CHYTRIDS VS. AMPHIBIANS: EMERGING DISEASE OR HISTORY OF NATURAL SELECTION?

A Thesis

By

Jorge Luis Esquivel

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APPROVED BY:

Robert Wayne Van Devender
Chairperson, Thesis Committee

Shea R. Tuberty
Member, Thesis Committee

John Walker
Member, Thesis Committee

Steven W. Seagle
Chairperson, Department of Biology

Edelma D. Huntley
Dean, Graduate Studies and Research

ABSTRACT

CHYTRIDS VS. AMPHIBIANS: EMERGING DISEASE OR HISTORY OF NATURAL SELECTION?

(December 2007)

Jorge Luis Esquivel, B. S., Universidad Nacional Autónoma, Costa Rica

B. S., Universidad Estatal a Distancia, Costa Rica

M.S., Appalachian State University

Thesis Chairperson: Robert Wayne Van Devender

Quantity of skin peptides and effectiveness of those peptides in suppressing the growth of *Batrachochytrium dendrobatidis* were analyzed for 134 individuals of 24 species of amphibians from the Southern Appalachian temperate zone of eastern North America and several Costa Rica sites in the tropics. Skin secretions ranged from 29.79 to 6298.33μg/g across species. In the Appalachians three species of *Plethodon* with direct development produced more peptide (average = 329.26μg/g) than did three species with indirect development; but that result was driven by the very low amount of peptide (average = 49.18μg/g) produced by one species, *Desmognathus quadramaculatus*. This result supported the hypothesis that peptide secretions are adaptive responses related to developmental type. In Costa Rica four species of salamanders with direct development produced more peptides (average = 2152.27μg/g) than fourteen species of frogs (average = 199.20μg/g). Phylogenetic patterns in peptide content were observed. Peptide contents of frogs varied interspecifically. Hylid frogs (Family Hylidae) had significantly more peptides than did leptodactylids (Family

Leptodactylidae) with indirect development. Two indirect development hylids (*Hyla microcephala* and *H. ebraccata*) and one direct development leptodactylid (*Eleutherodactylus stejnegerianus*) secreted more than 300μg/g of peptide. Most peptide content results supported the hypothesis that peptides would show phylogenetic constraints with uniform peptide concentrations within each amphibian family.

In most cases, inhibitory responses of peptide mixtures were not supportive of the developmental and phylogenetic constraint hypotheses. Strong inhibitory responses of the peptide mixtures were present in species with direct development and indirect development. Only frogs from Family Hylidae had inhibitory effects on *Batrachochytrium dendrobatidis* consistent with the phylogenetic constraint hypothesis. The two hylids assayed (*Hyla microcephala* and *H. ebraccata*) produced strong inhibitory effects with minimal inhibitory concentrations of 3.06μg/ml. For *Hyla microcephala,* inhibition increased in proportion to peptide concentration suggesting that novel peptides with unusually strong and persistent strong antimicrobial activity are present in this species. For indirect development amphibians increased peptide content was associated with increased effectiveness of peptide. Inhibitory responses at low peptide concentration were scattered among amphibians from different phylogenetic groups and different developmental strategies. This result suggests that antimicrobial peptide distribution is probably not a product of selection related to history of exposure to pathogens similar to BD. However, a negative relationship between minimal inhibitory concentrations and peptide contents for indirect developers is consistent with long selective pressure from pathogens similar to chytrids. Presence of peptides effective against *Batrachochytrium dendrobatidis* indicates the importance of further study to clarify the role of this innate immune system mechanism in nature. Peptide secretions against

Batrachochytrium dendrobatidis may be an important factor in understanding the dynamics of some population declines in amphibians. More research is also necessary to clarify other biological roles of the peptides in the skin secretions of amphibians.

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DEDICATION

God gave me a life that has been fulfilled with people who unconditionally love me and care for me and give me support. I dedicate this work to my loving wife Fern Perkins, who was there for me when I began to speak English and who supported me and guided me through my career in this country. I hope to live the rest of my life sharing with you our biology love and goals. I also dedicate this to my parents Rodrigo Esquivel and Patricia Sibaja who never doubted of my goals and ambitions and let me get to where I am today. Their effort in life rewarded them with a united family that will always make me do my best to accomplish what they did and to make them proud. Without you both I would not have anything. Also my work is dedicated to my brothers and sister, Rodrigo, Arturo, Adrian, and Gabriela, because they were always supportive and always reminded me of what family is all about, I love you guys. Finally, I dedicate my thesis to my fiends and brothers in Costa Rica, Parra, Sabo, Jupa, Chime, Milton, Panchis, that despite of all that we have gone through we are still together and supportive of our lives.

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Amphibians

Amphibians are ectothermic vertebrates, with fluctuating body temperatures that depend on their surrounding environment (Duellman and Trueb, 1986; Zug *et al.*, 2001). Amphibian eggs are laid in gelatinous capsules and are prone to desiccation, so terrestrial species must rely on moisture in their habitat (Duellman and Trueb, 1986; Zug *et al.*, 2001). The typical amphibian larva hatches using specialized epidermal cells that secrete a gelatinous dissolving substance that aids in exiting the egg capsule (Duellman and Trueb, 1986; Zug *et al.*, 2001). The larva then reaches open water to finish development (Duellman and Trueb, 1986). This need for open water for part or all of the life cycle is called indirect development (ID) (Duellman and Trueb, 1986; Zug *et al.*, 2001).

