutes after resuspension in APBS or dH_2O indicate that cell numbers significantly decreased in the dH_2O hypotonic environment (*$p < 0.05$ by unpaired, two-tailed Student's t test), but were not significantly changed upon resuspension in isotonic APBS ($p > 0.05$). Panels C and D show the mean ± standard error of three identical experiments. In some cases, standard errors are not observable because they are within the limits of the data symbol.

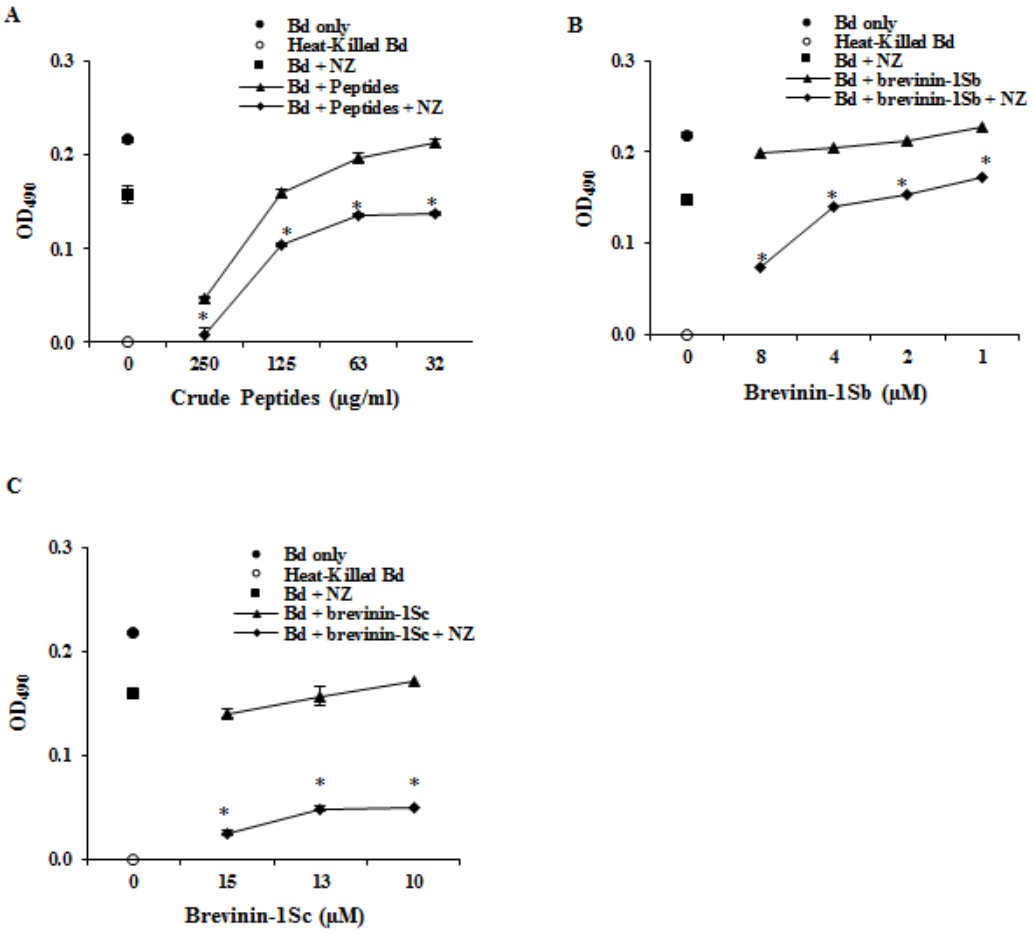


Figure 4-5. Nikkomycin Z and amphibian antimicrobial peptides inhibit *B. dendrobatidis* growth in a cooperative manner. (A) *B. dendrobatidis* cells were cultured in equal parts tryptone broth and HPLC water either alone ("Bd only"), with serial dilutions of *R. sphenocephala* antimicrobial peptides in HPLC water ("Bd + Peptides"), with 2 µM nikkomycin Z in HPLC water ("Bd + NZ"), or with both serial dilutions of *R. sphenocephala* antimicrobial peptides and 2 µM nikkomycin Z ("Bd + Peptides + NZ"). A negative control of dead *B. dendrobatidis* cells ("Heat-Killed Bd") was also included in each assay. *B. dendrobatidis* growth in the presence of NZ and peptides was significantly reduced compared to the growth in the presence of either NZ or peptides alone (*$p < 0.05$). In similar assays, I replaced a natural mixture of *R. sphenocephala* peptides with (B) purified brevinin-1Sb or (C) purified brevinin-1Sc. Each panel is representative of at least three experiments. Optical densities are the mean values ± standard errors for five replicates. In some cases, standard errors are not observable because they are within the limits of the data symbol.

Discussion

Effects of nikkomycin Z on *B. dendrobatidis* growth and morphology

Based on loss of cell viability and failure of additional growth following the removal of nikkomycin Z, the MIC of this drug against *B. dendrobatidis* is 250 μM. To my knowledge, this is the first report of nikkomycin Z effectiveness against a chytrid fungus. The minor increase in optical density of cells during initial culture with nikkomycin Z is best explained by increased cell size due to greater osmotic pressure.

In the absence of a normally functioning cell wall, fungal cells are highly susceptible to osmotic lysis (Ganesan et al. 2004). To examine this hypothesis, I conducted osmotic lysis assays and observed significantly greater lysis among cells treated with nikkomycin Z. This explains the reduced growth that *B. dendrobatidis* cells exhibit when cultured with high nikkomycin Z concentrations. While less substantial than in nikkomycin Z-treated *B. dendrobatidis,* I observed some lysis in dH_2O conditions in positive control cells without drug exposure. This is best explained by the fact that, at 5 days of culture, many of the original zoospores have grown into zoosporangia, released their zoospore contents, and reached the end of their lifespan. Both the newly released zoospores that lack a cell wall and the mature zoosporangia at the end of their lifespan may be susceptible to lysis in hypotonic environments, although not as susceptible as cells at the same stage treated with nikkomycin Z.

It is attractive to hypothesize that zoospores are the most vulnerable *B. dendrobatidis* life stage to antimicrobial agents like antifungal drugs and antimicrobial peptides due to their lack of a cell wall. The use of nikkomycin Z to prevent or delay the formation of a cell wall may increase the amount of time *B. dendrobatidis* cells are

sensitive to any antimicrobial agents that rely on the absence of a cell wall in order to disrupt the cell membrane or enter the intracellular space to exert their antimicrobial effects. Thus, the addition of nikkomycin Z to existing antifungal protocols may improve the likelihood of survival for infected amphibians.

Combined effects of nikkomycin Z and amphibian antimicrobial skin peptides

My observation of such a cooperative effect between nikkomycin Z and *R. sphenocephala* AMPs is significant because nikkomycin Z may be used to reduce *B. dendrobatidis* infection loads of many amphibian species by working cooperatively with the peptides that are naturally present. Further, this additive effect was observed using a concentration of nikkomycin Z far below its MIC. Since 2 μM nikkomycin Z can significantly inhibit *B. dendrobatidis* growth when combined with amphibian peptides, the current price of nikkomycin Z may not prohibit its use in treatments for infected amphibians if it is used at sub-MIC levels in a cocktail with other antifungal drugs like itraconazole, which is both effective against *B. dendrobatidis* by itself (Nichols and Lamirande 2000) and capable of synergism with nikkomycin Z against other species of fungi (Hector and Schaller 1992, Li and Rinaldi 1999, Ganesan et al. 2004).

The possible role of nikkomycin Z in the inhibition of host cell invasion

Recent studies highlight the importance of *B. dendrobatidis* germ tubes in invading host cell tissues and suggest that these structures have a cell wall (Greenspan et al. 2012, Van Rooij et al. 2012). The germ tube of an encysted zoospore stains with calcofluor white (Fig. 4-6), which demonstrates that chitin may be a major component of

this cell structure essential for invasion of amphibian keratinocytes. Additionally, the formation of the cell wall appears to happen immediately prior to germ tube-mediated host cell invasion. Delaying or preventing this cell wall formation may prevent the ability of the pathogen to invade the skin. Thus, nikkomycin Z activity may decrease the formation of these invading structures, thus inhibiting the mechanism by which the fungus enters host cells.

The use of nikkomycin Z to promote immune responses against *B. dendrobatidis*

Several studies indicate that the lymphocyte-mediated immune responses to this pathogen are impaired (Berger et al. 1998, Pessier et al. 1999, Berger et al. 2005b). *B. dendrobatidis* cells and supernatants treated with nikkomycin Z have a significantly reduced ability to inhibit lymphocyte proliferation, indicating that the immunosuppressive factor is sensitive to nikkomycin Z (Fites et al. 2013). Further, it is a common practice for fungal pathogens to mask pathogen-associated molecular patterns (PAMPs) in their cell walls to evade immune detection (Goodridge et al. 2009, Chai et al. 2010). Nikkomycin Z treatment may expose *B. dendrobatidis* PAMPs, increasing immune recognition in infected amphibians treated with nikkomycin Z. This also suggests that nikkomycin Z-treated *B. dendrobatidis* cells may be useful as an immunization tool since these cells appear to lack the immunosuppressive factor and may have increased cell-surface PAMP exposure, increasing the probability of pathogen recognition and an effective adaptive immune response to this deadly pathogen.

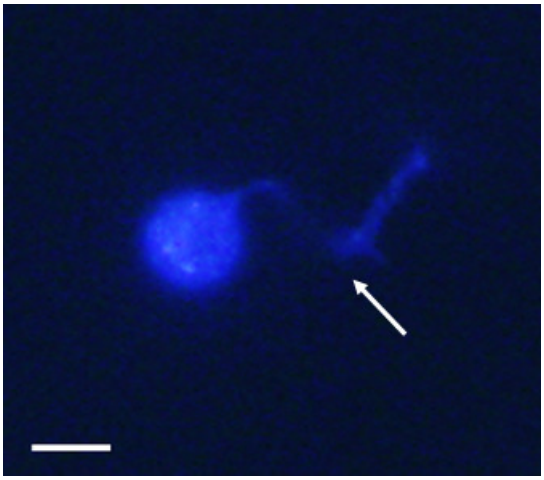


Figure 4-6. An encysted zoospore stained by calcofluor white allows visualization of the cell wall and a structure that appears to be germ tube. The proposed germ tube structure is labeled by an arrow. This suggests that chitin is a prevalent component of the germ tubes that are required for fungal invasion of host keratinocytes. Scale bar = 5 μm.

CHAPTER V

EFFECTS OF THE ANTIFUNGAL DRUGS AMPHOTERICIN B AND CHLORAMPHENICOL ON *BATRACHOCHYTRIUM DENDROBATIDIS* AND THEIR IMPACTS ON AMPHIBIAN INNATE IMMUNITY[2]

Abstract

Clinical trials testing potential antifungal drugs are needed to identify novel drugs to treat amphibians infected with this *B. dendrobatidis*. In this study, I quantified the minimal inhibitory concentrations (MIC) of chloramphenicol, amphotericin B, and itraconazole against *B. dendrobatidis*. I also found that treatment with chloramphenicol or amphotericin B significantly reduced *B. dendrobatidis* infection in naturally-infected southern leopard frogs (*Rana sphenocephala*) without host mortality, although neither drug was capable of complete fungal clearance. Long-term exposure of *R. sphenocephala* to these drugs did not inhibit antimicrobial peptide synthesis; however, I observed that chloramphenicol inhibited the growth of multiple *R. sphenocephala* skin bacterial isolates *in vitro* at concentrations below the MIC against *B. dendrobatidis*. Such results indicate that treatment with chloramphenicol might dramatically alter the protective natural skin microbiome when used as an antifungal agent. This study represents the first examination of alternative antifungal drug treatments on amphibian innate immune defenses.

[2] This chapter has been adapted from a manuscript entitled "An exploration of amphotericin B and chloramphenicol as alternative drugs for treatment of chytridiomycosis and their impacts on innate skin defenses" that has been re-submitted to *Applied & Environmental Microbiology* following minor modifications.

Introduction

The emerging fungal pathogen *B. dendrobatidis* causes chytridiomycosis, an amphibian skin disease characterized by disrupted skin functions. Due to the worldwide emergence of this pathogen, amphibians brought into captivity from wild populations are frequently infected with *B. dendrobatidis* and require quarantine and antifungal treatment before introduction into captive colonies. Currently, the most common treatment uses itraconazole, but this method is time-consuming, labor-intensive, and can be toxic (Garner et al. 2009, Woodhams et al. 2012b). For these reasons, clinical trials identifying other possible antifungal regimens are essential.

Amphotericin B has been identified recently as an antifungal drug active against *B. dendrobatidis* (Berger et al. 2009, Martel et al. 2011). Its mechanism lies in its ability to bind to ergosterol in fungal cell membranes (Gray et al. 2013). Another potential anti-*B. dendrobatidis* drug is chloramphenicol, which is known for its inhibition of prokaryotic ribosomes, preventing bacterial protein synthesis (Gale and Folkes 1953). Its antifungal mechanism is not well understood. Nevertheless, there are reports of chloramphenicol treatments resulting in cures of infected frogs (Bishop et al. 2009, Young et al. 2012), although its anti-chytrid properties were not quantified *in vitro*.

Whenever amphibian skin is treated with antifungal drugs, innate immune components present in the skin including ecologically important symbiotic skin bacteria and antimicrobial peptides, are also exposed to these compounds. Symbiotic skin bacteria have been identified as a potential defense against *B. dendrobatidis* in several species via the production and constitutive secretion of metabolites that inhibit *in vitro* growth of the fungus (refer to Chapter II). The interactions of these bacteria with their

host must be considered when antimicrobial agents are applied to the skin. Further, many species produce AMPs in dermal granular glands that are an important part of their innate immune defenses, which act as a first line of defense against a variety of infectious microorganisms, including *B. dendrobatidis* (Rinaldi et al. 2002, Rollins-Smith et al. 2002a, Rollins-Smith 2009). Thus, it is worthwhile to investigate possible effects of antifungal drugs on these natural innate skin defenses.

Here, I quantify the *in vitro* effects of amphotericin B and chloramphenicol against *B. dendrobatidis*. I also demonstrate that these drugs do not inhibit AMP production in *R. sphenocephala*, indicating that this innate immune defense is not impaired by antifungal drug treatment. However, I found that chloramphenicol is toxic to multiple skin bacterial isolates of *R. sphenocephala*, indicating that it may severely affect the innate skin microbiome defense. On the other hand, I did not find inhibitory effects on skin bacteria from amphotericin B or itraconazole. This study represents the first examination of impacts of antifungal on known amphibian innate skin defenses against the same pathogen. I also explored the effects of continuous exposure of naturally infected *R. sphenocephala* to either amphotericin B or chloramphenicol on infection level and mass throughout the drug treatments.

Material and Methods

Organisms

Rana sphenocephala (N = 35, Charles D. Sullivan Co., Nashville, TN) were collected in spring 2011. Using qPCR (methods in Chapter II), I determined that they were naturally infected with *B. dendrobatidis* ranging from 140 to over 200,000 zoospore

equivalents and they ranged in weight from 8.9 to 30.3 grams. For additional animal husbandry details, refer to Chapters II and III. *Batrachochytrium dendrobatidis* strain JEL197 (Longcore et al. 1999) was used in all *in vitro* experiments. Fresh subcultures of this strain were maintained throughout the length of experimentation. Both liquid and plate cultures were stored at 20-21°C with 1% tryptone as a nutrient source.

In vitro B. dendrobatidis growth inhibition by antifungal drugs

I conducted these assays using the same methods described for growth inhibition assays with *B. dendrobatidis* and bacterial supernatants presented in Chapter II, with the following modifications: Zoospores were cultured with or without addition of 50 µl serial dilutions of antifungal solutions in sterile HPLC-grade water (Fisher Scientific, Pittsburgh, PA). The antifungal drugs tested include amphotericin B (Fisher Scientific, Pittsburgh, PA), chloramphenicol (Sigma-Aldrich, St. Louis, MO), and itraconazole (Sporanox®, Centocor Ortho Biotech Products, Raritan, NJ).

Bacterial growth inhibition by antifungal drugs

I conducted these assays using the same methods described for growth inhibition assays with bacteria and antimicrobial peptides presented in Chapter III, with the following modifications: Instead of AMPs, the antifungal drugs itraconazole, amphotericin B, and chloramphenicol at various concentrations were diluted in HPLC water and cultured with bacterial isolates. The isolates used in these studies were isolated from the skin of adult *R. sphenocephala* using the swabbing and isolation method described in Chapter II.

Effects of antifungal drug exposure on antimicrobial peptide synthesis

I randomly divided *R. sphenocephala* (N = 14) into two groups (*B. dendrobatidis* infection levels were not significantly different between groups, unpaired two-tailed Student's t test, $p > 0.05$) and injected each frog to induce peptide secretion using 20 nmol norepinephrine per gbw as described in Chapter II. One group was maintained in a combination antifungal drug treatment of 200 µg/ml chloramphenicol and 40 µg/ml amphotericin B for 56 days. The second group was maintained in dechlorinated tap water for the same period. On Day 56, frogs were injected again with 20 nmol/gbw norepinephrine to induce peptides. Following the first and second injections, secreted peptides were collected, enriched, and quantified as described in Chapter II.

MALDI-TOF mass spectrometry

The presence or absence of previously described AMPs in individual skin secretions was determined by matrix-assisted laser-desorption ionization time-of-flight (MALDI-TOF) mass spectrometry as described in Chapter II.

***In vivo* antifungal treatments and quantification of *B. dendrobatidis* infection levels**

I randomly divided naturally infected *R. sphenocephala* (N = 34) into three groups and continually treated with 15 µg/ml amphotericin B (N = 12), 200 µg/ml chloramphenicol (N = 12), or dechlorinated tap water without antifungal drugs (N = 10). I swabbed frogs on days 0, 14, and 28 and used qPCR to assess infection intensity in terms of zoospore equivalents as described in Chapter II. DNA was extracted, and qPCR assays were performed on using methods described in Chapter II.

Statistical comparisons

I used unpaired, two-tailed Student's t tests and one-way ANOVA with Tukey post hoc tests as described in figure legends. In all statistical analyses, $p < 0.05$ was considered statistically significant. When more than one Student's t-test was performed within the same set of data, p values were adjusted with the Bonferroni correction (Bland and Altman 1995). Zoospore numbers and peptide concentrations compared in this study were log-transformed to normalize data and meet assumptions of homogeneity of variances for parametric statistics (Bland and Altman 1996, Manikandan 2010).

Results

Effects of antifungal drugs on *B. dendrobatidis* growth *in vitro*

I found that concentrations of itraconazole (Sporanox ®) as low as 20 ng/ml (Fig. 5-1A) consistently inhibited ≥90% of *B. dendrobatidis* growth in replicated trials. This concentration is considerably lower than the currently reported MIC of <1.56 μg/ml (Berger et al. 2009). Amphotericin B prevented *B. dendrobatidis* zoospore growth between 0.8 and 1.6 μg/ml in replicated trials (Fig. 5-1B). This is similar to the MIC's of 0.8 μg/ml and 3.125 μg/ml reported by Martel et al. (2011) and Berger et al. (2009), respectively. Chloramphenicol at 800 μg/ml completely inhibited growth of *B. dendrobatidis* zoospores (Fig. 5-1C) and concentrations as low as 12.5 μg/ml still inhibited up to 90% of growth.