Many amphibians, however, have direct development (DD) and complete development to metamorphosis within the egg capsules before hatching as miniature adults (Zug *et al.*, 2001). Direct development allowed amphibians to escape the need for free open water for completion of their life cycle (Zug *et al.*, 2001). This type of developmental strategy is characteristic of salamanders of Family Plethodontidae, Subfamily Plethodontinae, tribes Plethodontini and Bolitoglossini and in leptodactylid frogs in Genus *Eleutherodactylus* (Zug *et al.*, 2001). These groups have experienced great evolutionary success by having DD because these lineages escaped intense competition and predation in

aquatic environments (Duellman and Trueb, 1986; Zug *et al.*, 2001). Evolution of terrestrial forms required modifications in the animal's anatomy, physiology, and behavior (Duellman and Trueb, 1986). Amphibian skin is highly permeable and serves as a major respiratory/gas exchange organ as well as regulatory organ for ionic and osmotic balance (Boutilier, 1988; Zug *et al.*, 2001). In larvae (life stage similar physiologically and morphologically to fish) and strictly aquatic forms, gills are responsible for respiration (Duellman and Trueb, 1986; Zug *et al.*, 2001). Some amphibians (especially lungless salamanders of Family Plethodontidae) use the pharynx and mouth lining for gas exchange. These terrestrial species must lose water over respiratory surfaces so they require moist environments to avoid desiccation (Savage, 2002; Zug *et al.*, 2001). Amphibians rehydrate through skin in contact with a wet/moist surface (Duellman and Trueb, 1986).

Living amphibians have two types of skin epidermal glands: mucous and granular or poison glands (Savage, 2002; Zug *et al.*, 2001). Mucous glands secrete mucopolysaccharides that keep the skin moist for cutaneous respiration (Duellman and Trueb, 1986; Zug *et al.*, 2001). Granular glands secrete noxious to lethal substances that aid in defense against predators and have antimicrobial activity (Brodie, 1983; Savage, 2002; Zug *et al.*, 2001).

Amphibian Population Declines

Animal biodiversity has been decimated globally for numerous reasons, which are commonly associated with human beings. Amphibians have become the focus of research and conservation efforts due to their endangered status. The first global assessment of amphibians (GAA) presented amphibians as the most threatened vertebrate group on earth (IUCN, 2006). Of the 5918 amphibian species assessed, over a third (38.2%) are critically endangered, endangered, vulnerable, or near threatened (Figure 1). For birds and mammals these categories include just 12% and 23% of species, respectively (IUCN, 2006). At least 165 amphibian species are thought to be extinct and at least 43% of amphibian species have declined in populations (IUCN, 2006). Latin American countries have the most threatened species of amphibians, but the highest proportions of threatened species is on Caribbean islands (IUCN, 2006).

Amphibians are considered bioindicators of environmental health because their permeable skin, terrestrial habits, constant need for humidity or open water, and unshelled eggs expose them to most environmental factors (Blaustein and Kiesecker, 2002). All these characteristics make amphibians susceptible to stressors in either aquatic or terrestrial ecosystems. Amphibian declines seemed to be tied to one of six causes: alien species, over-exploitation, land use change (habitat loss), global change (including increased UV radiation and global warming), pesticides (including all toxic chemicals and heavy metals), and emerging infectious diseases (Blaustein and Kiesecker, 2002; Carey, 1993; Cohen, 2001; Collins and Storfer, 2003; Lannoo, 2005; Young *et al.*, 2001). All these causes or hypotheses are grouped into two classes based on our knowledge or understanding of the problem. Class

I hypotheses include three well known mechanisms that probably affect all species: alien species, over-exploitation, and land use change. Class II includes the three less well understood hypotheses which are more specific to amphibians: (global change, pesticides, and emerging infectious diseases) (Collins and Storfer, 2003). Combination of causes from Class I and II hypotheses can act together to increase population declines (Araújo *et al.*, 2006; Berger *et al.*, 2004; Bosch *et al.*, 2006; Carey and Bryant, 1995; Davidson *et al.*, 2007; Pounds *et al.*, 2006; Whitfield *et al.*, 2007). Emerging diseases seem to, have been the most important cause for amphibian decline in the past decade (Daszak *et al.*, 1999). Changing environmental conditions probably made it easier for emerging diseases and pathogens to increase and spread (Daszak and Cunningham, 2003; Daszak *et al.*, 2004b; Kriger *et* al., 2007; Williams *et al.*, 2002). These emerging diseases are decimating amphibian populations leading to extinctions (Daszak *et al.*, 1999; Lips *et al.*, 2003; Lips *et al.*, 2005; Norris, 2007). The amphibian chytrid fungus (*Batrachochytrium dendrobatidis*) is the most important pathogen causing disease in amphibians and population declines.

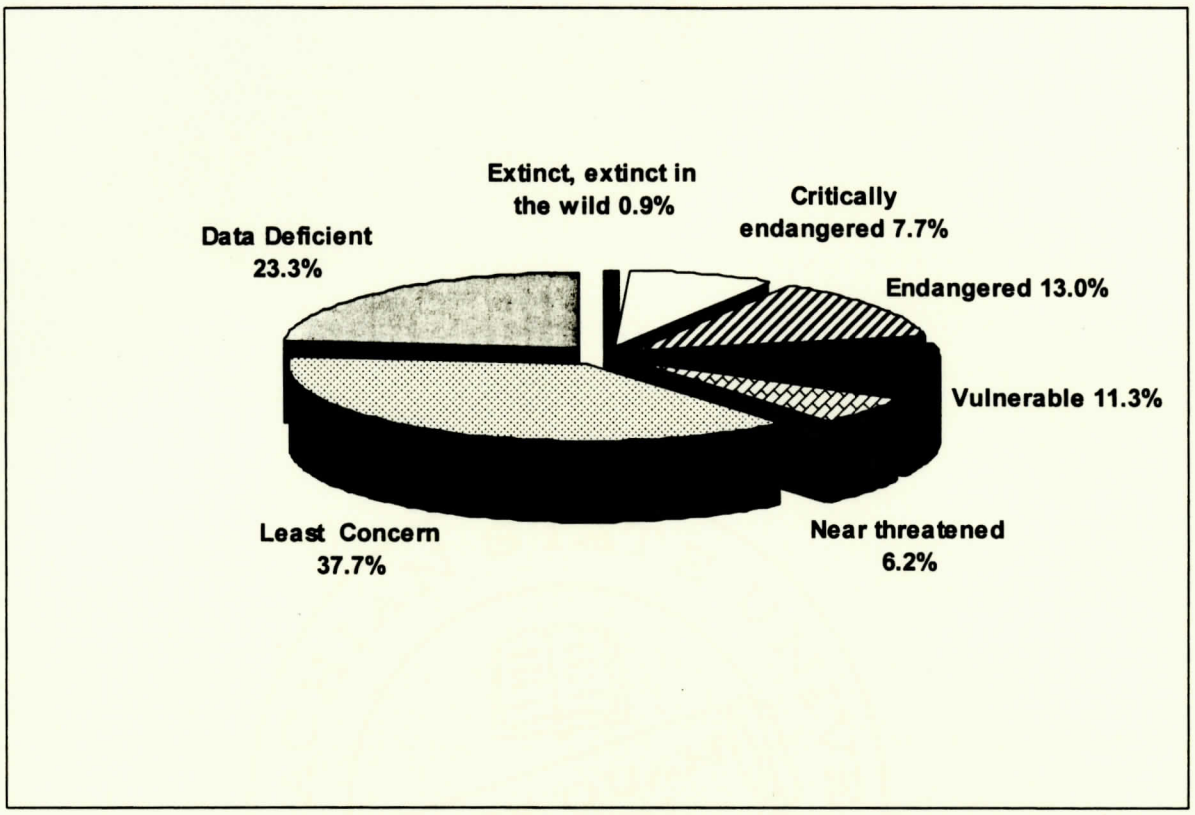

Figure 1. Summary of Red List status for amphibians. (Modified from IUCN, 2006).

Chytrids

The amphibian chytrid fungus *Batrachochytrium dendrobatidis* (BD) is responsible for decimating amphibian populations for the past two decades. As more naive populations are exposed to this pathogen, the number of declining populations keeps increasing (Berger *et* al., 1998; Longcore *et al.*, 1999). This fungal pathogen is part of the Emerging Infectious Diseases Hypothesis for amphibian population declines (Collins and Storfer, 2003). It is probably the most dangerous threat faced by any animal group today. The amphibian chytrid belongs to Phylum Chytridiomycota, a group of fungi that shares many characteristics with animals. The Phylum's only class, Chytridiomycetes, contains five orders: Chytridiales (includes BD), Monoblepharidales, Neocallimastigales, Rhizophydialies and Spizellomycetales (Alexopoulos and Mims, 1979; Barr, 1990). Most chytrids are saprophytes found in moist/wet soil or open water decomposing keratin, cellulose, pollen, chitin, or any other decaying material. Some chytrids are parasites of plants, animals, algae, nematodes, rotifers and other fungi (Alexopoulos and Mims, 1979; Barr, 1990; Berger *et al.*, 1998).

Chytrids are characterized by their asexual, haploid, reproductive cells called zoospores. Zoospores are formed in a zoosporangium and released through an opening in the sporangial wall. Zoospores are motile, single cells that possess one posteriorly oriented whiplash flagellum. This flagellum is similar to some protistans, and chytrids have been included in Kingdom Protista (Alexopoulos and Mims, 1979; Barr, 1990). Chytrids require a water film for zoospore dispersal; so members of this phylum are considered aquatic (Alexopoulos and Mims, 1979; Barr, 1990; James *et al.*, 2000). When zoospores find a suitable substrate, they grow to form a thallus in or on the substrate and form a sporangium.

When conditions are poor, zoospores can encyst and form a resting structure. Chytrids may release male and female zoospores either at different times or synchronously. Species can have sexual reproduction in several different manners. Male and female zoospores can fuse to form a diploid resting spore or sporangium. Thalli or sporangia can also fuse to form diploid resting stages. Resting stages can survive harsh environmental factors and maintain viability for long periods of time. When the environment is suitable again, the resting stage germinates, grows and releases more zoospores (Alexopoulos and Mims, 1979; Barr, 1990).

Amphibian chytrid (BD)

The name *Batrachochytrium dendrobatidis* (BD) comes from the Greek word *batracho* which means frog and *chytr,* which means earthen pot. The species name was taken out of *Dendrobates*, genus of the poison dart frog *Dendrobates azureus* from which the fungus was first isolated (Longcore *et al.*, 1999). It was first reported decimating amphibian populations in Costa Rica in the 90's (Lips, 1998), but the earliest record is from 1938 in southern Africa (Weldon *et al.*, 2004). Within the Order Chytridiales, which contains a number of other parasites (Alexopoulos and Mims, 1979; Barr, 1990), BD is the first chytrid known parasitizing a vertebrate (Berger *et al.*, 1998). The life cycle of BD doesn't deviate from other chytrids; it reproduces asexually and clonally (Morehouse *et al.*, 2003). The cycle starts when a zoospore encysts in the keratinized stratum corneum and stratum granulosum layers of the epidermis (Figure 2) of an amphibian or the keratinized mouth parts of tadpoles (Marantelli *et al.*, 2004) forming a germling (Berger *et al.*, 2005). Rhizoids appear to produce a thallus which can produce one or more zoosporangia. Zoosporangia release zoospores to

the environment through one or more discharge papillae that open through the sporangial wall (Berger *et al.*, 2005) (Figure 3). Sexual reproduction and resting stages of this particular chytrid fungus have not been observed in laboratory or in the environment. Evidence of local endemism and recombination in isolates of the Sierra Nevada of California suggests that sexual reproduction may take place occasionally in this species (Morgan *et al.*, 2007). Since other chytrid fungi have sexual reproduction, (by fusing zoospores, thalli, or sporangia, producing long lasting, protective, resting stages afterwards) (Alexopoulos and Mims, 1979; Barr, 1990). Several alternative sexual pathways might be possible in BD's life cycle (Figure 3). If any of these alternative pathways for sexual reproduction occur, resting stages should be formed,; and controlling dispersal into naive populations will be much more difficult (Morgan *et al.*, 2007).