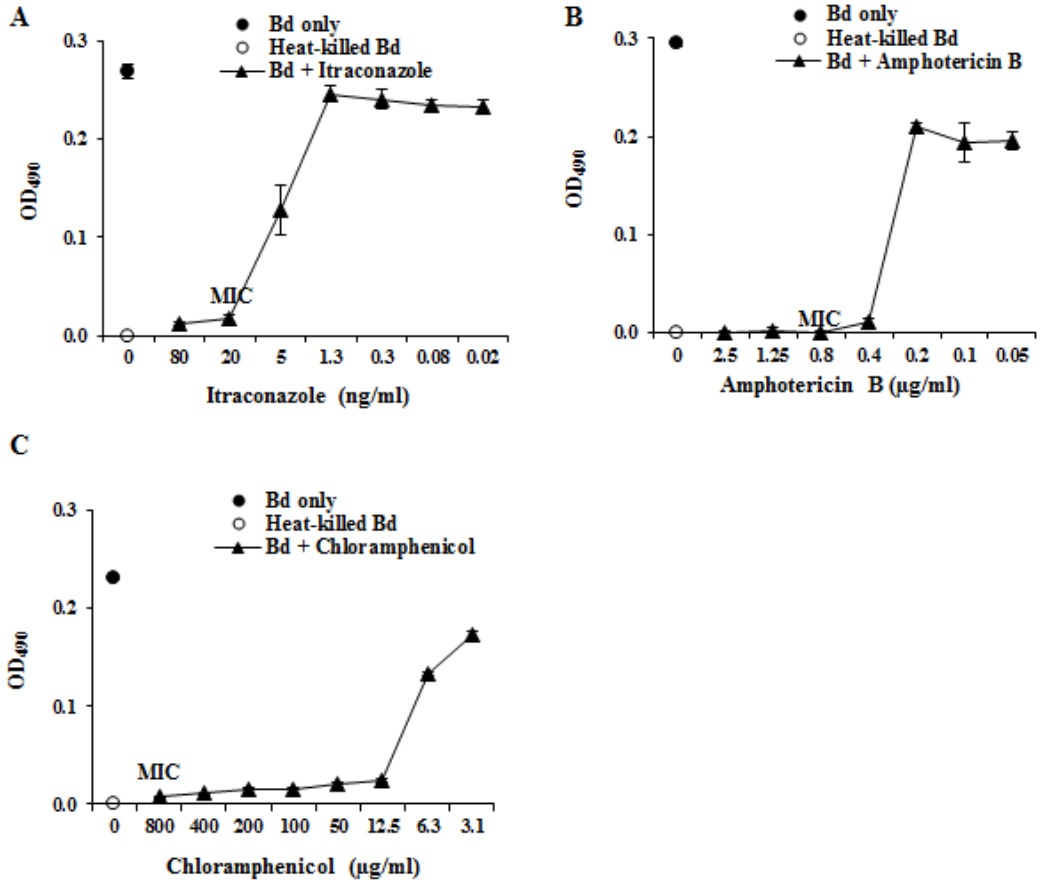


Figure 5-1. Itraconazole, amphotericin B, and chloramphenicol inhibit *B. dendrobatidis* growth *in vitro*. *B. dendrobatidis* in tryptone broth and HPLC water ("Bd only") or in tryptone broth with various concentrations of (A) itraconazole, (B) amphotericin B, or (C) chloramphenicol in HPLC water. Each data point represents the mean ± standard error (SEM) of five replicates. I tested multiple concentrations of each drug and the lowest antifungal drug concentration at which no *B. dendrobatidis* growth (defined as not significantly different, $p > 0.05$, from the negative control by an unpaired two-tailed Student's t test) is labeled as the minimum inhibitory concentration (MIC). Some error bars are not observable because they are within the limits of the data symbol. Data in each panel are representative of at least three similar experiments.

Bactericidal activity of chloramphenicol against amphibian skin symbionts

Prior to my study, it was unclear whether use of antifungal drugs would unintentionally deprive amphibians of the symbiotic skin bacteria important for innate defense. Therefore, examining the effects of chloramphenicol, amphotericin B, and itraconazole on natural *R. sphenocephala* skin symbionts is an important step to consider in developing new antifungal drug regimens to combat chytridiomycosis. I conducted growth inhibition assays testing these drugs against multiple *R. sphenocephala* skin isolates of varying morphologies. Chloramphenicol significantly inhibited the growth of every isolate tested (N = 10) at concentrations at and below the MIC of this drug against *B. dendrobatidis*, while neither amphotericin B or itraconazole inhibited the growth of any of the same isolates tested (Fig. 5-2A).

Chloramphenicol and amphotericin B do not inhibit antimicrobial peptide synthesis

Previous studies have showed that, following norepinephrine injection, peptide levels recover to pre-injection amounts in leopard frogs by 50 days post-injection (Pask et al. 2013). To assess whether continual exposure to chloramphenicol and amphotericin B impacts peptide synthesis, I induced peptides from *R. sphenocephala* on days 0 and 56. Between injections, frogs were kept in either dechlorinated tap water or in chloramphenicol and amphotericin B. Peptide levels after the second injection were not significantly different between groups or time points (Fig. 5-2B). All brevinin AMPs previously reported in this species (Conlon et al. 1999) were identified in secretions from individual frogs using MALDI-TOF mass spectrometry following drug treatment (Fig. 5-2C).

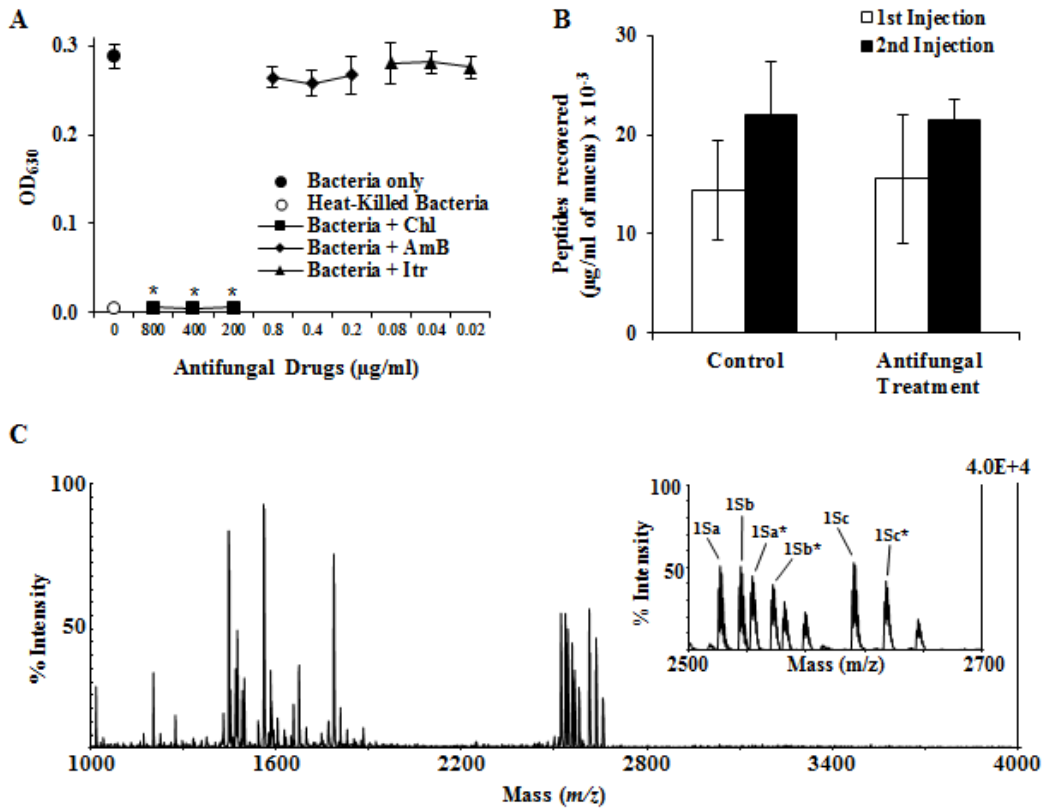


Figure 5-2. Effects of antifungal drugs on bacterial growth and on antimicrobial peptide synthesis. (A) Representative experiment of a growth inhibition assay testing each of the three antifungal drugs against multiple morphologically-distinct *R. sphenocephala* skin isolates. Every isolate that I tested (N = 10) was significantly inhibited by chloramphenicol (unpaired two-tailed Student's t test, *$p < 0.05$), while itraconazole and amphotericin B did not significantly impair growth of the same isolates at any concentration tested (unpaired two-tailed Student's t test, $p > 0.05$). (B) The peptide amounts measured before ("1st Injection) and after ("2nd Injection) continual exposure to both drugs ("Antifungal Treatment") or dechlorinated tap water ("Control") were not significantly different by one-way ANOVA with Tukey post hoc test ($p > 0.05$). (C) Representative spectrum of *R. sphenocephala* skin secretions (N = 5) following treatment with 200 µg/ml chloramphenicol and 40 µg/ml amphotericin B. Known peptides are labeled in the inset: brevinin-1Sa (1Sa, *m/z* = 2521, brevinin-1Sb (1Sb, *m/z* = 2535), and brevinin-1Sc (1Sc, *m/z* = 2612), while asterisks (*) mark sodium adducts of each peptide, respectively.

Antifungal activity against *B. dendrobatidis in vivo*

To assess the efficacy of continuous amphotericin B or chloramphenicol treatment, I divided naturally infected *R. sphenocephala* into three groups that were not significantly different in infection level (one-way ANOVA, $p > 0.05$). For 28 days, control frogs were in dechlorinated tap water without antifungal drugs. Experimental frogs were treated by either amphotericin B (15 µg/ml) or chloramphenicol (200 µg/ml). This chloramphenicol dosage was chosen because it inhibited ≥ 95% of *B. dendrobatidis* growth *in vitro* and was less inhibitory than the MIC to most bacterial isolates tested. Both drugs significantly reduced infections, but neither resulted in complete clearance (Fig. 5-3A). All groups experienced a net weight loss, but both antifungal-treated groups lost significantly less weight than controls (Fig. 5-3B). No frogs died in the treatments.

Discussion

Effects of antifungal drugs on *B. dendrobatidis* growth *in vitro*

This is the first report of an *in vitro* MIC against *B. dendrobatidis* for chloramphenicol, which has successfully cured amphibians in a small number of trials with low sample sizes (Bishop et al. 2009, Young et al. 2012). I confirmed the MIC of amphotericin B and more specifically defined the MIC for itraconazole. It is important to note that the itraconazole (Sporanox ®) uses hydroxypropyl-β-cyclodextrin to improve solubility. Additional components in the mixture include hydrochloric acid, propylene glycol, purified water, sodium hydroxide, and sodium saccharin. The possibility exists that this solvent may contribute to the lethal effects to hosts during *in vivo* studies and it may also add to the inhibitory effect upon *B. dendrobatidis* growth *in vitro*.

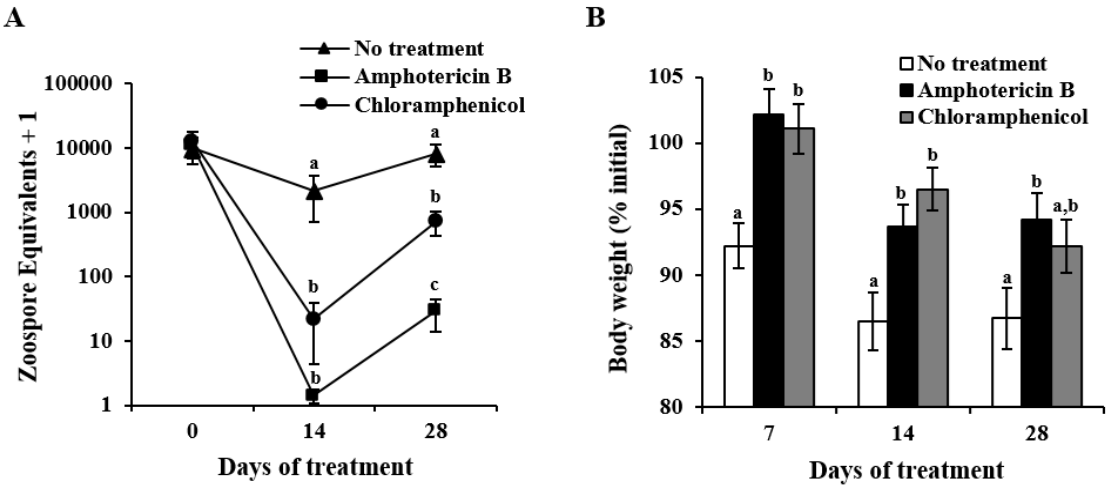


Figure 5-3. Amphotericin B and chloramphenicol reduce infection intensities on *B. dendrobatidis*-infected *R. sphenocephala*. (A) Treatment with either 200 µg/ml chloramphenicol (N = 12) or 15 µg/ml amphotericin B (N = 12) significantly reduced zoospore loads compared to untreated control frogs (N = 10). (B) Frogs in either antifungal drug-treated group lost significantly less weight compared to untreated frogs. For panels A and B, zoospore loads and frog weights between antifungal drug-treated groups and the control group were compared by one-way ANOVA with Tukey post hoc tests where different letters at each time point denote significance ($p < 0.05$).

Antifungal drug effects on *R. sphenocephala* innate immune skin defenses

Continual exposure to chloramphenicol and amphotericin B at concentrations which significantly reduced the burden of *B. dendrobatidis* infections for 56 days did not prevent antimicrobial peptide synthesis in *R. sphenocephala*. Because chloramphenicol inhibits growth of bacteria including the ten isolates that I tested, it is likely that the skin microbiota were reduced in the treated frogs. It has been suggested that antibiotic treatment inhibits peptide synthesis (Mangoni et al. 2001). My results do not support this observation. This will be an increasingly important point as this study and others (Bishop et al. 2009, Young et al. 2012) begin recommending continuous immersion antifungal treatments (i.e. two weeks or more) as a desirable alternative to the currently accepted daily itraconazole regimen. Though peptide defenses appear unimpaired by drug treatment, I observed significant inhibitory activity by chloramphenicol against ten *R. sphenocephala* skin bacterial isolates tested. Similarly, continual exposure to antibacterial agents like chloramphenicol may also impact microbiota in the gut that are essential for proper digestion and nutrient absorption, adversely affecting survival (Kupferberg 1997, Stevens and Hume 1998). These will be important considerations for investigators who undertake antifungal drug treatments of amphibians with the goal of returning them to the wild, especially for any species that naturally harbors known anti-*B. dendrobatidis* skin symbionts.

Effects of antifungal treatments *in vivo*

Amphotericin B and chloramphenicol significantly reduced but did not completely eliminate *B. dendrobatidis* infection, despite treatment solutions being

refreshed twice weekly to ensure continued drug efficacy. This phenomenon of significant inhibitory *in vitro* activity correlating with a decrease of *B. dendrobatidis* levels rather than a complete eradication has been reported before, and may be a result of fungistatic effects (Muijsers et al. 2012). This suggests that combination therapies may be the best focus for future studies, as they may be more likely to result in fully cured amphibians while preventing the development of resistant fungal strains. Despite the concerns itraconazole poses for some species and life stages, it has a history of clinical success and should continue to be used until successful alternative therapies can be found.

Another consideration is that both amphotericin B and chloramphenicol have reports of toxicity (Page 1991, Laniado-Laborín and Cabrales-Vargas 2009, reviewed in Muijsers et al. 2012). Specifically, chloramphenicol has been linked to bone marrow toxicity in humans (Rosenthal and Blackman 1965) and leukemia in toads (El-Mofty et al. 2000), while amphotericin B has caused observable toxic side effects in toad tadpoles at µg/ml concentrations (Martel et al. 2011). However, these effects were observed when drugs were administered at concentrations above those used in this study. I did not observe any lethal effects in the 28 day treatments, suggesting that toxicity may not result from the concentrations used or may not occur in adults of this species. However, further study of histopathology following treatment with these drugs is needed to determine the existence of any non-lethal toxic side effects, including the potential for anemia and nephrotoxicity.

CHAPTER VI

DISCUSSION AND FUTURE DIRECTIONS

Discussion

My dissertation work focused on two main goals: understanding the roles that skin bacteria and antimicrobial peptides play in defense against the amphibian fungal pathogen *B. dendrobatidis* (Fig. 6-1) and exploring new antifungal drug treatment protocols, with a specific focus on interactions between drugs and innate immune components (Fig. 6-2).

Using *R. sphenocephala*, I was the first to examine a protective role for the skin microbiome independent of any protection from antimicrobial peptides against the fungal pathogen *B. dendrobatidis* and identified new species of amphibian skin symbionts with the ability to inhibit or enhance the growth of this fungus. I also characterized a new antimicrobial peptide, Temporin-1S, in *R. sphenocephala,* elucidated the ontogeny of peptide development in this species, and described the effects of norepinephrine-stimulated peptide depletion on resident skin bacteria levels. I completed studies of three alternative antifungal drugs to determine their usefulness in treatment regimens while also focusing on their interactions with skin bacteria and AMPs. Finally, I developed three new methods that will be useful in future experiments in my field. These are (1) an antibiotic cocktail regimen to reduce amphibian skin bacteria (Chapter II), (2) norepinephrine-induced granular gland depletion (Appendix A), and (3) differential filtration to enrich for individual *B. dendrobatidis* life stages (Appendix B).

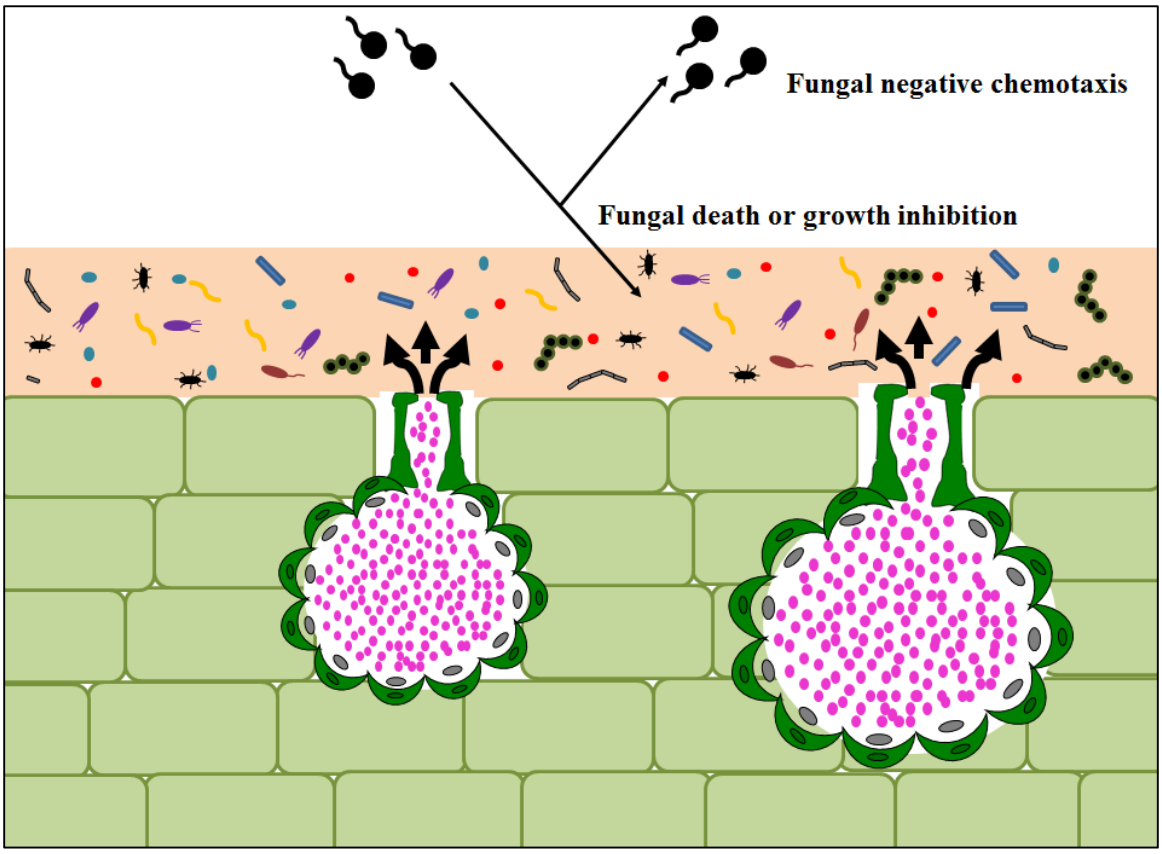


Figure 6-1. Skin innate immune defenses protect *R. sphenocephala* against the fungal pathogen *B. dendrobatidis*. Antimicrobial peptides in dermal granular glands are secreted onto the amphibian host's skin within a layer of mucus, which also hosts diverse symbiotic bacteria. Together, these defenses protect *R. sphenocephala* through various mechanisms, which may include triggering negative chemotaxis of the fungal zoospores away from host skin, inhibiting their growth, or causing their death. Image proportions are not necessarily drawn to scale.