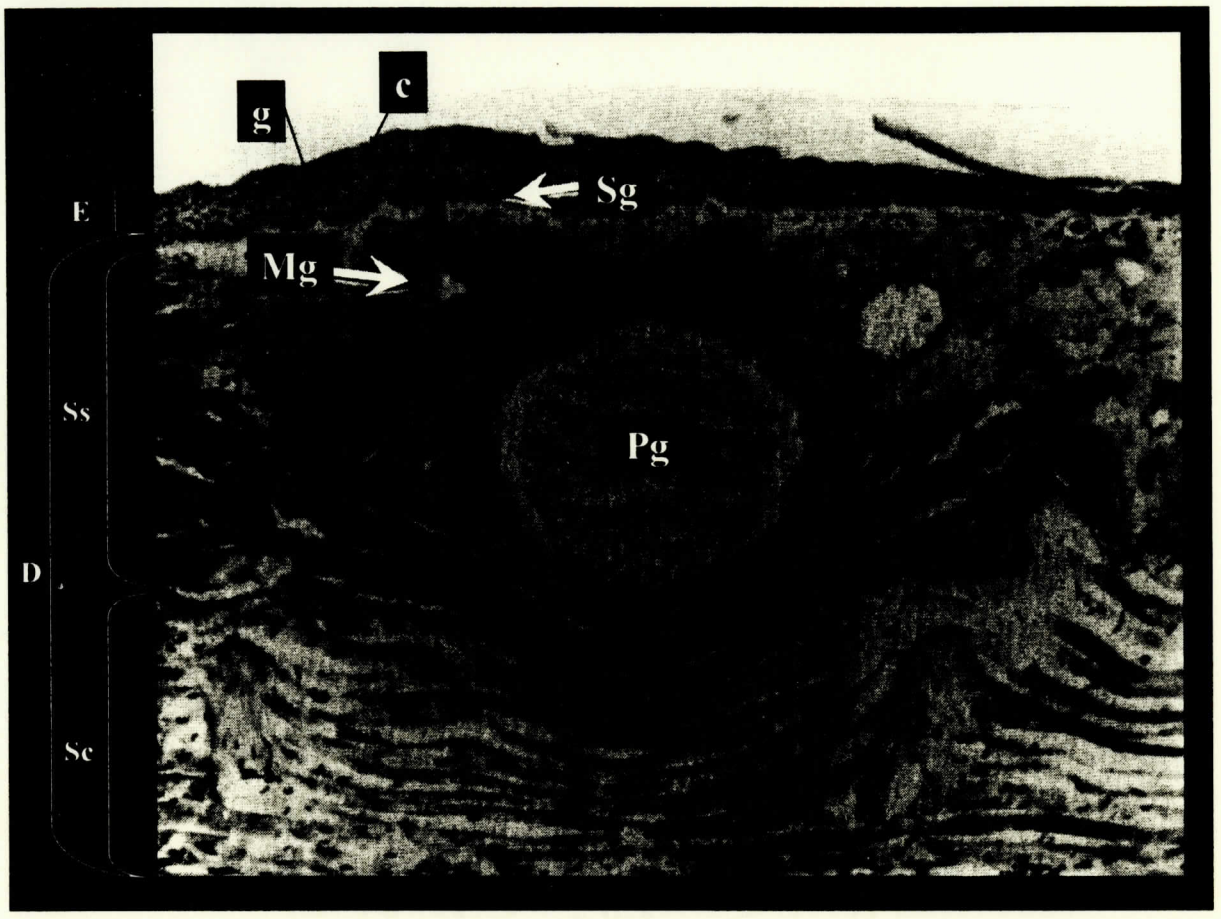

Figure.2. Structure of a cross section of the ventral skin of a marine toad *Bufo marinus*. Abbreviations are E=epidermis, D=Dermis, Ss=stratum spongiosum, Sc=stratum compactum, Sg=stratum germinativum, g=stratum granulosum, c=stratum corneum, Mg=mucous gland, Pg=poison or granular gland (Zug *et al.*, 2001).

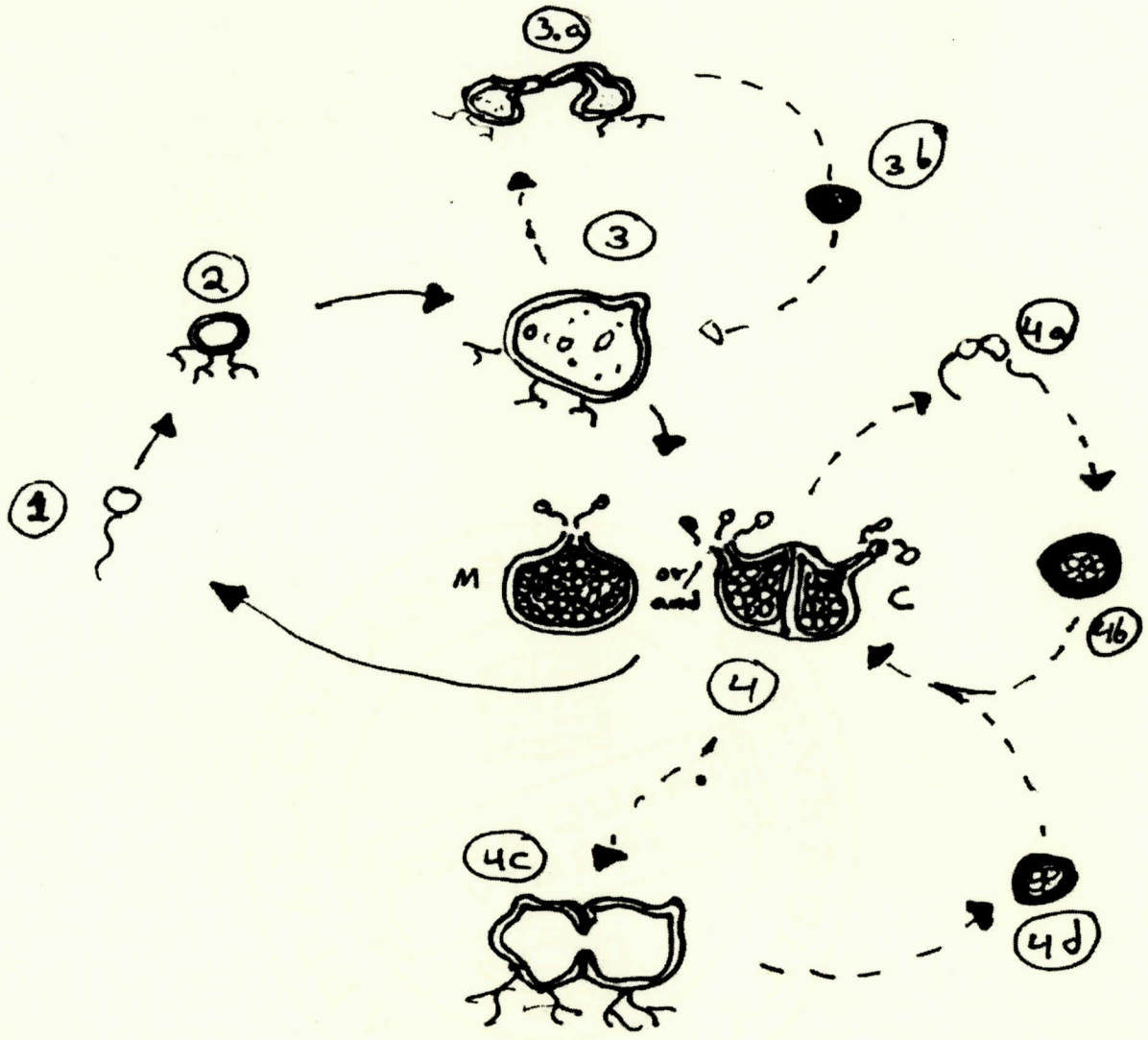

Figure 3. *Batrachochytrium dendrobatidis* life cycles. Known stages are connected by solid arrows and possible sexual pathways are indicated by dashed arrows. Where 1=zoospore, 2=encysted zoospore (germling) forming rhizoids, 3=thalli, 4=zoosporangia releasing zoospores, M=monocentric zoosporangium, C=colonial zoosporangia, 3a=fusing thalli, 4a=plasmogamy or fusing zoospores, 4c=fusing zoosporangia and 3b, 4b, 4d=resting stages (Adapted from Berger *et al.*, 2005).

Several aspects of the life history of BD enhance its ability to attack amphibians. Zoospores of BD survive longer (24 h) than those of most chytrids and remain infective (Piotrowski *et al.*, 2004). BD zoospores seem to lack the chemotaxis towards the host seen in other parasitic chytrids (Piotrowski *et al.*, 2004). BD can survive up to 7 weeks in pure water and still release zoospores (Johnson and Speare, 2003). BD can grow at temperatures between from 4 and 26°C but does best between 17 and 25°C. Zoosporangia can survive 8 days at 30°C, up to 96 hours at 32°C, 4 hours at 37°C, and above 37°C survival becomes a matter of minutes. Desiccation for at least 3 hours is required to kill the fungus (Johnson *et al.*, 2003, Woodhams *et al.*, 2003). UV light does not affect BD in any stage of the life cycle and high sugar percentages (>2%) in media inhibits the growth of BD (Johnson *et al.*, 2003). The optimal pH for the fungus is 6-7; zoospores survive poorly under pH of 6 (Johnson *et al.*, 2003), no growth occurs under pH of 5, and growth can be observed up to pH of 10 (Johnson and Speare, 2005). Also, BD produces proteases that degrade casein and gelatin and might be the enzymes that degrade the keratin in amphibian's skin (Johnson *et al.*, 2003).

Amphibians from altitudes of more than 500m are more susceptible to chytrid infections (Lips, 1998; Lips *et al.*, 2003). However, amphibians that inhabit low altitudes have been found infected too (Stuart *et al.* 2004). When amphibians are infected with BD they acquire chytridiomycosis. Symptoms include epidermal hyperkeratosis (thickening of the keratinized layers of the skin), hyperplasia (cell overproduction), lethargy, anorexia, skin lesions, and skin sloughing. In most cases the chytrid infection leads to death (Nichols *et al.*, 1998; Pessier *et al.*, 1999).

Some amphibians may be carriers that spread Bd. They can have BD without showing symptoms or can shed the infection. These abilities make these species natural carriers and dispersers of BD in the environment (Daszak, *et* al., 2004a; Garner *et al.*, 2006; Hanselmann *et al.*, 2004; Mazzoni *et al.*, 2003; Weldon *et al.*, 2004). *Ambystoma tigrinum* can slough the infected skin until no infection is observed (Davidson *et al.*, 2003). Sloughed contaminated skin in the water can carry zoospores, and zoosporangia that can infect other amphibians in the habitat. North American bullfrogs *Rana catesbeiana* can tolerate the infection without dying (Daszak, *et* al., 2004*a*; Garner *et al.*, 2006; Hanselmann *et al.*, 2004; Mazzoni *et al.*, 2003). Farming and export *R. catesbeaina* may be the most important channels of dispersal into many naive amphibian populations (Berger *et al.*, 1998; Daszak, *et* al., 2004*a*).