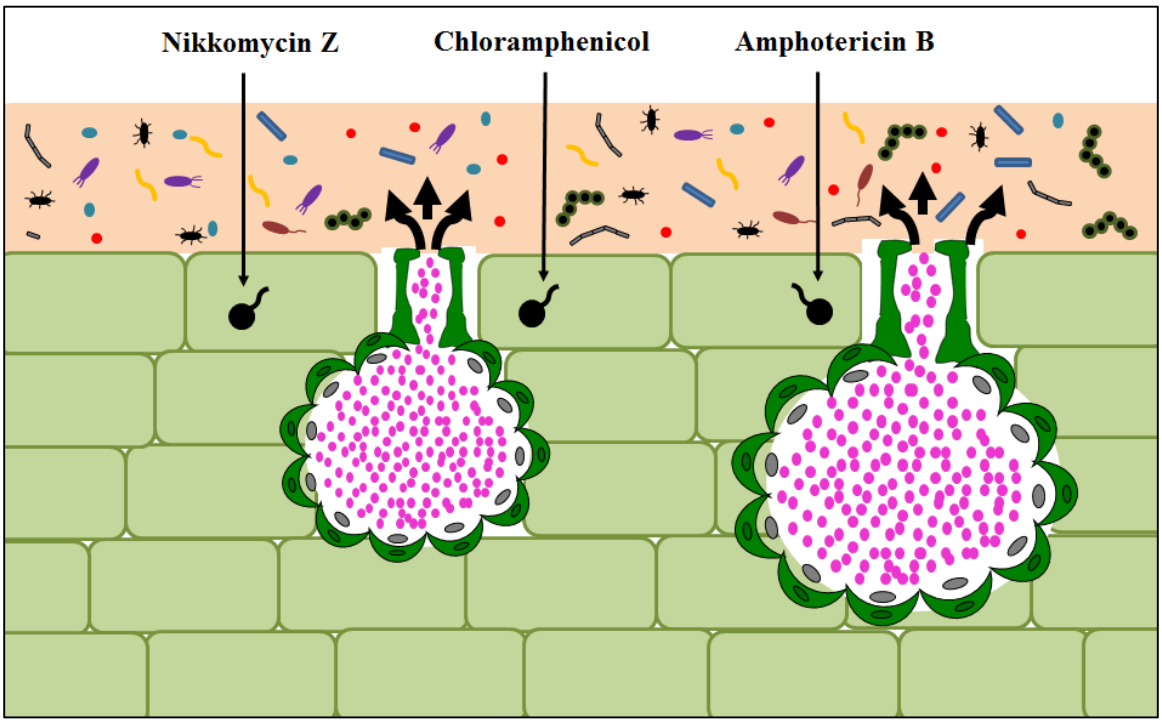


Figure 6-2. Nikkomycin Z, chloramphenicol, and amphotericin B are alternative drugs to treat amphibians infected with *B. dendrobatidis*. Despite the presence of antimicrobial peptides and symbiotic bacteria, *B. dendrobatidis* can still establish infection in many amphibians. This may be a result of one or more of the innate defenses being weakened or missing in a specific species or life stage. As a result, treatment with antifungal drugs is often necessary to eliminate the infection in captive animals, a process particularly important when bringing infected amphibians from the wild into protected environments within zoos, wildlife refuges, and conservation centers. My work has identified Nikkomycin Z to be a new antifungal drug effective against *B. dendrobatidis*. Further, I have extended the known understanding of chloramphenicol and amphotericin B as antifungal drugs against *B. dendrobatidis* by focusing on their effects on innate immune components present in the skin and conducting the first properly controlled clinical trials. Image proportions are not necessarily drawn to scale.

Skin bacteria contribute to anti-*B. dendrobatidis* defense in *R. sphenocephala*

To examine the role of the skin microbiome in protection of *R. sphenocephala* against *B. dendrobatidis* independent of AMP defenses, I used juveniles at one week post-metamorphosis after confirming their lack of skin peptides at this early developmental time point. I used an antibiotic cocktail to reduce skin bacteria from post-metamorphic juveniles ('metamorphs'). Following this treatment, I conducted an infection experiment on metamorphs with or without skin bacteria.

Reduction of skin bacteria resulted in increased *B. dendrobatidis* levels compared to control metamorphs with intact skin bacteria. I also collected isolates from the skin of these metamorphs prior to infection with *B. dendrobatidis* to study their natural skin microbiome. The 16S rDNA genes of these isolates were sequenced and 39 OTU's were identified. Supernatant analyses showed that several of these isolates constitutively secrete factors that inhibit growth of the fungus, although supernatants from three OTU's enhanced *B. dendrobatidis* growth. This study is the first observation of isolates from the phylum *Deinococcus-Thermus* on amphibian skin. Additionally, there were examples of species within the same genus or family that either enhanced or inhibited *B. dendrobatidis* growth, offering a greater depth of understanding of the diversity of the amphibian skin microbiome. Finally, every isolate tested from γ–*Proteobacteria* inhibited *B. dendrobatidis* growth, highlighting this lineage as one that invites further study.

This work adds to the growing body of knowledge of amphibian skin bacteria. It indicates additional diversity in the form of new taxonomic lineages present and in the various effects these bacteria have on *B. dendrobatidis* growth. By conducting these

experiments in very young metamorphs at a fragile life stage, I faced additional challenges to animal survival in the experiment that would most likely not have occurred with older animals. However, the benefits of using newly metamorphosed juveniles was the ability to study bacterial defenses in the absence of AMPs, a novel approach. My results indicate that the skin microbiome may be quite significant at this early time point and may protect *R. sphenocephala* at a time when their other defenses against this pathogen are limited.

AMPs contribute to anti-*B. dendrobatidis* defense in *R. sphenocephala*

Three antimicrobial peptides were previously described in this species, but their activity against *B. dendrobatidis* had not been determined. My studies confirmed the presence of these AMPs in *R. sphenocephala* adults by MALDI-TOF and tandem mass spectrometry and I conducted computational analyses to confirm the existence of a fourth AMP, Temporin-1S. I also showed that natural peptide mixtures effectively inhibited *B. dendrobatidis* growth and reported MICs for each of the individual purified synthetic peptides, which inhibited *B. dendrobatidis* at micromolar concentrations.

In the course of these experiments, I came upon a very interesting phenomenon. In two cohorts of *R. sphenocephala,* antimicrobial peptides were not expressed at significant levels early in post-metamorphosis development. This was unexpected because of previous reports of AMPs in tadpoles of two other species (Clark et al. 1994, Wabnitz et al. 1998). By investigating the presence of known peptides in skin secretions at various time points, I determined that *R. sphenocephala* skin peptide expression matures by 12 weeks post-metamorphosis. A delayed peptide development may explain

the vulnerability of juveniles of many species to *B. dendrobatidis*. My study of the peptide frequencies in *R. sphenocephala* from two populations indicated that while brevinin-1Sa was commonly expressed in both populations, additional peptides were expressed more frequently in one population than the other. This may be a result of differences in the genes for AMPs in these populations or due to differences in the environment which induce differential expression of the AMP genes.

Because of my interest in the interaction between antimicrobial peptides and symbiotic skin bacteria, I conducted experiments to determine if skin bacteria are susceptible to skin peptide activity, especially due to laboratory manipulations that result in high concentrations of peptides being secreted onto the skin. I found that while a few bacterial isolates were totally unaffected by peptide activity in *in vitro* assays, the growth of most isolates was impaired by antimicrobial peptides. This suggests that peptides may play a role in preventing symbiotic bacterial overgrowth on amphibian skin in addition to their roles in defense against pathogens. Additionally, I found that norepinephrine-induced peptide secretion, a common laboratory practice, significantly reduces the level of culturable skin bacteria on *R. sphenocephala*. This is an important finding as it shows that any future experiments using this technique to deplete peptides in animal experiments will also need to include proper controls to separate the effects of reduced bacteria vs. depleted peptides.

Alternative drug therapies to treat chytridiomycosis

In the search for novel anti-*Bd* therapies, I have determined that amphotericin B, chloramphenicol, and nikkomycin Z inhibit *Bd* growth *in vitro* with MICs of 0.8, 800,

and 500 μg/ml (250 μM), respectively. Continuous exposure to amphotericin B or chloramphenicol significantly reduced fungal burden of severely infected *R. sphenocephala* and decreased their weight loss over time compared to control, untreated frogs. I found that long-term treatment with amphotericin B and chloramphenicol did not inhibit antimicrobial peptide synthesis, although multiple *R. sphenocephala* skin bacterial isolates were sensitive to chloramphenicol *in vitro*, indicating that its use may significantly alter the skin microbiome.

My studies with the chitin synthase inhibitor nikkomycin Z indicate that exposure of *B. dendrobatidis* cells to sub-MIC concentrations resulted in significantly increased cell diameter, reduced replication capacity of whole cell cultures, and inhibited zoospore maturation, most likely by impairing cell wall synthesis. This drug also cooperates with *R. sphenocephala* natural peptide mixtures and synthetic purified antimicrobial peptides from this species to inhibit *B. dendrobatidis* growth *in vitro*.

These studies are significant due to a number of novel aspects. First, they represent the first assessment of drug impacts on the important amphibian innate skin defenses of antimicrobial peptides and symbiotic skin bacteria. I also conducted the first examination of cooperative activity between a drug and amphibian antimicrobial peptides. This is also the first time nikkomycin Z has been examined for effectiveness against a fungus from the phylum Chytridiomycota, which also includes *Synchytrium endobioticum*, a potato pathogen that causes black scab disease (Dickson 1922), and *Batrachochytrium salamandrivorans*, a recently discovered pathogen whose species name literally means "salamander-devouring" due to the destructive pathological effects

it has on host skin (Martel et al. 2013). These are all important additions to the literature surrounding the search for novel therapies for chytridiomycosis and other fungal diseases.

Future Studies

One of the questions I set out to answer during the course of my dissertation work was this: What are the relative contributions of antimicrobial peptides and symbiotic skin bacteria to defense against *B. dendrobatidis* in a relatively resistant amphibian species? Unfortunately, I encountered several unexpected hurdles. First, I observed a significant loss in the numbers of culturable skin bacteria from frogs moved from the wild into the laboratory. I observed this phenomenon in several experiments with two different species. Although I was able to prevent this reduction with the use of mesocosm water containing environmental bacteria, it appeared costly to the animals in terms of survival and weight loss, a phenomenon recently observed by another group (Küng et al. 2014). A second problem arose when I discovered that the antimicrobial peptide defense appears to be entirely absent immediately following metamorphosis and requires several weeks to mature. Thus, my original experimental design for an infection study involving frogs with both, one, or neither innate skin defense proved impossible, in part because of the microbiome changes and delay in peptide development, but also because the only way to deplete amphibian skin of peptides (norepinephrine injection) also significantly reduces skin bacteria, making a bacteria-intact, peptide-depleted group impossible to obtain.

I have considered many ways in which some of these hurdles could be overcome. For example, use of mesocosms throughout the experiment rather than bringing animals into the laboratory would overcome the unnatural changes to the skin microbiome and

give the peptide defense time to mature. However, the mesocosms would need to be provided with a source of insects to feed the developing frogs, and it is possible for frogs to escape the mesocosm or for predators to enter it, which may result in virulent strains of *B. dendrobatidis* used in such an infection experiment to be transported into the wild. In order to overcome the reduction in skin bacteria observed upon norepinephrine-induced peptide depletion, I have considered the use of genetically-modified frogs that have had their antimicrobial peptide genes knocked out. This is not currently possible in *R. sphenocephala*, as the gene for only one antimicrobial peptide has been reported (brevinin-1Sb, GenBank accession number: DQ923159). However, an amphibian species with all known antimicrobial peptide genes reported could theoretically be used instead. Of course, a total lack of antimicrobial peptides could result in drastic changes to the amounts and types of bacteria comprising the skin microbiome. Likewise, rendering amphibians without skin bacteria, either by antibiotic reduction or rearing in germ-free conditions, could impact antimicrobial peptide development. These are caveats researchers must consider before using frogs modified in any of these ways. In short, determining the relative contributions of antimicrobial peptides and symbiotic skin bacteria to defense against *B. dendrobatidis* is a surprisingly complex question that requires further research.

Nevertheless, my work has opened many avenues for additional future studies. In terms of the symbiotic skin bacteria community, future analyses should focus on the dynamic interactions that make this defense effective. For example, an exploration of whether bacterial signaling through innate pathways (Toll-like receptors, Nod-like receptors, etc.) can stimulate AMP expression and/or secretion and how these peptides

impact the diversity of skin bacteria present would be an important addition to the body of knowledge surrounding these defenses. Likewise, a study of whether bacterial skin symbionts are capable of sensing *B. dendrobatidis* in their immediate vicinity and upregulating production of antifungal metabolites could shed light on the mechanisms at work in effective bacterial defenses. In particular, recently emerging mass spectrometry techniques have been used to study how different interspecies interactions alter secreted metabolomes (Traxler et al. 2013). This technique could be useful in identifying new metabolites secreted by different symbionts in co-culture with *B. dendrobatidis* cells or supernatant. More in-depth sequencing of the diversity and numbers of inhibitory skin bacteria present on multiple individuals is an important step in determining whether a certain threshold of inhibitory bacteria must be met to result in an effective defense against *B. dendrobatidis*. Similarly, an analysis of how the bacterial skin community changes with age or *B. dendrobatidis* infection status will improve understanding of the amphibian skin microbiome and its defensive properties.

Our identification of multiple OTU's with inhibitory activity against *B. dendrobatidis*, including four that inhibit ≥90% growth, provides candidate bacteria to screen for novel antifungal genes and their products, which may result in new human medicines. Additionally, for amphibian species whose normal symbionts lack significant antifungal activity, newly identified antifungal genes could be inserted into these symbionts to improve the likelihood of protection. Previous attempts to transfer bacteria species to a new host species have not been successful due to failure of the introduce species to become established and persist (Becker et al. 2012, Woodhams et al. 2012b, Küng et al. 2014). Of course, this opens the door for questions about what other impacts

such genetically-modified bacteria might have, both on the host and in the larger ecosystem, which may reduce any benefits from such treatment.

I observed brevinin-1Sa in 96% and 98% of *R. sphenocephala* from Wilson and Shelby Counties, respectively. While it was the most prevalent peptide expressed, its MIC against *B. dendrobatidis* was less potent than the MICs for brevinin-1Sb and brevinin-1Sc, which were less frequently expressed in both populations. Thus, it will be interesting for future studies to examine if peptides that are more potent against this pathogen appear more frequently in subsequent generations as host and pathogen continue to co-exist in the same geographic range. Likewise, it would be interesting to determine whether *B. dendrobatidis* infection impacts the amounts of inhibitory peptides that are secreted onto the skin. This might be accomplished if antimicrobial peptide concentration on the skin can be quantified through direct-sampling MALDI (Pask et al. 2012), an increasing possibility as surface-desorption techniques continue to improve and become increasingly quantitative (Ifa et al. 2008, Da Costa et al. 2013).

Known amphibian AMPs, including those from other ranid frogs, are active against several important human bacterial, fungal, and viral pathogens as well as endotoxins and human tumors (Goraya et al. 1998, 2000, VanCompernolle et al. 2005, Koszałka et al. 2011, Wang et al. 2012, Schadich et al. 2013). The possible identification of further peptides with human medicinal potential is clearly an important goal, and there are undeniably new peptides to be discovered and characterized in the skin of *R. sphenocephala*. Dr. David Friedman (Vanderbilt Proteomics Laboratory, Nashville, TN) used tandem mass spectrometry to obtain partial sequences for peptide peaks that I commonly observed in *R. sphenocephala* secretions. For one peptide (m/z = 1557), we

obtained sequence information corresponding to >80% of the peptide's mass: S-I/L-V-G-W-R-D-K/Q-I/L-D-S. In the second, eighth, and ninth positions, the identity of the amino acid was narrowed down to two possibilities. The uncertainty of the leucine or isoleucine in the second and ninth positions matters little for antimicrobial activity, as both are similar amino acids and common in AMPs. If the eighth amino acid is a lysine, the resulting peptide would be cationic, amphipathic, and align most closely with three temporin family peptides. Unfortunately, more sequence information is needed for this peptide and others to assess antimicrobial character.

In terms of antifungal drug studies, my work opens several new questions. First, I showed that nikkomycin Z can cooperate with amphibian antimicrobial peptides to inhibit *B. dendrobatidis* growth. Similar cooperative effects could also exist between drugs like nikkomycin Z and antifungal bacterial metabolites. Also, it is known that the antifungal drugs itraconazole and terbinafine hydrochloride induce the production of antimicrobial peptides in human skin cells (Kanda et al. 2011). Identifying antifungal drugs that have similar effects on amphibian epithelial cells may help in the search for novel therapeutic regimens. In my work with *R. sphenocephala* skin bacteria, I developed an antibiotic protocol to significantly reduce skin bacteria levels. In future, this protocol could be modified to include antifungal drugs like chloramphenicol, amphotericin B, and nikkomycin Z if the desired goal is reduction of skin bacteria on individuals already infected by *B. dendrobatidis*. This modification could prevent *B. dendrobatidis* overgrowth on skin once natural bacterial competitors are removed and prior to bioaugmentation with beneficial species.

APPENDIX A

NOREPINEPHRINE DEPLETION OF ANTIMICROBIAL PEPTIDES FROM THE SKIN GLANDS OF *XENOPUS LAEVIS*[3]

Abstract

Amphibian granular gland secretion can be stimulated in the laboratory by norepinephrine injection. I found that two injections of 80 nmol/g norepinephrine were necessary to fully deplete the AMP stores. One injection resulted in secretion of most stored peptides. A second injection, 2 days later, released a small amount of AMPs that were not compositionally different from those released by the first injection. A third injection, 4 days after the first, did not result in further AMP release. Periodic acid-Schiff staining indicated that mucus gland secretion was also induced by norepinephrine.

Introduction

Natural AMP secretion from dermal granular glands is a result of α-adrenergic nerve stimulation, which causes contraction of myoepithelial cells, forcing AMP granules onto the skin surface (Benson and Hadley 1969, Dockray and Hopkins 1975). To investigate the role of AMPs in protection from *B. dendrobatidis*, it is necessary to deplete the granular glands of AMPs in experimental subjects (Ramsey et al. 2010). However, the possible necessity of multiple norepinephrine injections for total peptide

[3] This chapter is adapted from the publication: Gammill WM, Fites JS, Rollins-Smith LA. (2012) Norepinephrine depletion of antimicrobial peptides from the skin glands of *Xenopus laevis*. Developmental & Comparative Immunology 37(1):19-27.

depletion of granular glands has not been investigated. It also remains unclear what other effects, such as mucus gland depletion, would result from repeated norepinephrine stimulation.

Materials and Methods

Organisms

I used twelve healthy outbred adult *Xenopus laevis* (Xenopus I, Dexter, MI) ranging in weight from 60 to 135 g. They were maintained in polystyrene containers in dechlorinated tap water at approximately 22 ºC. Three times a week, they were fed ground beef heart and their water was changed. Examination of skin histology following euthanasia did not show any signs of *B. dendrobatidis* infection.

Skin peptide collection and enrichment

Crude skin peptides were collected from *X. laevis* by norepinephrine injection as previously described (refer to Chapter II). In collaboration with J. Scott Fites, I randomly divided twelve frogs into four groups. For group 1 (N = 3), we administered a subcutaneous dorsal injection of APBS on Day 0 to serve as a control group. For groups 2 – 4 (N = 9), we administered subcutaneous injections of norepinephrine-HCL at 80 nmol norepinephrine/gbw dissolved in APBS on Day 0. On Day 2, we repeated this protocol with groups 3 and 4 (N = 6) by injecting a second time. On Day 4, we gave group 4 frogs (N = 3) a third injection, this time at 20 nmol norepinephrine/gbw. Following each injection, peptides were collected in 50 ml collection buffer.

Histology

Each frog was euthanized by IACUC-approved methods 24 hr after its final injection. I fixed two sections each of dorsal and ventral skin in 10% buffered formalin for 48 hr. These sections were stained by Hematoxylin and Eosin (H&E) to observe granular glands or Periodic acid-Schiff (PAS) to observe mucus glands. Staining was completed by the Translational Pathology Shared Resource (Vanderbilt University, Nashville, TN). I photographed slides under a microscope with an Olympus DP71 camera and DP Controller software, version 3.1.1.267 (Olympus Corporation).

To assess peptide contents remaining in *X. laevis* granular glands following each injection, I quantified the amount of granular gland material observable in H&E stained images. Because amphibian granular glands are round or elliptical in shape (Barbeau and Lillywhite 2005), the area of the cross-section of the contents in each granular gland was calculated by measuring the height and width of each cross-section to the nearest 10 μm and calculating an area (Area = (πLW)/4 where L = length and W = width) assuming an elliptical shape for each gland (Moy 1970, Lepri and Randall 1983, Barbeau and Lillywhite 2005). Once I determined the areas of the cross-sections, I divided the sum of the areas (μm^2) in each skin sample by the length (mm) of the skin section to give an area of contents per mm of skin. In all cases, 90-100 mm of skin were analyzed per treatment.

Mass spectrometry

MALDI-TOF mass spectrometry was used to identify and determine relative quantities of individual AMPs in the secretions collected following each norepinephrine or APBS injection in *X. laevis*. For details on the methodology, refer to Chapter II.

Results

Development of a method to deplete amphibian AMPs from granular glands

I carried out a series of APBS or norepinephrine injections in *X. laevis* in collaboration with J. Scott Fites (Vanderbilt University, Nashville, TN). Following one APBS (vehicle) injection, we observed low amounts (4,522 ± 1,116 μg/ml of mucus) of secreted peptides in comparison with the amount of peptides secreted following the first norepinephrine injection at 80 nmol norepinephrine/gbw, which resulted in 406,215 ± 20,843 μg/ml of mucus, a 90-fold increase (Fig. A-1). A second injection resulted in an additional low level of peptide release (30,010 ± 6,232 μg/ml of mucus), while a third at a lower concentration led to peptide release (4,179 ± 442 μg/ml of mucus) not significantly different from those injected with APBS. This demonstrates that in *X. laevis*, one injection of 80 nmol norepinephrine/gbw is sufficient for major peptide release, but a second appears necessary for maximal depletion, and a third is unnecessary.

Following peptide collection, enrichment, and quantification, I analyzed secretions from norepinephrine- and APBS-injected frogs with MALDI-TOF MS to identify the individual peptides obtained with each injection (Fig. A-2). The peptides secreted following one norepinephrine injection (Fig. A-2A) were comparable in composition to the peptides released by a second injection (Fig. A-2B). In two cases, there were no identifiable peptides recovered after a second injection, indicating that even one injection was sufficient for depletion of some individuals. I also confirmed that a third injection (Fig. A-2C) was unnecessary for maximal AMP depletion as it did not yield further identifiable peptides and resulted in spectra qualitatively similar to the spectra of secretions following APBS injection (Fig. A-2D).

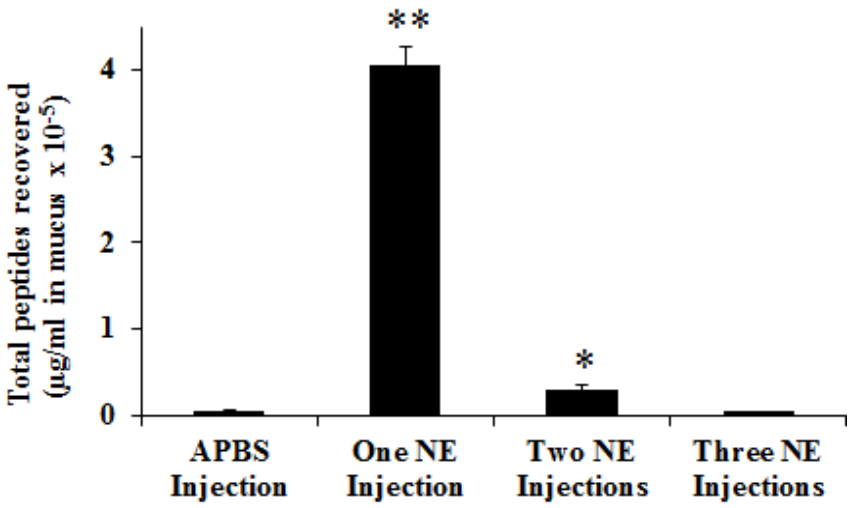


Figure A-1. Significant peptide release occurs following a single norepinephrine injection, but two injections are necessary for more complete depletion. Peptide release following injection of norepinephrine (NE) was greatest following one injection (N = 9 frogs, Day 0, 80 nmol NE/gbw). Additional peptides were recoverable after two injections of NE (N = 6 frogs, Day 2, 80 nmol norepinephrine/gbw), although at a significantly lower concentration. A third injection (N = 3 frogs, Day 4, 20 nmol NE/gbw) did not lead to a significant release of peptides ($p = 0.9191$) compared to the APBS-injected control (N = 3 frogs, Day 0). **$p < 0.0001$, *$p < 0.001$ compared to APBS-injected control by a two-tailed Student's *t* test after log transformation. One-way analysis of variance (ANOVA) with Tukey post hoc test confirmed *t* test results. Error bars represent standard errors. Some standard errors were too small to be visualized.

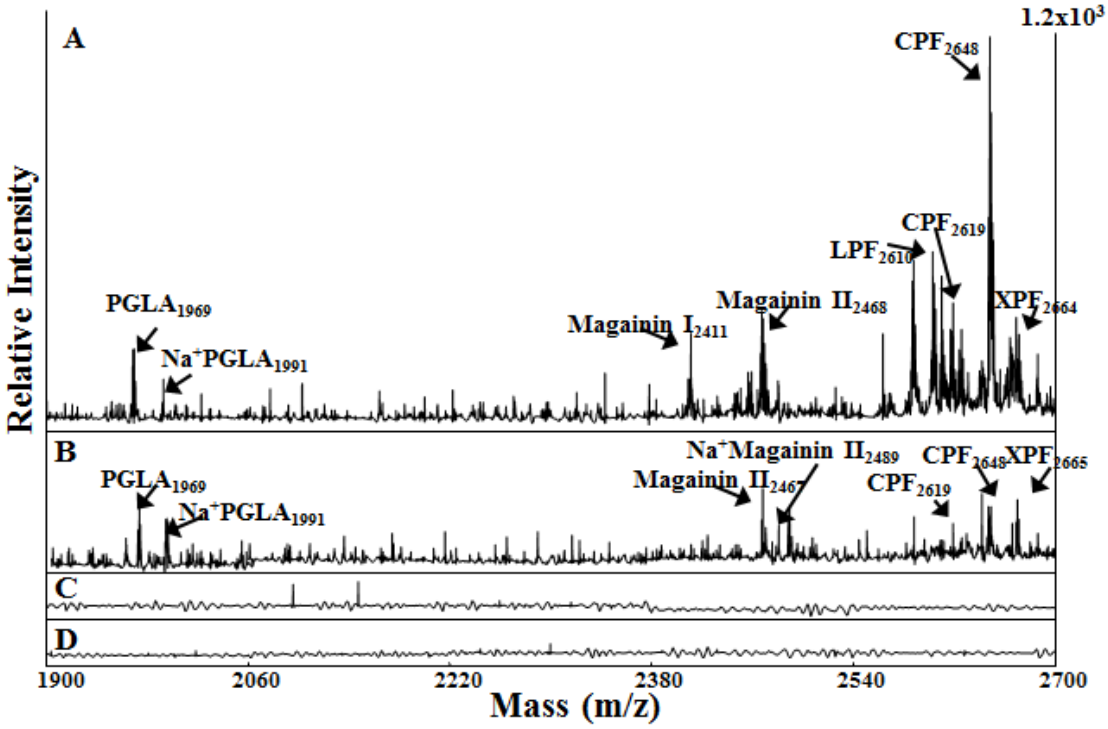


Figure A-2. The peptides released following a second injection of norepinephrine are compositionally similar to those released following the first injection. MALDI-TOF mass spectrometry analysis of AMPs collected following (A) one norepinephrine injection at 80 nmol norepinephrine/gbw, (B) two norepinephrine injections at 80 nmol norepinephrine/gbw, (C) two norepinephrine injections at 80 nmol norepinephrine/gbw followed by a final norepinephrine injection at 20 nmol norepinephrine/gbw, and (D) one APBS injection. Spectra (A – C) represent peptide secretions collected from the same frog after its first, second, and third norepinephrine injection, respectively, while the spectrum for the APBS control (D) represents a different frog. While variation was noted between different animals as described in the text, representative results are shown. Previously described antimicrobial peptides are labeled. PGLa = peptide with amino terminal glycine and carboxyl terminal leucinamide, CPF = caerulein precursor fragment, XPF = xenopsin precursor fragment, LPF = levitide precursor fragment, and Na^+ indicates an adduct of the peptide with a sodium ion.

Histology confirmed the results of peptide quantification and mass spectrometry analysis (Fig. A-3). Based on my quantification of granular gland contents, AMPs secreted following a second injection were mainly dorsal in origin, while there was no significant difference in the amount of material remaining in ventral glands following two or three injections (Fig. A-3I).

Norepinephrine induction of granular gland secretion triggers mucus gland activity

Dockray and Hopkins (1975) reported that *X. laevis* mucus glands do not secrete contents upon adrenaline injection, while Sjöberg and Flock (1976) showed mucus glands have adrenergic nerves as their sole source of stimulation. I used a PAS stain of skin fixed 24 hr after APBS or norepinephrine injection. I observed that dorsal and ventral glands generally contained a normal mucus level (Fig. A-4,A-D) after APBS injection. However, mucus glands responded to norepinephrine (Fig. A-4,E-P) by secretion, in agreement with Sjöberg and Flock's (1976) identification of α-adrenergic nerve terminals. After three norepinephrine injections, I observed a range of effects in which some glands were almost or completely empty (Fig. A-4,E-L, indicated by arrows), while others contained a normal level of mucus (Fig. A-4,K-P; indicated by "N"). Some mucus glands also sustained structural damage (Fig. A-4F, J, and O; indicated by asterisks) due to the intensity of multiple norepinephrine injections. This damage appeared to be in the form of ruptured gland walls, which suggested that the repeated norepinephrine stimulus caused significant physiological stress at a microscopic level. This may explain why Dockray and Hopkins (1975) did not observe an effect on the mucus glands, as they only used one injection at a relatively low concentration of 0.3 nmol norepinephrine/gbw.

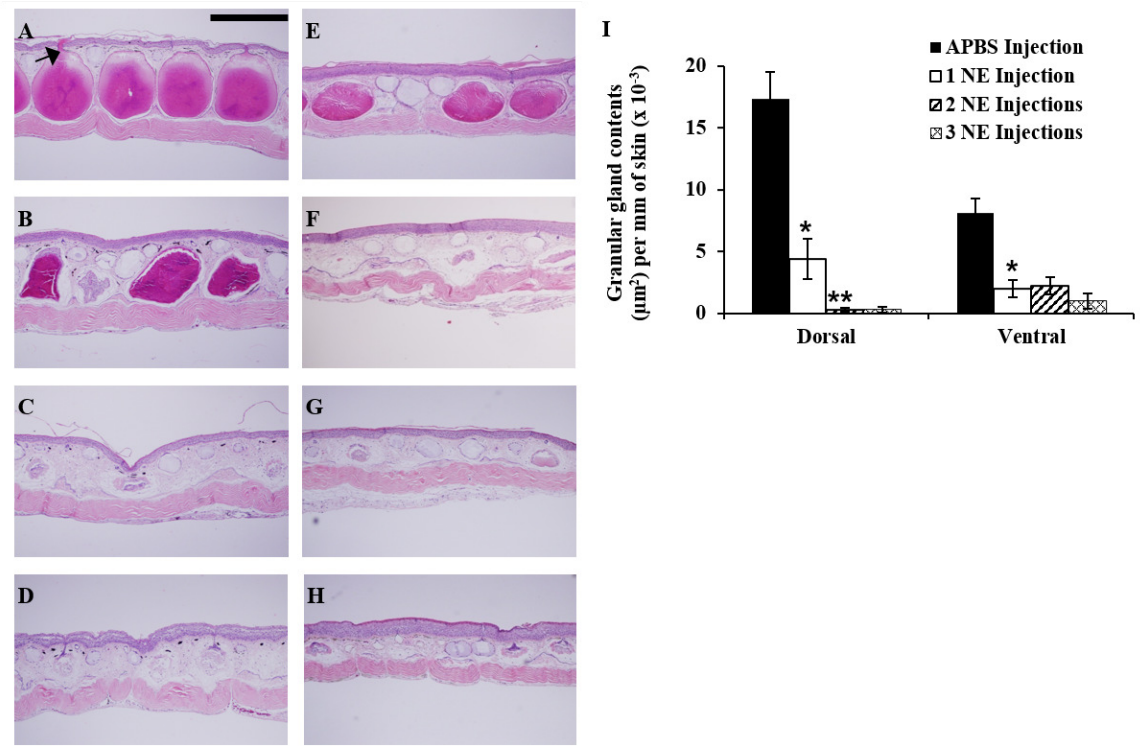


Figure A-3. Quantification of granular gland cross-sections indicate that a second norepinephrine injection is necessary for total granular gland depletion, but a third injection is unnecessary. Dorsal (A – D) and ventral (E –H) skin sections were stained with H&E stain to observe the granular gland contents following one APBS injection (A, E) or one (B, F), two (C, G), or three (D, H) norepinephrine injections. The scale bar is equivalent to 500 μm. Representative images are shown. (I) By quantifying the amount of granular gland contents remaining after each injection, I determined that peptides released following a second norepinephrine injection are primarily dorsal in origin. Between 150 and 160 glands per injections were analyzed. $^{**}p < 0.01$, $^{*}p < 0.05$ by a one-tailed Student's *t* test following log transformation. One-way analysis of variance (ANOVA) with Tukey post hoc test confirmed *t* test results. Error bars represent standard errors.

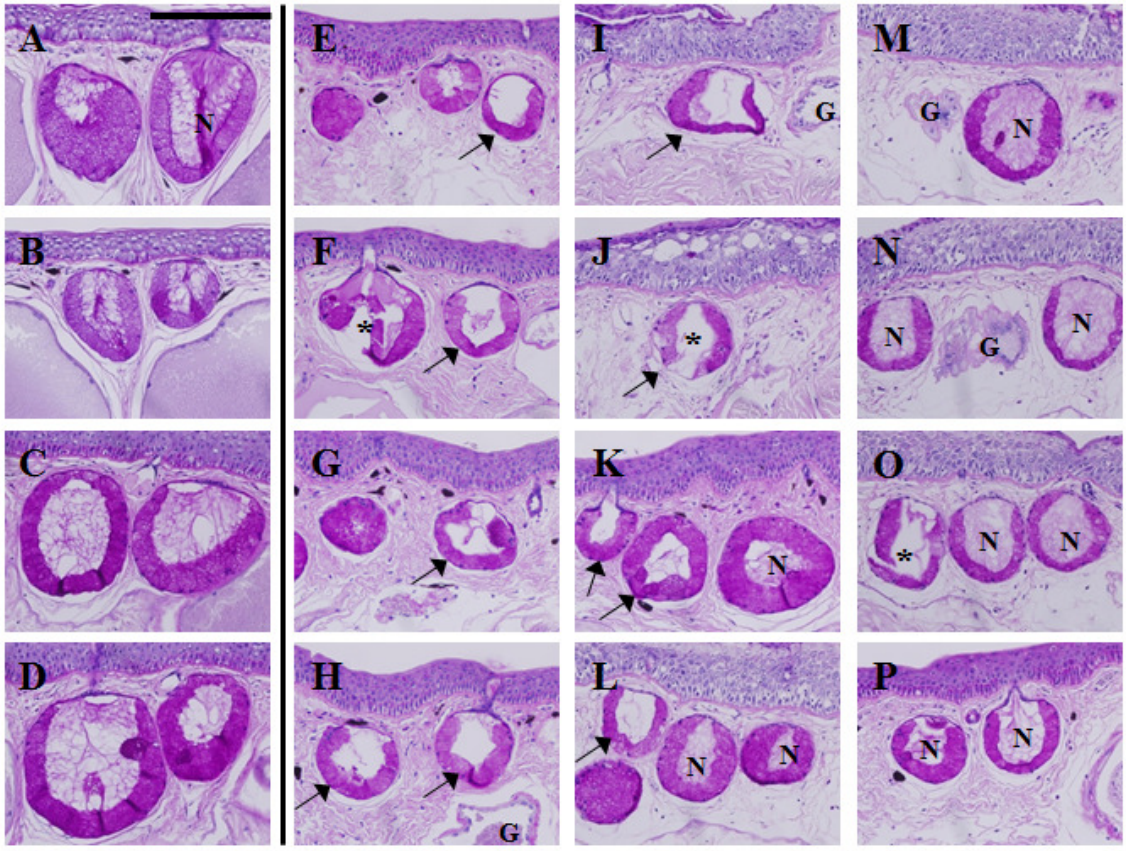


Figure A-4. Mucus glands are also stimulated by treatment with norepinephrine. PAS analysis of mucus glands indicated that mucus glands following APBS injection contained normal amounts of mucus in both dorsal (A-B) and ventral (C-D) glands. Following three norepinephrine injections, mucus gland secretion was evident, but at variable levels, from both dorsal (E-H,K,P) and ventral (I-J,L,M-O) glands. "N" indicates glands that have normal levels of mucus, arrows (↗) indicate glands that are mostly, if not entirely empty, and asterisks (*) indicate glands that show signs of structural damage. The scale bar is equivalent to 200 μm. Images shown are representative of larger sample sizes and dorsal and ventral glands are represented by an equal number of pictures.

Discussion

My data showed that one norepinephrine injection was sufficient to induce the release of most skin peptides. In fact, the amount collected after one injection was 20-fold higher than the amount released by norepinephrine signaling during a simulated predator attack (Ramsey et al. 2010). Thus, a second injection is necessary only when the desired result is near total peptide depletion. Analysis of ventral gland histology indicated that a low level of material (~7% of the contents following APBS injection) was still present after three dorsal injections, which may be due to distance of these glands from the injection site. Including a ventral injection in future protocols may result in elimination of any remaining peptides. Differences in the response of granular and mucus glands to the same treatment may lie in the distribution of nerve terminals. In granular glands, they are enmeshed within the myoepithelial cells with a direct connection to the secretory apparatus while in mucus glands, they are located outside the gland parenchyma, making indirect gland control via transmitter diffusion more likely.

Ultimately, granular glands provide an important chemical defense system against microbial invaders in the form of diverse AMPs. Continuing research on the function, regulation, and potential applications of these peptides is essential for devising conservation strategies for the many species that are currently facing the threat of chytridiomycosis. The methods presented here also provide a way to temporarily remove AMP defenses for *in vivo* experiments. Understanding how best to apply these methods to limit the stress to overall animal physiology is also important, as they are applied to a variety of species.

APPENDIX B

DIFFERENTIAL FILTRATION: A METHOD TO ENRICH INDIVIDUAL *BATRACHOCHYTRIUM DENDROBATIDIS* LIFE STAGES FROM HETEROGENEOUS CULTURES.

Abstract

As the scientific community continues to study the biological processes and pathogenic mechanisms of *B. dendrobatidis*, the ability to study different developmental stages of this fungus will become increasingly important. In this study, I developed a method to separate and enrich individual developmental stages of *B. dendrobatidis* using a system of differential filtration under vacuum. This method results in zoospore cultures of significantly higher purity than obtained with currently accepted protocols. Further, it allows for the partial enrichment of more mature life stages, including intermediate cells and zoosporangia. Recent studies in our laboratory (Fites et al 2013) showed that maturing *B. dendrobatidis* secrete factors that inhibit lymphocytes. However, the specific developmental stage at which the factors are produces is not yet determined. The development of this method for enrichment of intermediate stages meets a critical need to identify the specific stages of the *B. dendrobatidis* life cycle where host invasion machinery and secreted lymphotoxic factors are produced.

Introduction

Previous studies have focused on amphibian innate immune defenses against *B. dendrobatidis* as well as effective antifungal drugs to combat chytridiomycosis.

Antimicrobial peptides exert their effects by acting on the fungal cell membrane (Nicolas and Mor 1995, Zasloff 2002). Many antifungal drugs active against *B. dendrobatidis* target zoospore membranes or inhibit enzymes necessary for cell wall formation (Berger et al. 2009, Martel et al. 2011, Holden et al. 2014). Whether other known anti-*B. dendrobatidis* drugs or amphibian immune defenses like skin bacteria (Woodhams et al. 2007, Becker and Harris 2010, Woodhams et al. 2012a) and antibodies (Ramsey et al. 2010) primarily target zoospores or more mature life stages has yet to be determined. Further, *B. dendrobatidis* secretes one or more soluble factors, most likely components of the cell wall, which paralyze amphibian lymphocyte responses (Fites et al. 2013). As work to characterize and identify these immunosuppressive factors continues, the ability to examine their presence and potency among different developmental stages will prove essential to fully understanding how *B. dendrobatidis* modulates host immunity.