The African Clawed Frog (*Xenopus laevis*) may be the original host for Bd (Weldon *et al.*, 2004) and the first natural disperser of the fungus. This mainly aquatic frog carries the fungus as part of its skin flora and does not present clinical signs of chytridiomycosis in the wild. It does not display population declines and seems to have been infected with BD as early as 1938 (Weldon *et al.*, 2004). After 1935 huge numbers of *X. laevis* wild caught from southern Africa were exported around the world for pregnancy tests and as research subjects (Weldon *et al.*, 2004).

Another possible vector for water-bourne pathogens like BD are water birds, which might easily transport zoospores great distances (Czeczuga *et* al., 2004). In fact, 48 species of birds in Poland are known to carry 97 species of zoosporic fungi, including 21 species of Chytridiomycetes on their feathers (Czeczuga *et* al., 2004). Johnson and Spear (2005) found that one minute of contact between BD zoospores and feathers of both terrestrial and aquatic

birds was enough for zoospores to be transported and subsequently produce growth after an hour on culture media. Zoosporangia with protective cell wall, survived up to 3 hours in duck feathers and up to 2 hours in terrestrial bird feathers (Johnson and Spear, 2005). BD survival in feathers makes birds potential carriers of BD from a body of water to another and around the world if birds are migratory. Environmental pH is a key factor for survival of BD in media and in soil, with BD showing no growth in either if the pH is acidic (Johnson and Speare, 2003; Johnson and Spear, 2005). But when the pH is optimal, just creek bed sand with as low as 10% moisture content is sufficient to maintain survival, growth and viability of zoosporangia and zoospores after 12 weeks (Johnson and Spear, 2005). Soil is also potential vector for dispersing BD when used for plant nursery or construction.

Some treatments can kill 100% of BD in the laboratory., Exposure to 4% household bleach for 10 minutes, potassium permanganate for 10 minutes, 1% formaldehyde for 5 minutes, 70% ethanol for 5 minutes, desiccation for 2 hours and 40 minutes or heat at 60°C for 30 minutes will kill all BD (Johnson *et al.*, 2003; Webb *et al.*, 2007). Amphibians have natural adaptations that can inhibit the growth of pathogens like BD. Skin flora (cutaneous bacteria) of three salamander species (*Hemidactylium scutatum*, *Plethodon cinereus* and *Rana muscosa*), inhibited the growth of BD (Harris *et al.,* 2006; Woodhams *et al.*, 2007). Probiotic mixtures from skin of amphibians could potentially be used against BD in natural sites where the infection has reached (Harris *et al.,* 2006). Also, amphibians can inhibit pathogen skin infections using their innate and adaptive immune systems.

Skin secretions of amphibians (Antimicrobial peptides)

All modern amphibians seem to have immune systems similar to those reported in African clawed frogs (*Xenopus laevis*) and axolotls (*Ambystoma mexicanum*) (Carey *et al.*, 1999). Amphibians, like mammals, respond to antigenic stimuli with adaptive and innate immune systems. This study was based on the antimicrobial peptides secreted by the skin of amphibians, one of the skin defense mechanisms that belong to the innate immune system. The innate immune system produces general, non-specific and fast responses to all kinds of antigens when injury or attack occurs (Carey *et al.*, 1999). Innate immune responses are the first line of defense against digestive tract and skin pathogens, and are mostly done by antimicrobial peptides (Carey *et al.*, 1999). Also, the innate response aids in activation of the adaptive immune system. After antigen detection, the adaptive immune response activates and produces highly specific antibodies against that antigen (Carey *et al.*, 1999). Antibody responses are slower than innate responses, taking up to 24 hours for *X. laevis* to produce the most common antibody in amphibians IgM (Du Pasquier *et al.*, 1989).

Amphibian skin secretions probably evolved as a defense mechanism against predators (Brodie, 1983; Zug *et al.*, 2001). Secretions can be noxious and repel predators or toxic/lethal that can kill predators (Brodie, 1983; Zug *et al.*, 2001). Skin secretions of frogs can cause oral dyskinesia and sedative/anesthetic responses in the mouths of their snake predators increasing probability and numbers of frogs that escape (Cohen, 2001). Antimicrobial peptides are synthesized and stored in granular (poison) glands in the epidermal layer of the skin (Apponyi *et* al, 2004; Rollins-Smith *et al.*, 2005a,b,c) (Figure 2). All amphibian poison glands are similar but their secretions vary greatly in toxicity from

merely irritating to lethal (Zug *et al.*, 2001). Granular glands are surrounded by smooth muscles controlled by the sympathetic nervous system. An alarm response, due to stressors, injury, or hormonal stimuli produces stimulation of adrenergic receptors that lead to release gland contents onto the skin surface (Rollins-Smith *et al.*, 2005b; Savage, 2002). Electrical stimulation and injection of adrenergic agents like norepinephrine (NE) induce release of secretions as well (Apponyi *et al.*, 2004; Rollins-Smith *et al.*, 2005b). Some amphibians require two to three weeks to recharge their granular glands (Rollins-Smith *et al.*, 2005a,b). Antimicrobial peptides range from 10-46 amino acid residues. They are normally basic, hydrophobic, cationic (positively charged), and form amphipathic (polar at one end of the molecule (hydrophilic) and non-polar (hydrophobic) at the other end) α –helixes (Carey *et al.*, 1999; Rollins-Smith *et al.*, 2005a; Strandberg and Ulrich, 2004). Different families of peptides are secreted by amphibians and each amphibian species produces its own unique peptide mixture. (Rollins-Smith *et al.*, 2005a). These collections of peptides have different activity against gram positive and gram negative bacteria, fungi, protozoa, viruses (including HIV) and cancer cells having great medical importance (Amiche *et al.*, 2000; Apponyi *et al.*, 2004; Cohen, 2001; Conlon *et al.*, 2003; Pierre *et al.*, 2000; Rollins-Smith *et al.*, 2005b; VanCompernolle *et al.*, 2005).