Here, we describe a sterile method to enrich for individual developmental stages of *B. dendrobatidis*. This method uses filters of different pore sizes to separate cells based on size, resulting in significant enrichment of mature life stages separate from immature zoospores or highly pure zoospore cultures, depending on the filters used.

Material and Methods

Zoospore Enrichment

These experiments were conducted with *B. dendrobatidis* strain JEL197 (Longcore et al. 1999). For information on fungal culture conditions, refer to Chapter II. Zoospores were harvested from agar plates as previously described (Chapter II) with modifications described here: The volumes obtained directly from plates without use of a

filtration step were considered an unenriched zoospore population. To obtain a more pure culture, liquid from agar plates was filtered under vacuum through sterile nylon spectra/mesh filters with pore sizes of 5 or 20 μm (Spectrum Laboratories, Rancho Dominguez, CA) or through sterile nitrocellulose-membrane filters with a pore size of 3 μm (Millipore, Billerica, MA). The different classes of cells distinguished with a light microscope were zoospores, encysted zoospores (appearance of a germ tube-like structure), germlings/thalli (rhizoid-bearing cells), or mature zoosporangia with internal zoospores (Berger et al. 2005a). Purity of a given cell type was determined based on the number of those cells divided by the number of total cells. Cell yield was determined by comparing the cell counts of the filter-enriched fraction to a fraction obtained before enrichment. Experiments described in this section were performed by J. Scott Fites (Vanderbilt University, Nashville, TN).

Intermediate Cell Enrichment

To enrich for intermediate stages (germlings and thalli), I harvested zoospores from agar plates following 5-8 days of growth, using 20 μm pore filters as described above. I quantified cells in the filtrate by counts on a hemocytometer and resuspended them in 1% tryptone broth at 1×10^6 cells/ml. These were incubated in cell culture flasks at 19-21°C for 3 days. During this time, harvested zoospores developed into intermediate cells. Following incubation, I scraped the sides of the cell culture flask with a sterile cell scraper (BD Falcon, Bedford, MA) to release adherent cells into the media. I transferred the flask contents to wetted 20 μm pore filter paper and rinsed the flask with 5 ml additional tryptone broth which was also filtered. Then, I filtered the filtrate a second

time using 8 μm pore filter paper to retain intermediate cells while allowing zoospores to pass through. I recovered the intermediate cells by transferring the 8 μm pore filter paper to a conical with sterile forceps and resuspended the retained cells in sterile media. Cell cultures prior to filtration, cells in the filtrate, and cells resuspended from the filter were counted on a hemocytometer and assessed for life stage by microscopy.

Mature Cell Enrichment

To enrich for more mature stages, including intermediate cells and mature zoosporangia, I harvested zoospores with 20 μm pore filters and resuspended them at 1×10^6 cells/ml. These were incubated in cell culture flasks at 19-21°C for 4 days as the zoospores developed into intermediate cells (germlings and thalli) and mature cells (zoosporangia). To enrich for these cell types, I scraped the sides of a cell culture flask with a sterile cell scraper and transferred flask contents to a wetted 20 μm pore filter paper which I rinsed and transferred to a conical to resuspend retained cells in sterile media. Cell cultures prior to filtration, cells in the filtrate, and cells resuspended from the filter were counted on a hemocytometer and assessed for life stage by microscopy.

Results

Improved enrichment of *B. dendrobatidis* zoospores

Zoospores and the number of total cells washed from agar plate cultures of *B. dendrobatidis* were quantified without filtration and following filtration with 3, 5, and 20 μm pores. Without filtration, zoospore purity was only 70.4% ± 2.0% (Fig B-1A). In comparison, zoospore purity significantly increased when filter-enriching the population.

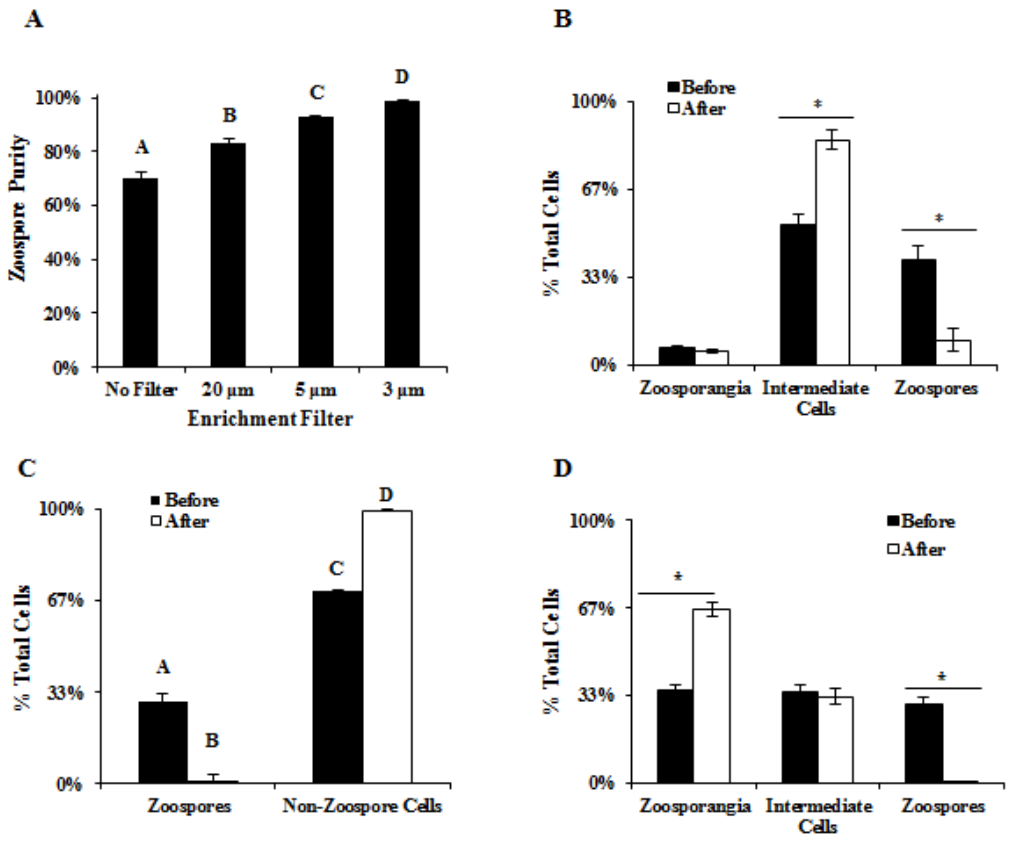


Figure B-1. A method to enrich for different *B. dendrobatidis* life stages by differential filtration. (A) Zoospore purity with or without filtration using different pore sizes is shown as mean ± SEM (one-way ANOVA with Tukey post hoc test, different letters represent significant differences, $p < 0.01$). (B) Use of a 20 µm pore filter and an 8 µm pore filter enriched for intermediate cell types and reduced the number of zoospores in the resulting product (two-tailed Student's t tests, $*p < 0.05$). (C) Use of a 20 µm pore filter enriched for all non-zoospores cell types (germlings, thalli, and zoosporangia) and reduced the number of zoospores in the resulting product (one-way ANOVA with Tukey post hoc test, different letters represent significant differences, $p < 0.05$). (D) Specifically, use of a 20 µm pore filter to enrich for all non-zoospores cell types resulted in a significant increase in zoosporangia and a significant decrease in zoospores, with the percentage of intermediate cells present before and after filtration largely unaffected. All panels show the mean of at least three experiments. Where more than one Student's t test was conducted within one data set, p values have been adjusted by the Bonferroni correction. All panels indicated the average percentage a given cell type among total cells counted before and after filtration. Error bars indicate standard error.

With 20 μm pores, zoospores were 83.3% ± 1.4% of the filtrate population. Even greater purity (92.9% ± 0.5%) was obtained when enriched with 5 μm pores, and the highest purity levels were achieved with the 3 μm pores (98.7 ± 0.5%), though yield was reduced with only 12.4% ± 3.7% of the original cells reaching the filtrate.

Separation and enrichment of intermediate *B. dendrobatidis* developmental stages

Following 3 days of incubation, zoospore cultures matured and contained a significant portion of intermediate cells (52.9% ± 4.3%), defined as encysted zoospores, germlings, and thalli. In order to enrich for these intermediate cell types, two filtration steps were necessary. First, I used filtration with a 20 μm pore filter to separate zoospores and intermediate cells from zoosporangia, which were retained by the filter. I filtered the filtrate from this procedure a second time using an 8 μm pore filter to separate intermediate cells retained by the filter from zoospores in the filtrate. Cells resuspended from the 8 μm pore filter were 85.4% ± 3.7% intermediate cells (Fig B-1B). This was approximately 5% ± 3% of the culture prior to filtration, with 1.8×10^7 cells ± 26% on average, indicating a useful cell yield despite significant cell loss from two filtrations.

Separation and enrichment of mature *B. dendrobatidis* developmental stages

Following 4 days of incubation, zoospore cultures matured and contained a significant proportion of cells beyond the zoospore stage (70.0% ± 2.7%), defined as encysted zoospores, germlings, thalli, and zoosporangia. With filtration with 20 μm pore filters, many maturing cells were retained while zoospores and some intermediate cells passed through the filter. Cells resuspended from the filter were 99.3% ± 0.3% maturing

cells (Fig. B-1C). The average cell yield was 5.8×10^7 cells ± 10%, which was approximately 38% ± 11% of the culture prior to filtration. In distinguishing among mature zoosporangia and intermediate cells at the encysted, germling, or thallus stages, I observed that the increased percentage of maturing cells retained by the 20 µm filter was specifically due to an enrichment in zoosporangia, as the percentage of intermediate cells was unaffected by filtration (Fig. B-1D). A second filtration step did not improve the purity level of this population of cells and reduced the cell yield.

Discussion

Enrichment of individual *B. dendrobatidis* life stages

Here, we present a novel method for enriching individual *B. dendrobatidis* life stages *in vitro* in high yields for further experimentation. The development of this method provides a way to obtain highly pure zoospore cultures. Some studies of the properties of zoospores have been conducted using cells collected from an agar plate without a filtration step to enrich for zoospores (Brutyn et al. 2012, McMahon et al. 2013). Our findings show that such cultures are ~30% more mature cells. Thus, results from these studies are inconclusive as to the role of zoospores or zoospore products.

Using differential filtration allows for the enrichment of mature stages, but we emphasize that some cells in the resulting culture may not be viable. Particularly, empty zoosporangia that have released their zoospore contents will be incapable of further growth. Further, differences in strain and culture conditions, including temperature, cell concentration, and type of media used, may result in different percentages of viable cells.

Improved understanding of host-pathogen interactions

This method will enable analysis and quantification of physiologic and pathogenic aspects specific to each fungal developmental stage. It will also allow more in-depth analysis of the antifungal activity of several immune system components of interest. For example, there is no information on which life stages are primarily targeted by bacterial metabolites and antibodies. And while AMPs are thought to be most effective against the zoospore stage, which lacks a cell wall, little is known about how they may affect more mature life stages. Further, *B. dendrobatidis* secretes a soluble inhibitory factor that impairs lymphocyte activity by causing apoptosis (Fites et al. 2013). Characterization of this factor in individual developmental stages will result in improved knowledge of *B. dendrobatidis* pathogenicity in addition to a better understanding of the interplay between the host immune system and each of the pathogen's developmental stages.

Development of improved antifungal drug regimens

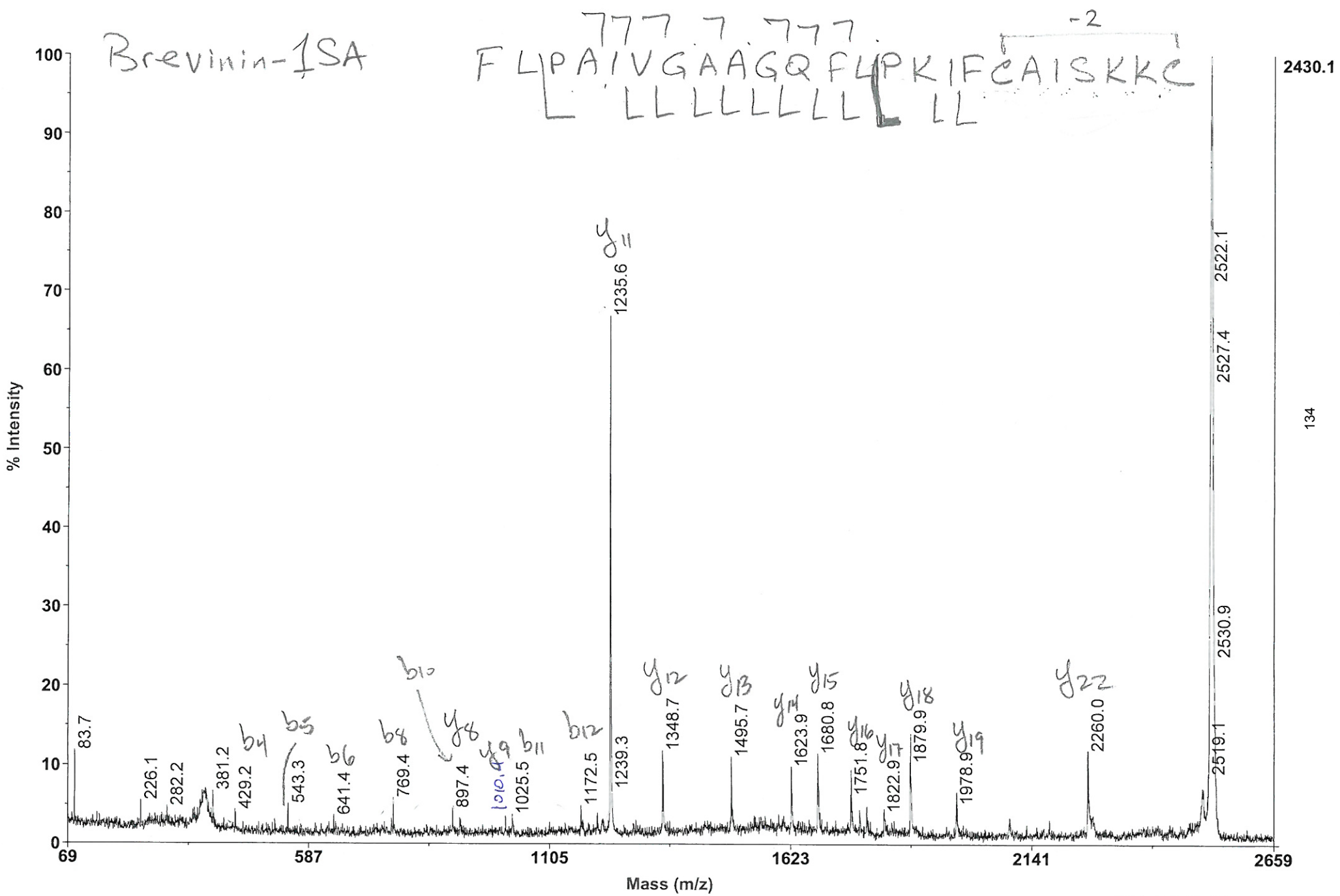
The ability to study specific developmental stages individually may result in the development of improved antifungal drug regimens. There are currently few treatments for amphibians infected with *B. dendrobatidis* and use of the most common drug of choice, itraconazole, involves a labor intensive, time consuming regimen with toxicity concerns (Garner et al. 2009, Woodhams et al. 2012b). The ability to separate and individually study different life stages of *B. dendrobatidis* may allow for the development of a cocktail with components that are active against multiple developmental stages of *B. dendrobatidis*, resulting in improved treatment regimens for captive amphibians.

APPENDIX C

TANDEM MASS SPECTROMETRY DATA

The following pages show fragmentation data collected during MALDI-TOF-TOF with notation by Dr. David Friedman (Vanderbilt Proteomics Laboratory, Nashville, TN). Fragmentation data was collected for brevinin-1Sa, brevinin-1Sb, brevinin-1Sc, and Temporin-1S. Brevinin-1Sb is labeled as "Brevinin-1BLa" because the two peptides have the same peptide sequence, despite being from two different amphibian species.

4700 MS/MS Precursor 2520.04 Spec #1[BP = 2523.9, 2430]

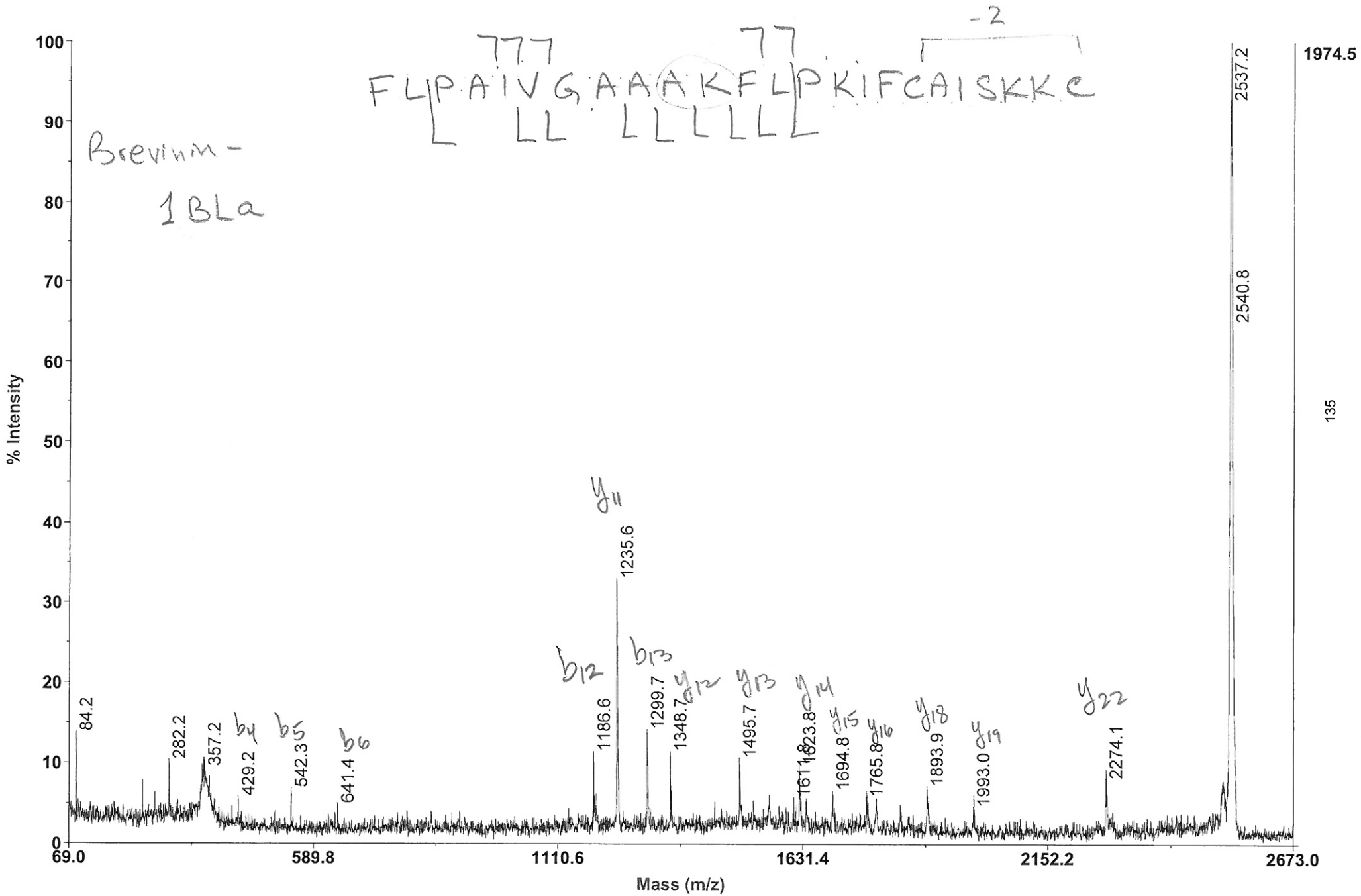


Printed: 13:58, February 15, 2012

T:\proteomics\instruments\local\proteomics 4700\D drive\toftof data\Proteomics Laboratory\Rollins-Smith\P2548 sample 23 2521.T2D