Three models have been proposed for the ability of these peptides to disrupt the pathogen's cell membrane. The **barrel-stave model** (Figure 4, A-D), involves assembly of transmembrane peptides side by side to form a pore. The **toroidal wormhole model** (Figure 4, A-E), includes a lipid coating for the pore. The **carpet model** (Figure 4, A-F), most used by amphibian peptides, suggests that peptides disrupt the membrane directly by producing a high density of peptides on the cell membrane (Strandberg and Ulrich, 2004). The

amphipathic α-helical structures of the peptide align along the membrane and tilt into the bilayer at an angle of 40° to penetrate it (Figure 4) (Strandberg and Ulrich, 2004). All peptides disrupt membrane structure and function and alter flux of ions and small molecules across the membrane and lead to cell lysis (Strandberg and Ulrich, 2004).

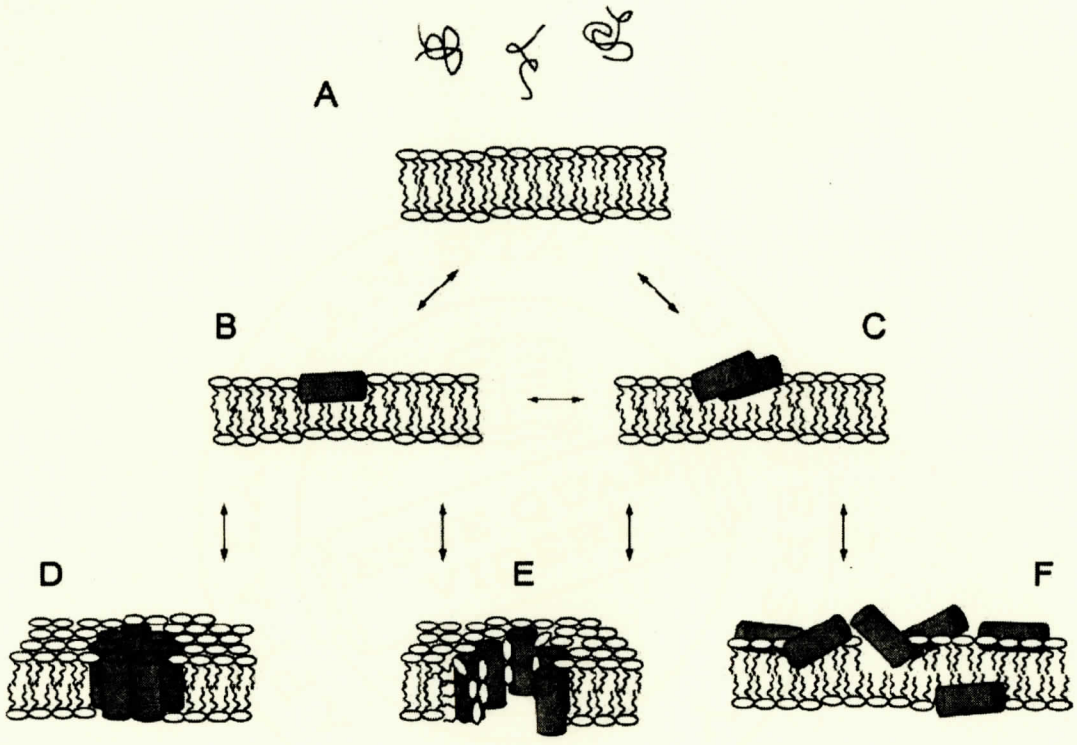

Figure 4. Models for action of antimicrobial peptides in killing pathogenic cells. Abbreviations are A=unstructured peptides approaching the cell membrane, B=peptide bound to the bilayer surface as a monomer with a well-defined structure, shown as a cylinder, C=peptides assembled on the cell membrane, D=barrel-stave model for formation of a pore across the membrane, E=toroidal wormhole model with pore lining, and F=carpet model with membrane disruption by local, unorganized, high concentration of peptides (Strandberg and Ulrich, 2004).

The quantity, composition and antimicrobial activity of peptides produced by each amphibian species is completely unknown. Peptides from several amphibian skins inhibit growth of BD (Amiche *et* al., 2000; Apponyi *et* al., 2004; Rollins-Smith *et al.*, 2002a,b,c; Woodhams *et al.*, 2006a,b). Peptide mixture quantity and potency against pathogens like BD are quite variable among amphibian species (Amiche *et* al., 2000; Apponyi *et al.*, 2004; Rollins-Smith *et al.*, 2002a,b,c; Woodhams *et al.*, 2006a,b). Growth inhibition of BD is maximal when peptide mixtures act synergistically (Rollins-Smith *et al.*, 2002b). All amphibians have similar skin structure, but some species are resistant to skin pathogens like BD by virtue of antimicrobial peptides. This dichotomy suggests several testable hypotheses about presence of antimicrobial peptides and BD susceptibility:

1) Antimicrobial activity is a random result of selective pressures on skin secretions unrelated to BD. Under this hypothesis, occurrence of antimicrobial peptides might occur in phylogenetic lineages but would not be influenced by geography or life history patterns.

2) Antimicrobial peptides in general and resistance to organisms like BD in particular, represent adaptive responses to exposure to waterborne pathogens encountered in the adult and larval environments. Under this hypothesis, aquatic species should have maximal exposure to pathogens and maximal antimicrobial activity of peptides. This group of species should include species with aquatic larvae and ID. Species with little contact with aquatic situations (DD) should have minimal antimicrobial activity to aquatic pathogens. The reproductive strategies of DD species should reduce selective pressure to antimicrobial activity.

3) Antimicrobial activity against BD would have a general selective advantage in a world filled with this sort of pathogen. Amphibian lineages with resistance should prosper

relative to those without resistance. Under this hypothesis, antimicrobial activity should show strong correlation to evolutionary history and sort according to the relationships among the amphibian species. Selection should favor retention of antimicrobial activity whenever it occurs.