Applied Biosystems 4700 Proteomics Analyzer 347007018

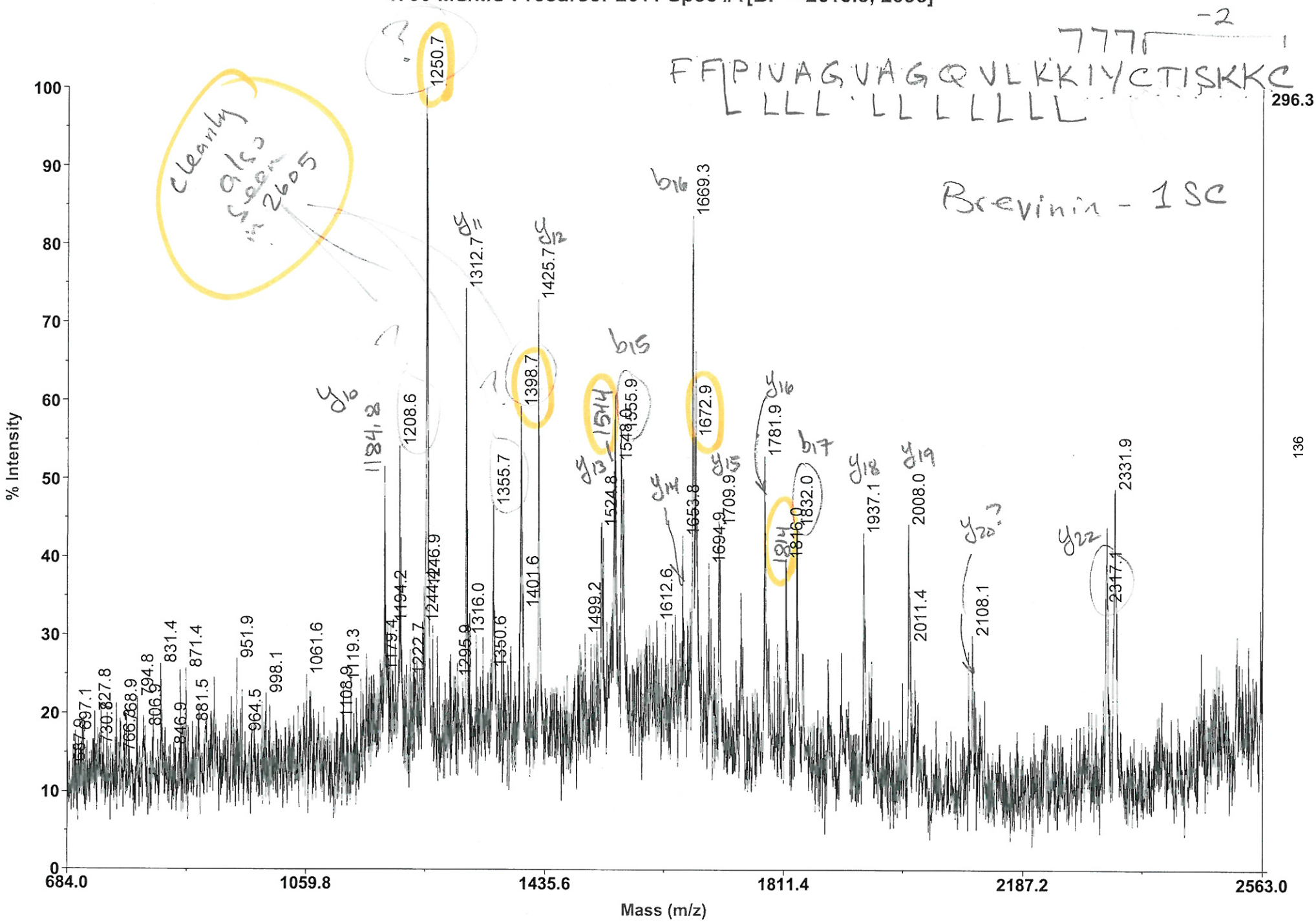
4700 MS/MS Precursor 2534.1 Spec #1[BP = 2538.5, 1975]



Printed: 14:08, February 15, 2012

T:\proteomics\instruments\local\proteomics 4700\D drive\toftof data\Proteomics Laboratory\Rollins-Smith\P2548 sample 23 2534.T2D

Applied Biosystems 4700 Proteomics Analyzer 347007018

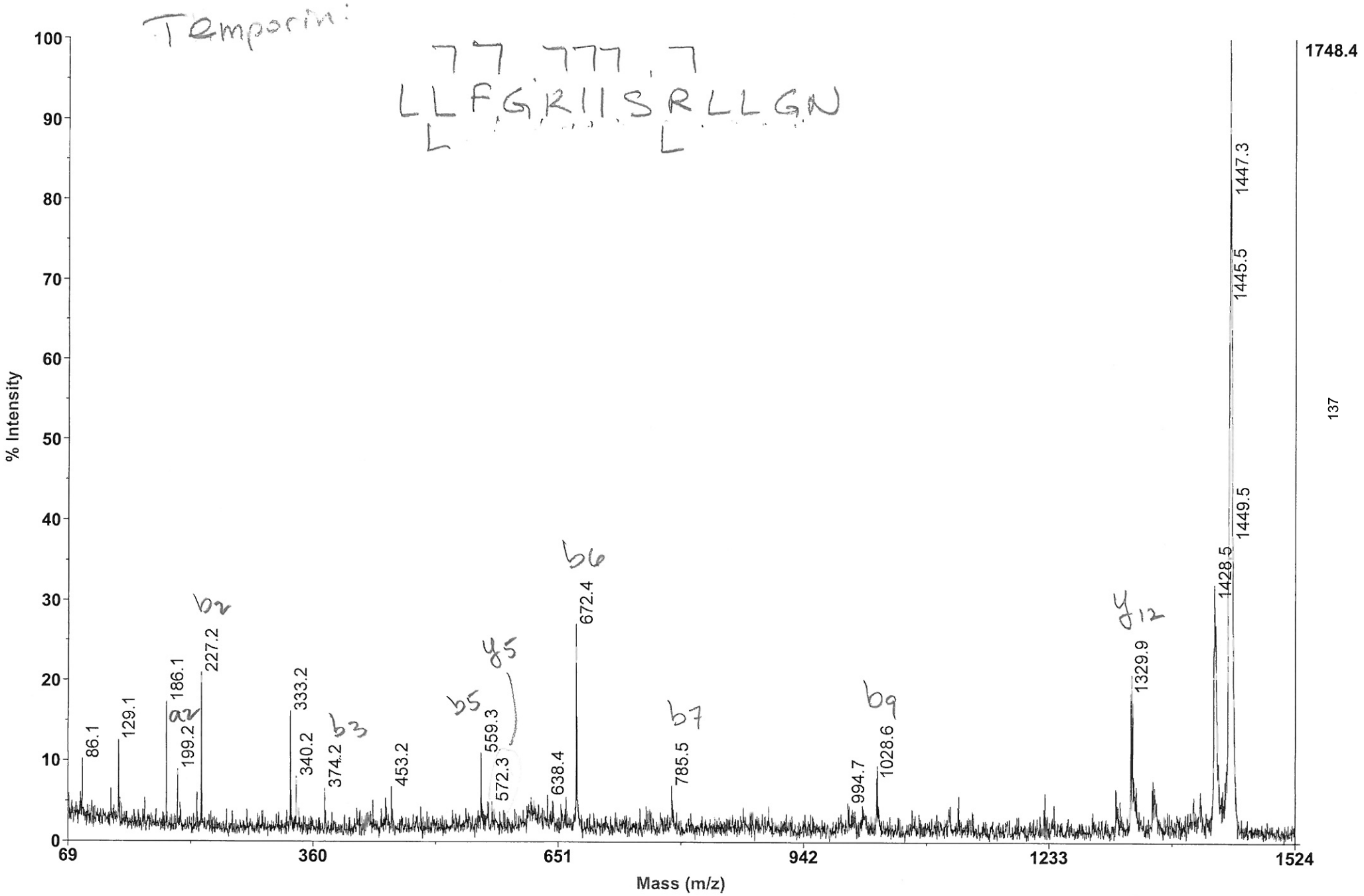


T:\proteomics\instruments\local\proteomics 4700\D drive\toftof data\Proteomics Laboratory\Rollins-Smith\P2548 sample 23 2611.T2D

Printed: 14:16, February 15, 2012

Applied Biosystems 4700 Proteomics Analyzer 347007018

4700 MS/MS Precursor 1443.7 Spec #1[BP = 1446.5, 1748]



Printed: 14:46, February 15, 2012

T:\proteomics\instruments\local\proteomics 4700\D drive\toftof data\Proteomics Laboratory\Rollins-Smith\P2548 sample 23 1443.7.T2D

APPENDIX D

LIST OF PUBLICATIONS

I. **Gammill WM**, Fites JS, Rollins-Smith LA. (2012) Norepinephrine depletion of antimicrobial peptides from the skin glands of *Xenopus laevis*. *Developmental & Comparative Immunology* 37(1):19-27.

II. Fites JS, Ramsey JP, **Holden WM**, Collier SP, Sutherland DM, Reinert LK, et al. (2013) The invasive chytrid fungus of amphibians paralyzes lymphocyte responses. *Science*. 342(6156):366-9.

III. **Holden WM**, Fites JS, Reinert LK, Rollins-Smith LA. (2014) Nikkomycin Z is an effective inhibitor of the chytrid fungus linked to global amphibian declines. *Fungal Biology*. 118(1), 48-60.

IV. **Holden WM**, Ebert AR, Canning PF, Rollins-Smith LA. An exploration of amphotericin B and chloramphenicol as alternative drugs for treatment of chytridiomycosis and their impacts on innate skin defenses. Submitted to *Applied & Environmental Microbiology.*

V. **Holden WM**, Hanlon SM, Woodhams DC, Chappell TM, Wells HL, Glisson SM, McKenzie VJ, Parris, MJ, Rollins-Smith LA. Symbiotic skin bacteria defend *Rana sphenocephala* juveniles against the fungus responsible for global amphibian declines. Submitted to *Global Change Biology*.

VI. **Holden WM**, Reinert LK, Hanlon SM, Chappell TM, Parris MJ, Rollins-Smith LA. Antimicrobial peptide defenses of southern leopard frogs, *Rana sphenocephala*, against the pathogenic chytrid fungus, *Batrachochytrium dendrobatidis*. *In Preparation*.

VII. **Holden WM***, Fites JS*, Rollins-Smith LA. Differential filtration: A method to enrich individual *Batrachochytrium dendrobatidis* life stages from heterogeneous cultures. *In Preparation*.

*These authors contributed equally to this work.

REFERENCES

1. Amiche M, Seon AA, Pierre TN, Nicolas P (1999) The dermaseptin precursors: a protein family with a common preproregion and a variable C-terminal antimicrobial domain. FEBS Letters. 456:352-356.

2. Asoodeh A, Zardini HZ, Chamani J (2012) Identification and characterization of two novel antimicrobial peptides, temporin-Ra and temporin-Rb, from skin secretions of the marsh frog (*Rana ridibunda*). J. Pept. Sci. 18:10-16.

3. Baek JH, Ji Y, Shin JS, et al. (2011) Venom peptides from solitary hunting wasps induce feeding disorder in lepidopteran larvae. Peptides. 32:568-572.

4. Barbeau TR, Lillywhite HB (2005) Body wiping behaviors associated with cutaneous lipids in hylid tree frogs of Florida. J. Exp. Biol. 208:2147-2156.

5. Becker CG, Fonseca CR, Haddad CF, et al. (2007) Habitat split and the global decline of amphibians. Science. 318:1775-1777.

6. Becker MH, Brucker RM, Schwantes CR, et al. (2009) The bacterially produced metabolite violacein is associated with survival of amphibians infected with a lethal fungus. Appl. Environ. Microbiol. 75:6635-6638.

7. Becker MH, Harris RN (2010) Cutaneous bacteria of the redback salamander prevent morbidity associated with a lethal disease. PLoS One. 5:e10957.

8. Becker MH, Harris RN, Minbiole KP, et al. (2012) Towards a Better Understanding of the Use of Probiotics for Preventing Chytridiomycosis in Panamanian Golden Frogs. Ecohealth. 8:501-506.

9. Bell SC, Alford RA, Garland S, et al. (2013) Screening bacterial metabolites for inhibitory effects against *Batrachochytrium dendrobatidis* using a spectrophotometric assay. Dis. Aquat. Organ. 103:77-85.

10. Benson BJ, Hadley ME (1969) *In vitro* characterization of adrenergic receptors controlling skin gland secretion in two anurans *Rana pipiens* and *Xenopus laevis*. Comp. Biochem. Physiol. 30:857-864.

11. Berger L, Speare R, Daszak P, et al. (1998) Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. Proc. Natl. Acad. Sci. USA. 95:9031-9036.

12. Berger L, Hyatt AD, Speare R, Longcore JE (2005a) Life cycle stages of the amphibian chytrid *Batrachochytrium dendrobatidis*. Dis. Aquat. Organ. 68: 51-63.

13. Berger L, Speare R, Skerratt L (2005b) Distribution of *Batrachochytrium dendrobatidis* and pathology in the skin of green tree frogs (*Litoria caerulea*) with severe chytridiomycosis. Dis. Aquat. Organ. 68: 65-70.

14. Berger L, Speare R, Marantelli G, Skerratt LF (2009) A zoospore inhibition technique to evaluate the activity of antifungal compounds against *Batrachochytrium dendrobatidis* and unsuccessful treatment of experimentally infected green tree frogs (*Litoria caerulea*) by fluconazole and benzalkonium chloride. Res Vet Sci 87:106-110.

15. Bernard JJ, Gallo RL (2011) Protecting the boundary: the sentinel role of host defense peptides in the skin. Cell. Mol. Life Sci. 68:2189-2199.

16. Bishop PJ, Speare R, Poulter R, et al. (2009) Elimination of the amphibian chytrid fungus *Batrachochytrium dendrobatidis* by Archey's Frog *Leiopelma archeyi*. Dis. Aquat. Org. 84:9-15.

17. Bland JM, Altman DG (1995) Multiple significance tests: the Bonferroni method. BMJ 310:170.

18. Bland JM, Altman DG (1996) The use of transformation when comparing two means. BMJ 312:1153.

19. Blaustein AR, Wake DB (1995) The puzzle of declining amphibian populations. Scientific American, 272:52-57.

20. Bollinger TK, Mao J, Schock D, et al. (1999) Pathology, isolation, and preliminary molecular characterization of a novel iridovirus from tiger salamanders in Saskatchewan. J. Wildl. Dis. 35:413-429.

21. Bosch J, Martínez-Solano I, García-París M (2001) Evidence of a chytrid fungus infection involved in the decline of the common midwife toad (*Alytes obstetricans*) in protected areas of central Spain. Biol. Conserv. 97:331-337.

22. Boyle DG, Boyle DB, Olsen V, et al. (2004) Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time Taqman PCR assay. Dis. Aquat. Organ. 60:141-148.

23. Briggs C, Burgin S (2004) Congo Red, an effective stain for revealing the chytrid fungus, *Batrachochytrium dendrobatidis*, in epidermal scrapings from frogs. Mycologist. 18:98-103.

24. Brucker RM, Harris RN, Schwantes CR, et al. (2008) Amphibian chemical defense: antifungal metabolites of the microsymbiont *Janthinobacterium lividum* on the salamander *Plethodon cinereus*. J. Chem. Ecol. 34:1422-1429.

25. Brucker RM, Baylor CM, Walters RL, et al. (2008b) The identification of 2,4-diacetylphloroglucinol as an antifungal metabolite produced by cutaneous bacteria of the salamander *Plethodon cinereus*. J. Chem. Ecol. 34:39-43.

26. Brutyn M, D'Herde K, Dhaenens M, et al. (2012) *Batrachochytrium dendrobatidis* zoospore secretions rapidly disturb intercellular junctions in frog skin. Fungal Genet. Biol. 49:830-837.

27. Burrowes PA, Joglar RL Green DE (2004) Potential causes for amphibian declines in Puerto Rico. Herpetologica. 60:141-154.

28. Caporaso JG, Kuczynski J, Stombaugh J, et al. (2010) QIIME allows analysis of high-throughput community sequencing data. Nature Methods 7:335-336.

29. Carey C, Cohen N, Rollins-Smith L (1999) Amphibian declines: an immunological perspective. Dev. Comp. Immunol. 23:459-472.

30. Carey C, Alexander MA (2003) Climate change and amphibian declines: is there a link? Divers. Distrib. 9:111-121.

31. Catenazzi A, Vredenburg VT, Lehr E (2010) *Batrachochytrium dendrobatidis* in the live frog trade of *Telmatobius* (Anura: *Ceratophryidae*) in the tropical Andes. Dis. Aquat. Organ. 92:187-191.

32. Cebra JJ (1999) Influences of microbiota on intestinal immune system development. Am. J. Clin. Nutr. 69:1046S-51S.

33. Chai LY, Netea MG, Sugui J, et al. (2010) *Aspergillus fumigatus* conidial melanin modulates host cytokine response. Immunobiology. 215:915-920.

34. Chaucheyras-Durand F, Durand H (2010) Probiotics in animal nutrition and health. Benef. Microbes 1:3-9.

35. Chryssanthou E (1997) *In vitro* susceptibility of respiratory isolates of *Aspergillus* species to itraconazole and amphotericin B. Acquired resistance to itraconazole. Scand. J. Infect. Dis. 29:509-512.

36. Clark DP, Durell S, Maloy WL, Zasloff M (1994) Ranalexin. A novel antimicrobial peptide from bullfrog (*Rana catesbeiana*) skin, structurally related to the bacterial antibiotic, polymyxin. J. Biol. Chem. 269:10849-10855.

37. Collins JP, Storfer A (2003) Global amphibian declines: sorting the hypotheses. Divers. Distrib. 9:89-98.

38. Collins JP, Crump ML, Lovejoy III TE (2009) *Extinction in our times: global amphibian decline*. Oxford University Press.

39. Collins JP (2010) Amphibian decline and extinction: What we know and what we need to learn. Dis. Aquat. Organ. 92:93-99.

40. Conlon TJ, Halverson T, Dulka J, et al. (1999) Peptides with antimicrobial activity of the brevinin-1 family isolated from skin secretions of the southern leopard frog, *Rana sphenocephala*. J. Pept. Res. 54:522-527.

41. Conlon JM, Kolodziejek J, Nowotny N (2004) Antimicrobial peptides from ranid frogs: taxonomic and phylogenetic markers and a potential source of new therapeutic agents. Biochim. Biophys. Acta. 1696:1-14.

42. Conlon JM, Woodhams DC, Raza H, et al. (2007) Peptides with differential cytolytic activity from skin secretions of the lemur leaf frog *Hylomantis lemur* (*Hylidae*: *Phyllomedusinae*). Toxicon. 50:498-506.

43. Conlon JM, Reinert LK, Mechkarska M, et al. (2013) Evaluation of the Skin Peptide Defenses of the Oregon Spotted Frog *Rana pretiosa* Against Infection by the Chytrid Fungus *Batrachochytrium dendrobatidis*. J. Chem. Ecol. 39:797-805.

44. Da Costa C, Reynolds JC, Whitmarsh S, et al. (2013) The quantitative surface analysis of an antioxidant additive in a lubricant oil matrix by desorption electrospray ionization mass spectrometry. Rapid Comm. Mass Spec. 27:2420-2424.

45. Daszak P, Cunningham AA, Hyatt AD (2003) Infectious disease and amphibian population declines. Divers. Distrib. 9:141-150.

46. Davidson C, Benard MF, Shaffer HB, et al. (2007) Effects of chytrid and carbaryl exposure on survival, growth and skin peptide defenses in foothill yellow-legged frogs. Environ. Sci. Tech. 41:1771-1776.

47. Denning DW, Venkateswarlu K, Oakley KL, et al. (1997) Itraconazole resistance in *Aspergillus fumigatus*. Antimicrob. Agents Chemother. 41:1364–1368.

48. Dickson BT (1922) Diseases of the Potato. Fourteenth Annual Report Quebec Society Protection Plants, 67-105.

49. Dobber R, Hertogh-Huijbregts A, RozingJ, et al. (1992) The involvement of the intestinal microflora in the expansion of CD4+ T cells with a naive phenotype in the periphery. Dev. Immunol. 2:141-150.

50. Docherty DE, Meteyer CU, Wang J, et al. (2003) Diagnostic and molecular evaluation of three iridovirus-associated salamander mortality events. J. Wildl. Dis. 39:556-566.

51. Dockray GJ, Hopkins CR (1975) Caerulein secretion by dermal glands in *Xenopus laevis.* J. Cell Biol. 64:724-733.

52. Donnelly MA, Crump ML (1998) Potential effects of climate change on two neotropical amphibian assemblages. Climate Change 39:541-561.

53. Dunn AJ (1988) Systemic interleukin-1 administration stimulates hypothalamic norepinephrine metabolism parallelling the increased plasma corticosterone. Life Sci. 43:429-435.

54. El-Mofty M, Abdelmeguid N, Sadek I, et al. (2000). Induction of leukemia in chloramphenicol-treated toads. East Mediterr. Health J. 6:1026-1034.

55. Feldhaar H (2011) Bacterial symbionts as mediators of ecologically important traits of insect hosts. Ecol. Entomol. 36:533-543.

56. Fellers GM, Green DE, Longcore JE (2001) Oral chytridiomycosis in the mountain yellow-legged frog (*Rana muscosa*). Copeia 2001:945-953.

57. Fisher MC, Henk DA, Briggs CJ et al. (2012) Emerging fungal threats to animal, plant and ecosystem health. Nature. 484:186-194.

58. Fites JS, Ramsey JP, Holden WM, et al. (2013) The invasive chytrid fungus of amphibians paralyzes lymphocyte responses. Science. 342:366-369.

59. Fox H (1981) Cytological and morphological changes during amphibian metamorphosis. Eds: Gilbert LI, Frieden E. New York: Plenum Press.

60. Fraune S, Bosch TC (2010) Why bacteria matter in animal development and evolution. Bioessays. 32:571-580.

61. Gagliardo R, Crump P, Griffith E, et al. (2008) The principles of rapid response for amphibian conservation, using the programmes in Panama as an example. Int. Zoo Yearb. 42:125-135.

62. Gale EF, Folkes JP (1953) The assimilation of amino-acids by bacteria. 15. Actions of antibiotics on nucleic acid and protein synthesis in *Staphylococcus aureus.* Biochem. J. 53:493-498.

63. Gallo RL, Nakatsuji T (2011) Microbial symbiosis with the innate immune defense system of the skin. J. Invest. Dermatol. 131:1974-1980.

64. Gammill WM, Fites JS, Rollins-Smith LA (2012) Norepinephrine depletion of antimicrobial peptides from the skin glands of *Xenopus laevis*. Dev. Comp. Immunol. 37:19–27.

65. Ganesan LT, Manavathu EK, Cutright JL, et al. (2004) *In-vitro* activity of nikkomycin Z alone and in combination with polyenes, triazoles or echinocandins against *Aspergillus fumigatus*. Clin. Microbiol. Infect. 10:961-966.

66. Garbeva P, Van Veen JA, Van Elsas JD (2004) Microbial diversity in soil: selection of microbial populations by plant and soil type and implications for disease suppressiveness. Annu. Rev. Phytopathol. 42:243-270.

67. Garner TW, Perkins MW, Govindarajulu P, et al. (2006) The emerging amphibian pathogen *Batrachochytrium dendrobatidis* globally infects introduced populations of the North American bullfrog, *Rana catesbeiana*. Biol. Lett. 2:455-459.

68. Garner TWJ, Garcia G, Carroll B, Fisher MC (2009) Using itraconazole to clear *Batrachochytrium dendrobatidis* infection, and subsequent depigmentation of *Alytes muletensis* tadpoles. Dis. Aquat. Organ. 83:257–260.

69. Gaughran JP, Lai MH, Kirsch DR, Silverman SJ (1994) Nikkomycin Z is a specific inhibitor of *Saccharomyces cerevisiae* chitin synthase isozyme Chs3 *in vitro* and *in vivo*. J. Bacteriol. 176:5857-5860.

70. Goodman AL, McNulty NP, et al. (2009) Identifying genetic determinants needed to establish a human gut symbiont in its habitat. Cell Host Microbe 6:279–89.

71. Goodrich-Blair H, Hussa E (2013) It Takes a Village: Ecological and Fitness Impacts of Multipartite Mutualism. Ann. Rev. Microbiol. 67:161-178.

72. Goodridge HS, Wolf AJ, Underhill DM (2009) Beta-glucan recognition by the innate immune system. Immunol. Rev. 230:38-50.

73. Gosner KL (1960) A simplified table for staging anuran embryos and larvae with notes on identification. Herpetologica. 16:183–190.

74. Gottlieb D, Carter HE, Legator M, Gallicchio V (1954) The Biosynthesis of Chloramphenicol I.: Precursors Stimulating the Synthesis. J. Bacteriol. 68:243.

75. Goraya J, Knoop FC, Conlon JM (1998) Ranatuerins: Antimicrobial Peptides Isolated from the Skin of the American Bullfrog, *Rana catesbeiana*. Biochem. Biophys. Res. Comm. 250:589-592.

76. Goraya J, Wang Y, Li Z, et al. (2000) Peptides with antimicrobial activity from four different families isolated from the skins of the North American frogs *Rana luteiventris*, *Rana berlandieri* and *Rana pipiens*. Eur. J. Biochem. 267:894-900.

77. Gray KC, Palacios DS, Dailey I, et al. (2012) Amphotericin primarily kills yeast by simply binding ergosterol. Proc. Natl. Acad. Sci. USA. 109:2234-2239.

78. Greenspan SE, Longcore JE, Calhoun AJ (2012) Host invasion by *Batrachochytrium dendrobatidis*: fungal and epidermal ultrastructure in model anurans. Dis. Aquat. Organ. 100:201-210.

79. Gunduz EA, Douglas AE (2009) Symbiotic bacteria enable insect to use a nutritionally inadequate diet. Proc. R. Soc. B. Biol. Sci. 276:987-991.

80. Hajishengallis G, Lambris JD (2012) Complement and dysbiosis in periodontal disease. Immunobiology. 217:1111-1116.

81. Hanlon SM, Parris MJ (2012) The impact of pesticides on the pathogen *Batrachochytrium dendrobatidis* independent of potential hosts. Arch. Environ. Contam. Toxicol. 63:137-143.

82. Hanlon SM, Kerby JL, Parris MJ (2012) Unlikely Remedy: Fungicide Clears Infection from Pathogenic Fungus in Larval Southern Leopard Frogs (*Rana sphenocephala*). PloS One. 7:e43573.

83. Harris RN, Brucker RM, Walke JB, et al. (2009a) Skin microbes on frogs prevent morbidity and mortality caused by a lethal skin fungus. ISME J. 3:818-824.

84. Harris RN, Lauer A, Simon MA, et al. (2009b) Addition of antifungal skin bacteria to salamanders ameliorates the effects of chytridiomycosis. Dis. Aquat. Organ. 83:11-16.

85. Haslam IS, Roubos EW, Mangoni ML, et al. (2013) From frog integument to human skin: dermatological perspectives from frog skin biology. Biol. Rev. doi: 10.1111/brv.12072.

86. Hayes TB, Collins A, Lee M, et al. (2002) Hermaphroditic, demasculinized frogs after exposure to the herbicide atrazine at low ecologically relevant doses. Proc. Natl. Acad. Sci. USA. 99:5476-5480.

87. Hayes T, Haston K, Tsui M, et al. (2003) Atrazine-induced hermaphroditism at 0.1 ppb in American leopard frogs (*Rana pipiens*): laboratory and field evidence. Environ. Health Perspect. 111:568-575.

88. Hector RF (1993) Compounds active against cell walls of medically important fungi. Clin. Microbiol. Rev. 6:1-21.

89. Hector RF, Schaller K (1992) Positive interaction of nikkomycins and azoles against *Candida albicans in vitro* and *in vivo*. Antimicrob. Agents Chemother. 36: 1284-1289.

90. Hector RF, Zimmer BL, Pappagianis D (1990) Evaluation of nikkomycins X and Z in murine models of coccidioidomycosis, histoplasmosis, and blastomycosis. Antimicrob. Agents Chemother. 34:587-593.

91. Henao-Mejia J, Elinav E, Jin C, et al. (2012) Inflammasome-mediated dysbiosis regulates progression of NAFLD and obesity. Nature. 482:179-185.

92. Hill MJ (1997) Intestinal flora and endogenous vitamin synthesis. Eur. J. Cancer Prev. 6:S43-S45.

93. Hill WA, Newman SJ, Craig L, et al. (2010) Diagnosis of *Aeromonas hydrophila*, *Mycobacterium* species, and *Batrachochytrium dendrobatidis* in an African Clawed Frog (*Xenopus laevis*). JAALAS. 49:215-220.

94. Hird DW, Diesch SL, McKinnell RG, et al. (1981) *Aeromonas hydrophila* in wild-caught frogs and tadpoles (*Rana pipiens*) in Minnesota. Lab. Animal Sci. 31:166-169.

95. Holden WM, Fites JS, Reinert LK, Rollins-Smith LA (2014) Nikkomycin Z is an effective inhibitor of the chytrid fungus linked to global amphibian declines. Fungal Biology. 118:48-60.

96. Hooper LV, Littman DR, Macpherson AJ (2012) Interactions between the microbiota and the immune system. Science. 336:1268-1273.

97. Hubbard GB (1981) *Aeromonas hydrophila* infection in *Xenopus laevis*. Lab Animal Sci. 31:297-300.

98. Ifa DR, Manicke NE, Rusine AL, Cooks RG (2008) Quantitative analysis of small molecules by desorption electrospray ionization mass spectrometry from polytetrafluoroethylene surfaces. Rapid Comm. Mass Spec. 22:503-510.

99. IUCN Red List for Amphibians: Analysis of Data. (2008) Accessed: 13 February 2014. http://www.iucnredlist.org/initiatives/amphibians/analysis

100. James TY, Kauff F, Schoch CL, et al. (2006) Reconstructing the early evolution of Fungi using a six-gene phylogeny. Nature. 443:818-822.

101. Johnson PT, McKenzie VJ, Peterson AC, et al. (2011) Regional Decline of an Iconic Amphibian Associated with Elevation, Land-Use Change, and Invasive Species. Conserv. Biol. 25:556-566.

102. Kanda N, Kano R, Ishikawa T, Watanabe S (2011) The antimycotic drugs itraconazole and terbinafine hydrochloride induce the production of human β-defensin-3 in human keratinocytes. Immunobiology. 216:497-504.

103. Kang S, Denman SE, Morrison M, et al. (2010) Dysbiosis of fecal microbiota in Crohn's disease patients as revealed by a custom phylogenetic microarray. Inflamm. Bowel Dis. 16:2034-2042.

104. Knapp RA, Corn PS, Schindler DE (2001) The introduction of nonnative fish into wilderness lakes: good intentions, conflicting mandates, and unintended consequences. Ecosystems. 4:275-278.

105. Kriger KM, Hines HB, Hyatt AD, et al. (2006) Techniques for detecting chytridiomycosis in wild frogs: comparing histology with real-time taqman PCR. Dis. Aquat. Organ. 71:141-148.

106. Koszałka P, Kamysz E, Wejda M, et al. (2011). Antitumor activity of antimicrobial peptides against U937 histiocytic cell line. Acta Biochimica Polonica, 58:111-117.

107. Kueneman JG, Parfrey LW, Woodhams DC, et al. (2013) The amphibian skin-associated microbiome across species, space and life history stages. Mol. Ecol. doi: 10.1111/mec.12510.

108. Küng D, Bigler L, Davis LR, et al. (2014) Stability of Microbiota Facilitated by Host Immune Regulation: Informing Probiotic Strategies to Manage Amphibian Disease. PLoS One. 9:e87101.

109. Kupferberg SJ (1997) The role of larval diet in anuran metamorphosis. Am. Zool. 37:146-159.

110. La Marca E, Reinthaler HP (1991). Population changes in *Atelopus* species of the Cordillera de Mérida, Venezuela. Herpetol. Rev. 22:125-128.

111. La Marca E, Lips KR, Lötters S, et al. (2005) Catastrophic Population Declines and Extinctions in Neotropical Harlequin Frogs (Bufonidae: *Atelopus*) 1. Biotropica. 37:190-201.

112. Le Guennec R, Reynes J, Mallié M, et al. (1995) Fluconazole- and itraconazole-resistant *Candida albicans* strains from AIDS patients: multilocus enzyme electrophoresis analysis and antifungal susceptibilities. J. Clin. Microbiol. 33:2732-2737.

113. Lam BA, Walke JB, Vredenburg VT, Harris RN (2010) Proportion of individuals with anti-*Batrachochytrium dendrobatidis* skin bacteria is associated with population persistence in the frog *Rana muscosa*. Biol. Conserv. 143:529-531.

114. Lam BA, Walton DB, Harris RN (2011) Motile zoospores of *Batrachochytrium dendrobatidis* move away from antifungal metabolites produced by amphibian skin bacteria. EcoHealth. 8:36-45.

115. Laniado-Laborín R, Cabrales-Vargas MN (2009) Amphotericin B: side effects and toxicity. Rev. Ibero. Micol. 26:223-227.

116. Laurance WF, McDonald KR, Speare R (1996) Epidemic disease and the catastrophic decline of Australian rain forest frogs. Conserv. Biol. 10:406-413.

117. Lefcort H, Meguire RA, Wilson LH, Ettinger WF (1998) Heavy metals alter the survival, growth, metamorphosis, and antipredatory behavior of Columbia spotted frog (*Rana luteiventris*) tadpoles. Arch. Environ. Contam. Toxicol. 35:447-456.

118. Lehtinen RM, Galatowitsch SM, Tester JR (1999) Consequences of habitat loss and fragmentation for wetland amphibian assemblages. Wetlands. 19:1-12.

119. Leibowitz SF, Diaz S, Tempel D (1989) Norepinephrine in the paraventricular nucleus stimulates corticosterone release. Brain Res. 496:219-227.

120. Leivas PT, Savaris M, Lampert S, Lucas EM (2013) Predation of *Odontophrynus americanus* (Anura: *Odontophrynidae*) by the invasive species *Lithobates catesbeianus* (Anura: *Ranidae*) in an Araucaria Forest remnant in Southern Brazil. Herp. Notes 6:603-606.

121. Lepri JJ, Randall JA (1983) Hormonal regulation of sandbathing in male kangaroo rats (*Dipodomys merriami*). Behav. Neural Biol. 37:256-64.

122. Ley RE, Turnbaugh PJ, Klein S, et al. (2006) Microbial ecology: human gut microbes associated with obesity. Nature. 444:1022-1033.

123. Leyden J (1998) Pharmacokinetics and pharmacology of terbinafine and itraconazole. J. Am. Acad. Dermatol. 5:S42-47.

124. Li RK, Rinaldi MG (1999) *In vitro* antifungal activity of nikkomycin Z in combination with fluconazole or itraconazole. Antimicrob. Agents Chemother. 43:1401-1405.

125. Lips KR, Diffendorfer J, Mendelson III, JR, Sears MW (2008). Riding the wave: reconciling the roles of disease and climate change in amphibian declines. PLoS Biol. 6:e72.

126. Liu X, Rohr JR, Li Y (2013) Climate, vegetation, introduced hosts and trade shape a global wildlife pandemic. Proc. R. Soc. B. Biol. Sci. 280(1753).

127. Longcore JE, Pessier AP, Nichols DK (1999) *Batrachochytrium dendrobatidis* gen. et sp. nov., a chytrid pathogenic to amphibians. Mycologia. 91:219-227.

128. Luckey TD (1972) Introduction to intestinal microecology. Am. J. Clin. Nutr. 25:1292-1294.

129. Machin D, Cheung YB, Parmar M (2006) *Survival analysis: a practical approach*. Wiley.com. Accessed: 13 February 2014.

130. Mangoni ML, Miele R, Renda TG, et al. (2001) The synthesis of antimicrobial peptides in the skin of Rana esculenta is stimulated by microorganisms. FASEB J. 15:1431-1432.

131. Manikandan S (2010) Data transformation. J Pharmacol Pharmacother 1:126-127.

132. Marcum RD, St. Hilaire S, Murphy PJ, Rodnick KJ (2010) Effects of *Batrachochytrium dendrobatidis* infection on ion concentrations in the boreal toad *Anaxyrus* (*Bufo*) *boreas*. Dis. Aquat. Organ. 91:17-21.

133. Martel A, Rooij PV, Vercauteren G, et al. (2011). Developing a safe antifungal treatment protocol to eliminate *Batrachochytrium dendrobatidis* from amphibians. Med. Mycol. 49:143-149.

134. Martel A, Spitzen-van der Sluijs A, Blooi M, et al. (2013) *Batrachochytrium salamandrivorans* sp. nov. causes lethal chytridiomycosis in amphibians. Proc. Natl. Acad. Sci. USA. 110:15325-15329.

135. Mazmanian SK, Liu CH, Tzianabos AO, Kasper DL (2005) An immunomodulatory molecule of symbiotic bacteria directs maturation of the host immune system. Cell. 122;107-118.

136. Mazzoni R, Cunningham AA, Daszak P, et al. (2003) Emerging Pathogen in Wild Amphibians and Frogs (*Rana catesbeiana*) Farmed for International Trade. Emerg. Infect. Dis. 9:995-998.

137. MbangKollo D, deRoos R (1983) Comparative effects of epinephrine, norepinephrine, and a gentle handling stress on plasma lactate, glucose, and hematocrit levels in the American bullfrog (*Rana catesbeiana*). Gen. Comp. Endocrinol. 49:167-175.

138. McCallum ML (2007) Amphibian decline or extinction? Current declines dwarf background extinction rate. J. Herpetol. 41:483-491.

139. McCarthy PJ, Troke PF, Gull K (1985) Mechanism of action of nikkomycin and the peptide transport system of *Candida albicans*. J. Gen. Microbiol. 131:775-80.

140. McClanahan Jr L, Baldwin R (1969) Rate of water uptake through the integument of the desert toad, *Bufo punctatus*. Comp. Biochem. Physiol. 28:381-9.

141. McKenzie VJ, Bowers RM, Fierer N, et al. (2012) Co-habiting amphibian species harbor unique skin bacterial communities in wild populations. ISME J. 6:588-596.

142. McMahon TA, Romansic JM, Rohr JR (2013) Non-monotonic and monotonic effects of pesticides on the pathogenic fungus *Batrachochytrium dendrobatidis* in culture and on tadpoles. Environ. Sci. Technol. 47:7958-7964.

143. McMenamin SK, Hadly EA, Wright CK (2008) Climatic change and wetland desiccation cause amphibian decline in Yellowstone National Park. Proc. Natl. Acad. Sci. USA. 105:16988-16993.

144. McReynolds JR, Donowho K, Abdi A, et al. (2010) Memory-enhancing corticosterone treatment increases amygdala norepinephrine and Arc protein expression in hippocampal synaptic fractions. Neurobiol. Learn. Mem. 93:312-21.

145. Mechkarska M, Ahmed E, Coquet L, et al. (2010) Antimicrobial peptides with therapeutic potential from skin secretions of the Marsabit clawed frog *Xenopus borealis* (*Pipidae*). Comp Biochem Physiol C: Toxicol & Pharmacol 152:467-72.

146. Miller DL, Rajeev S, Brookins M, et al. 2008. Concurrent infection with Ranavirus, *Batrachochytrium dendrobatidis*, and *Aeromonas* in a captive anuran colony. J. Zoo Wildlife Med. 39:445-449.

147. Möhrle V, Roos U, Bormann C (1995) Identification of cellular proteins involved in nikkomycin production in *Streptomyces tendae* Tü901. Mol. Microbiol. 15:561-571.

148. Monheit JE, Cowan DF, Moore DG (1984) Rapid detection of fungi in tissues using calcofluor white and fluorescence microscopy. Arch. Pathol. Lab. Med. 108:616-618.

149. Moran NA (2007) Symbiosis as an adaptive process and source of phenotypic complexity. Proc. Natl. Acad. Sci. USA. 104:8627-8633.

150. Morin PJ (1983) Predation, competition, and the composition of larval anuran guilds. Ecol. Monogr. 53:119-138.

151. Moy RF (1970). Histology of the subauricular and rump glands of the pronghorn (*Antilocapra americana Ord*). Am. J. Anat. 129:65-87.

152. Muegge BD, Kuczynski J, Knights D, et al. (2011) Diet drives convergence in gut microbiome functions across mammalian phylogeny and within humans. Science. 332:970-974.

153. Muijsers M, Martel A, Van Rooij P, et al. (2012) Antibacterial therapeutics for the treatment of chytrid infection in amphibians: Columbus's egg? BMC Vet. Res. 8:175.

154. Murphy PJ, St-Hilaire S, Bruer S, et al. (2009) Distribution and pathogenicity of *Batrachochytrium dendrobatidis* in boreal toads from the Grand Teton area of western Wyoming. Ecohealth. 6:109-120.

155. Nichols DK, Lamirande EW (2000) Treatment of cutaneous chytridiomycosis in blue-and-yellow poison dart frogs (*Dendrobates tinctorius*) (abstract). pp. 51. In: Proceedings of Getting the Jump on Amphibian Diseases, Cairns, Australia.

156. Nicolas P, Mor A (1995) Peptides as weapons against microorganisms in the chemical defense system of vertebrates. Annu. Rev. Microbiol. 49:277-304.

157. Nutkins JC, Williams DH (1989) Identification of highly acidic peptides from processing of the skin prepropeptides of *Xenopus laevis*. Eur. J. Biochem. 181:97-102.

158. Olson ME, Gard S, Brown M, et al. (1992) *Flavobacterium indologenes* infection in leopard frogs. J. Am. Vet. Med. Assoc. 201:1766.

159. Ortiz-Santaliestra ME, Rittenhouse TA, Cary TL, Karasov WH (2013) Interspecific and postmetamorphic variation in susceptibility of three North American anurans to *Batrachochytrium dendrobatidis*. J. Herpetol. 47:286-292.

160. Osherov N, Kontoyiannis DP, Romans A, May GS (2001) Resistance to itraconazole in *Aspergillus nidulans* and *Aspergillus fumigatus* is conferred by extra copies of the *A. nidulans* P-450 14α-demethylase gene, pdmA. J. Antimicrob. Chemother. 48:75–81.

161. Osusky M, Osuska L, Hancock RE, et al. (2004) Transgenic potatoes expressing a novel cationic peptide are resistant to late blight and pink rot. Transgenic Res. 13:181-90.

162. Page SW (1991) Chloramphenicol 1. Hazards of use and the current regulatory environment. Aus. Vet. J. 68:1-2.

163. Panda NK (1997) Itraconazole - A Potent Antifungal Drug. IJO & HNS 49:293-4.

164. Parker JM, Mikaelian I, Hahn N, Diggs HE (2002) Clinical diagnosis and treatment of epidermal chytridiomycosis in African clawed frogs (*Xenopus tropicalis*). Comp. Med. 52:265-268.

165. Pask JD, Woodhams DC, Rollins-Smith LA (2012) The ebb and flow of antimicrobial skin peptides defends northern leopard frogs (*Rana pipiens*) against chytridiomycosis. Glob. Change Biol. 18:1231-1238.

166. Pask JD, Cary TL, Rollins-Smith LA (2013) Skin peptides protect juvenile leopard frogs (*Rana pipiens*) against chytridiomycosis. J Exp Biol 216:2908-16.

167. Pennisi E (2009) Life and death play out on the skin of frogs. Science. 326:507-8.

168. Pessier AP, Nichols DK, Longcore JE, Fuller MS (1999) Cutaneous chytridiomycosis in poison dart frogs (*Dendrobates* spp.) and White's tree frogs (*Litoria caerulea*). J. Vet. Diagn. Invest. 11:194-199.

169. Peterson JD, Wood MB, Hopkins WA, et al. (2007) Prevalence of *Batrachochytrium dendrobatidis* in American bullfrog and southern leopard frog larvae from wetlands on the Savannah River Site, South Carolina. J. Wildl. Dis. 43:450-460.

170. Pfaller MA, Messer SA, Boyken L, et al. (2008) *In vitro* survey of triazole cross-resistance among more than 700 clinical isolates of *Aspergillus* species. J. Clin. Microbiol. 46:2568-2572.

171. Picco AM, Collins JP (2008) Amphibian commerce as a likely source of pathogen pollution. Conserv. Biol. 22:1582-1589.

172. Pinjon E, Moran GP, Jackson CJ, et al. (2003) Molecular mechanisms of itraconazole resistance in *Candida dubliniensis*. Antimicrob. Agents Chemother. 47:2424-2437.

173. Pounds JA, Crump ML (1994) Amphibian declines and climate disturbance: the case of the golden toad and the harlequin frog. Conserv. Biol. 8:72-85.

174. Pounds JA, Bustamante MR, Coloma LA, et al, (2006) Widespread amphibian extinctions from epidemic disease driven by global warming. Nature 439:161-7.

175. Putnam ML, Miller ML (2007) *Rhodococcus fascians* in herbaceous perennials. Plant Disease 91:1064-1076.

176. Rachowicz LJ, Vredenburg VT (2004) Transmission of *Batrachochytrium dendrobatidis* within and between amphibian life stages. Dis. Aquat. Organ. 61:75-83.

177. Rachowicz LJ, Hero J, Alford RA, et al. (2005) The novel and endemic pathogen hypotheses: competing explanations for the origin of emerging infectious diseases of wildlife. Conserv. Biol. 19:1441-1448.

178. Ramakrishnan L, Valdivia RH, McKerrow JH, Falkow S (1997) *Mycobacterium marinum* causes both a long-term subclinical infection and acute disease in the leopard frog (*Rana pipiens*). Infect. Immun. 65:767-773.

179. Ramsey JP, Reinert LK, Harper LK, et al. (2010) Immune defenses against *Batrachochytrium dendrobatidis*, a fungus linked to global amphibian declines, in the South African clawed frog, *Xenopus laevis*. Infect. Immun. 78:3981-3992.

180. Ramsey JP, Mercurio A, Holland JA, et al. (2013) The cutaneous bacterium *Janthinobacterium lividum* inhibits the growth of *Trichophyton rubrum in vitro*. Int. J. Dermatol. 1-4.

181. Resnick NM, Maloy WL, Guy HR, Zasloff M (1991 A novel endopeptidase from *Xenopus* that recognizes a-helical secondary structure. Cell. 66:541-554.

182. Retallick RW, McCallum H, Speare R, (2004) Endemic infection of the amphibian chytrid fungus in a frog community post-decline. PLoS Biol. 2:e351.

183. Richards-Hrdlicka KL, Richardson JL, Mohabir L (2013) First survey for the amphibian chytrid fungus *Batrachochytrium dendrobatidis* in Connecticut (USA) finds widespread prevalence. Dis. Aquat. Organ. 102:169-180.

184. Rinaldi AC (2002) Antimicrobial peptides from amphibian skin: an expanding scenario. Curr. Opin. Chem. Biol. 6:799-804.

185. Rollins LA, McKinnell RG (1980) The influence of glucocorticoids on survival and growth of allografted tumors in the anterior eye chamber of leopard frogs. Dev. Comp. Immunol. 4:283-294.

186. Rollins-Smith LA (1998) Metamorphosis and the amphibian immune system. Immunological Reviews. 166:221-230.

187. Rollins-Smith LA (2001) Neuroendocrine-immune system interactions in amphibians: Implications for understanding global amphibian declines. Immunologic. Res. 23:273-280.

188. Rollins-Smith LA, Carey C, Longcore J, et al. (2002a). Activity of antimicrobial skin peptides from ranid frogs against *Batrachochytrium dendrobatidis*, the chytrid fungus associated with global amphibian declines. Dev. Comp. Immunol. 26:471-479.

189. Rollins-Smith LA, Doersam JK, Longcore JE, et al. (2002b) Antimicrobial peptide defenses against pathogens associated with global amphibian declines. Dev. Comp. Immunol. 26:63–72.

190. Rollins-Smith LA, Reinert LK, Miera V, Conlon JM (2002c) Antimicrobial peptide defenses of the Tarahumara frog, *Rana tarahumarae*. Biochem. Biophys. Res. Commun. 297:361-367.

191. Rollins-Smith LA, Carey C, Conlon JM, et al. (2003) Activities of temporin family peptides against the chytrid fungus (*Batrachochytrium dendrobatidis*) associated with global amphibian declines. Antimicrob Agents Chemother. 47:1157-1160.

192. Rollins-Smith LA, Conlon JM (2005) Antimicrobial peptide defenses against chytridiomycosis, an emerging infectious disease of amphibian populations. Dev. Comp. Immunol. 29:589-598.

193. Rollins-Smith LA, Reinert LK, O'Leary CJ, et al. (2005) Antimicrobial peptide defenses in amphibian skin. Integr. Comp. Biol. 45:137-142.

194. Rollins-Smith LA, Woodhams DC, Reinert LK, et al. (2006) Antimicrobial peptide defenses of the mountain yellow-legged frog (*Rana muscosa*). Dev. Comp. Immunol. 30:831–842.

195. Rollins-Smith LA (2009) The role of amphibian antimicrobial peptides in protection of amphibians from pathogens linked to global amphibian declines. Biochim. Biophys. Acta. 1788:1593-1599.

196. Rollins-Smith LA, Ramsey JP, Reinert LK, et al. (2009) Immune defenses of *Xenopus laevis* against *Batrachochytrium dendrobatidis*. Front Biosci (Schol Ed), 1:68-91.

197. Rollins-Smith LA, Ramsey JP, Pask JD, et al. (2011). Amphibian immune defenses against chytridiomycosis: impacts of changing environments. Integr. Comp. Biol. 51:552-562.

198. Rollins-Smith LA, Woodhams DC (2012) Amphibian immunity: staying in tune with the environment. In Eco-Immunology (ed. G.E. Demas and R.J Nelson), pp. 92-143. Oxford, United Kingdom: Oxford University Press.

199. Ron SR, Duellman WE, Coloma LA, Bustamante MR (2003) Population decline of the Jambato toad *Atelopus ignescens* (Anura: *Bufonidae*) in the Andes of Ecuador. J. Herpetol. 37:116-126.

200. Rosenberg E, Zilber-Rosenberg I (2011) Symbiosis and development: the hologenome concept. Birth Defects Res. Part C: Embryo Today. 93:56-66.

201. Rosenblum EB, Stajich JE, Maddox N, Eisen MB (2008) Global gene expression profiles for life stages of the deadly amphibian pathogen *Batrachochytrium dendrobatidis*. Proc. Natl. Acad. Sci. USA. 105:17034-17039.

202. Rosenblum EB, Poorten TJ, Settles M, et al. (2009) Genome-wide transcriptional response of *Silurana* (*Xenopus*) *tropicalis* to infection with the deadly chytrid fungus. PLoS One 4:e6494.

203. Rosenthal RL, Blackman A (1965) Bone-marrow hypoplasia following use of chloramphenicol eye drops. JAMA. 191:136-137.

204. Rovito SM, Parra-Olea G, Vásquez-Almazán CR, et al. (2009) Dramatic declines in neotropical salamander populations are an important part of the global amphibian crisis. Proc. Natl. Acad. Sci. USA. 106:3231-3236.

205. Roy D (2002) Amphibians as environmental sentinels. J. Biosci. 27:187-188.

206. Royet J, Gupta D, Dziarski R (2011) Peptidoglycan recognition proteins: modulators of the microbiome and inflammation. Nature Rev. Immunol. 11:837-851.

207. Ruiz-Herrera J, Ortiz-Castellanos L (2010) Analysis of the phylogenetic relationships and evolution of the cell walls from yeasts and fungi. FEMS Yeast Res. 10:225-243.

208. Schadich E, Mason D, Cole AL (2013) Neutralization of bacterial endotoxins by frog antimicrobial peptides. Microbiol. Immunol. 57:159-161.

209. Schloegel L, Hero J-M, Berger L, et al. (2006) The decline of the sharp-snouted day frog (*Taudactylus acutirostris*): The first documented case of extinction by infection in a free-ranging wildlife species? Ecohealth. 3:35-40.

210. Schloegel LM, Daszak P, Cunningham AA, et al. (2010) Two amphibian diseases, chytridiomycosis and ranaviral disease, are now globally notifiable to the World Organization for Animal Health (OIE): an assessment. Dis. Aquat. Organ. 92:101-108.

211. Schloegel LM, Toledo LF, Longcore JE, et al. (2012) Novel, panzootic and hybrid genotypes of amphibian chytridiomycosis associated with the bullfrog trade. Mol. Ecol. 21:5162-5177.

212. Sjoberg E, Flock A (1976) Innervation of skin glands in the frog. Cell Tiss. Res. 172:81-91.

213. Song Y, Ji S, Liu W, et al. (2013) Different expression profiles of bioactive peptides in *Pelophylax nigromaculatus* from distinct regions. Biosci. Biotechnol. Biochem. 77:1075-1079.

214. Sparling DW, Fellers GM, McConnell LL (2001) Pesticides and amphibian population declines in California, USA. Environ. Toxicol. Chem. 20:1591-1595.

215. Stevens CE, Hume ID (1998) Contributions of microbes in vertebrate gastrointestinal tract to production and conservation of nutrients. Physiol. Rev. 78: 393-427.

216. Stuart SN, Chanson JS, Cox NA, et al. (2004) Status and trends of amphibian declines and extinctions worldwide. Science. 306:1783-1786.

217. Sun Z, Fan Y, Zha Q, Zhu MY (2010) Corticosterone up-regulates expression and function of norepinephrine transporter in SK-N-BE(2)C cells. J. Neurochem. 113:105-116.

218. Tamboli CP, Neut C, Desreumaux P, Colombel JF (2004) Dysbiosis in inflammatory bowel disease. Gut. 53:1-4.

219. Taylor SK, Williams ES, Mills KW (1999) Experimental exposure of Canadian toads to *Basidiobolus ranarum*. J. Wildl. Dis. 35:58-63.

220. Tennessen JA, Blouin MS (2007) Selection for antimicrobial peptide diversity in frogs leads to gene duplication and low allelic variation. J. Molec. Evol. 65:605-615.

221. Tennessen JA, Woodhams DC, Chaurand P, et al. (2009) Variations in the expressed antimicrobial peptide repertoire of northern leopard frog (*Rana pipiens*) populations suggest intraspecies differences in resistance to pathogens. Dev. Comp. Immunol. 33:1247-1257.

222. Tennessen JA, Blouin MS (2010) A revised leopard frog phylogeny allows a more detailed examination of adaptive evolution at ranatuerin-2 antimicrobial peptide loci. Immunogenetics. 62:333-343.

223. Thekkiniath JC, Zabet-Moghaddam M, San Francisco SK, San Francisco MJ. (2013) A novel subtilisin-like serine protease of *Batrachochytrium dendrobatidis* is induced by thyroid hormone and degrades antimicrobial peptides. Fungal Biol. 117:451-461.

224. Traxler MF, Watrous JD, Alexandrov T, et al. (2013) Interspecies interactions stimulate diversification of the *Streptomyces coelicolor* secreted metabolome. mBio. 4:e00459-13.

225. Trejo WH, Bennett RE (1963) *Streptomyces nodosus* sp. n., the amphotericin-producing organism. J. Bacteriology. 85:436-439.

226. Vaarala O (2013) Human intestinal microbiota and type 1 diabetes. Curr. Diabetes Reports. 13:601-607.

227. Van Rooij P, Martel A, D'Herde K, et al. (2012) Germ tube mediated invasion of *Batrachochytrium dendrobatidis* in amphibian skin is host dependent. PLoS One 7:e41481.

228. VanCompernolle SE, Taylor RJ, Oswald-Richter K, et al. (2005) Antimicrobial peptides from amphibian skin potently inhibit human immunodeficiency virus infection and transfer of virus from dendritic cells to T cells. J Virol. 79:11598-11606.

229. Verschuere L, Rombaut G, Sorgeloos P, Verstraete W (2000) Probiotic bacteria as biological control agents in aquaculture. Microbiol. Mol. Biol. Rev. 64:655-671.

230. Voyles J, Berger L, Young S, et al. (2007) Electrolyte depletion and osmotic imbalance in amphibians with chytridiomycosis. Dis. Aquat. Organ. 77:113-118.

231. Voyles J, Young S, Berger L, et al. (2009) Pathogenesis of chytridiomycosis, a cause of catastrophic amphibian declines. Science. 26:582-585.

232. Voyles J, Rosenblum EB, Berger L (2011) Interactions between *Batrachochytrium dendrobatidis* and its amphibian hosts: a review of pathogenesis and immunity. Microb. Infect. 13:25-32.

233. Voyles J, Vredenburg VT, Tunstall TS, et al. (2012) Pathophysiology in mountain yellow-legged frogs (*Rana muscosa*) during a chytridiomycosis outbreak. PLoS One 7: e35374.

234. Vredenburg VT, Knapp RA, Tunstall TS, Briggs CJ (2010) Dynamics of an emerging disease drive large-scale amphibian population extinctions. Proc. Natl. Acad. Sci. USA. 107:9689-9694.

235. Wabnitz PA, Walters H, Tyler MJ, et al. (1998) First record of host defence peptides in tadpoles. The magnificent tree frog *Litoria splendida*. J. Pept. Res. 52:477-481.

236. Wake DB, Vredenburg VT (2008) Colloquium paper: are we in the midst of the sixth mass extinction? A view from the world of amphibians. Proc. Natl. Acad. Sci. USA. 105:11466–11473.

237. Walker SF, Bosch J, et al. (2010) Factors driving pathogenicity vs. prevalence of amphibian panzootic chytridiomycosis in Iberia. Ecol. Lett. 13:372-382.

238. Wang G, Li X, Wang Z (2009) APD2: the updated antimicrobial peptide database and its application in peptide design. Nucleic Acids Res. 37:D933-D937.

239. Wang C, Li HB, Li S, et al. (2012) Antitumor effects and cell selectivity of temporin-1CEa, an antimicrobial peptide from the skin secretions of the Chinese brown frog (*Rana chensinensis*). Biochimie, 94:434-441.

240. Weinstein PD, Cebra JJ (1991) The preference for switching to IgA expression by Peyer's patch germinal center B cells is likely due to the intrinsic influence of their microenvironment. J. Immunol. 147:4126-4135.

241. Weldon C, du Preez LH (2004) Decline of the Kihansi Spray Toad, from the Udzungwa Mountains, Tanzania. Froglog. 62:2–3.

242. Weldon C, du Preez LH, Hyatt AD, et al. (2004) Origin of the amphibian chytrid fungus. Emerg. Infect. Dis. 10:2100-2105.

243. Wiesner J, Vilcinskas A (2010) Antimicrobial peptides: the ancient arm of the human immune system. Virulence. 1:440-464.

244. Woodhams DC, Voyles J, Lips KR, et al. (2006) Predicted disease susceptibility in a Panamanian amphibian assemblage based on skin peptide defenses. J. Wildl. Dis. 42:207-218.

245. Woodhams DC, Vredenburg VT, Simon MA, et al. (2007) Symbiotic bacteria contribute to innate immune defenses of the threatened mountain yellow-legged frog, *Rana muscosa*. Biol. Cons. 138:390-398.

246. Woodhams DC, Alford RA, Briggs CJ, et al. (2008) Life-history trade-offs influence disease in changing climates: strategies of an amphibian pathogen. Ecology. 89:1627-1639.

247. Woodhams DC, Kenyon N, Bell SC, et al. (2010) Adaptations of skin peptide defences and possible response to the amphibian chytrid fungus in populations of Australian green-eyed treefrogs, *Litoria genimaculata*. Divers. Distrib. 16:703-12.

248. Woodhams D, Bigler L, Marschang R (2012a) Tolerance of fungal infection in European water frogs exposed to *Batrachochytrium dendrobatidis* after experimental reduction of innate immune defenses. BMC Vet Res. 8: 197.

249. Woodhams DC, Geiger CC, Reinert LK, et al. (2012b) Treatment of amphibians infected with chytrid fungus: learning from failed trials with itraconazole, antimicrobial peptides, bacteria, and heat therapy. Dis. Aquat. Organ. 98:11-25.

250. Young S, Speare R, Berger L, Skerratt LF (2012) Chloramphenicol with fluid and electrolyte therapy cures terminally ill green tree frogs (*Litoria caerulea*) with chytridiomycosis. J. Zoo Wildlife Med. 43:330-337.

251. Zairi A, Tangy F, Bouassida K, Hani K (2009) Dermaseptins and magainins: antimicrobial peptides from frogs' skin—new sources for a promising spermicides microbicides—a mini review. BioMed Res. Internat. Article ID: 452567.

252. Zasloff M (2002) Antimicrobial peptides of multicellular organisms. Nature. 415:389-395.