These hypotheses can be tested by comparing antimicrobial activity of: 1) amphibians with aquatic larvae (ID) with those without aquatic larvae (DD); 2) Amphibians from different regions and climates; and 3) Amphibians from several different evolutionary units. Two areas are ideal for these tests, Costa Rica and the Southern Appalachians. Costa Rica is the country with highest amphibian diversity in Mesoamerica and ranks as the tenth most diverse country in the world (Young *et al.*, 2004). Also, Costa Rica includes the first documented case of population die-offs caused by BD (Lips, 1998). The Southern Appalachians of North Carolina, USA, have the greatest new world salamander diversity and the region ranks seventh in amphibian diversity in the world (Lannoo, 2005; Young *et al.*, 2004). Appalachian amphibian populations haven't declined due to BD infections as is the case in other regions. Both regions represent areas with high amphibian diversity and susceptibility to population declines by BD infections.

MATERIALS AND METHODS

Batrachochytrium dendrobatidis (Isolate JEL 423: from *Phyllomedusa lemur,* El Cope, Panama), was obtained in agar medium from Dr. Joyce Longcore (University of Maine, Orono) and cultured in sterile conditions under a hood (LABCONCO Class II Purifier Biosafety Cabinet) in the Mycology laboratory of the Department of Biology at Appalachian State University. Amphibian chytrid fungi were grown in two 100ml flasks filled with H Broth medium. Each stock solution was inoculated with 3/4ml of BD solution and incubated at room temperature for one week. After incubation, stocks were refrigerated at 5°C and renewed every two months to assure fungal viability. All old cultures, materials and solutions used for BD work were autoclaved at 125°C for 45 minutes before disposal.

Zoospores of BD were transferred to Petri dishes ¾ filled with TGHL agar media. Each Petri dish was inoculated with 3/4ml of the H Broth chytrid stocks, closed, sealed with parafilm and incubated for 9-11 days at room temperature. Plates were then flooded with three milliliters of sterile H Broth for 30 minutes to release zoospores. Broth with zoospores was vacuum filtered through a sterile 20µm pore diameter nylon spectra/mesh (Spectrum Laboratories Inc. 146510) to separate zoospores from thalli and zoosporangia. Fifty µl of the zoospore filtrate were mixed with 50µl of 0.4% Trypan Blue and counted using a hemocytometer kit (Hausser Scientific 1483) to estimate zoospores/ml. Zoospore filtrate was diluted with H Broth to a final concentration of 10^7zoospore/ml for growth inhibition assays.

Appalachian Collections

Six species of plethodontid salamanders were collected in Watauga and Caldwell counties, North Carolina, USA in the Southern Appalachian Mountains. *Plethodon yonahlossee* (*n*=10), *Plethodon montanus* (*n*=9) and *Plethodon cylindraceus* (*n*=10) had DD. *Desmognathus quadramaculatus* (*n*=12), *Desmognathus orestes* (*n*=11) and *Eurycea wilderae* (*n*=9) had ID. Individuals were put in separate Zip lock bags and transported to the Animal Care Facility at Appalachian State University (IACUC protocol #05-7). Salamanders were stored individually in plastic containers with moist paper towels at 18°C for 21 days to allow regeneration of peptides lost during collection (Rollins-Smith *et al.*, 2005b). Salamanders were fed one small cricket daily. Skin secretions of the amphibians were induced by injection of norepinephrine (NE) solution. Individuals were massed, placed in Zip lock bags, and injected with NE solution (0.01ml/g of amphibian) using a 50µl Hamilton syringe #408 with a 27 gauge one inch needle. Larger animals were injected using a one milliliter syringe with a 27 gauge one inch needle. Animals were injected through the bag to avoid loss of peptide when handling. Fifty milliliters of Collecting Buffer were added to each bag for 15 minutes to dissolve all skin secretions. Animals were rinsed with water and returned to their containers. To avoid contamination and to preserve peptides, one milliliter of 50% HCl was added to each sample bag. Collection fluids were transferred to a 50ml syringe and pushed through two activated C-18 SepPak cartridges (Waters Corp. WAT020515) with a 50ml syringe. Before extraction cartridges were activated with 10ml of methanol and 10ml of Buffer A and stored in 50ml centrifuge tubes with two milliliters of Buffer A.

Costa Rican Collections

Costa Rican amphibians (14 frogs and 4 salamanders) were collected from Santa Ana Conservation Center (Santa Ana, San Jose, Costa Rica), Tapantí-Macizo de la Muerte National Park (Cartago, Costa Rica), and sites in land administered by Friends of the Osa FOO and in the property of Porfirio Sanchez Marenco (Osa, Puntarenas, Costa Rica). Species were selected based on availability, abundance and number of SepPak cartridges. Data recorded for each individual were: latitude, longitude and elevation with a GPS unit (Model SporTrak Magellan using NAD27 UTM function); site name; province; species and number of animals used for each sample (*n*). Since most frogs collected in Costa Rica were too small to yield the necessary 300µg of total peptide, two or three individuals were included in a sample. Skin secretions of the Costa Rican amphibians were extracted in the field. SepPak cartridges were activated and transported to Costa Rica in Zip lock plastic bags in a cooler with ice. Release of skin peptides and processing the solutions was the same as with the Appalachian samples except that fluids were forced through the SepPak cartridges using a Peptide Injection Gun (PIG) (Figure 5).

Peptide injection gun (PIG).—This very useful new tool was added to the methodology. SepPak filters can get dirty and clogged. For this reason, pushing the peptides through the SepPak cartridges with a 50µl syringe sometimes was quite energy and time consuming. A caulking gun was modified to provide pressure when forcing the fluids through the SepPak cartridges. A 50ml syringe is placed in a plastic pipe section with rubber rings that hold in

position the syringe and cartridges. The PIG is also, light, easy to carry and cheap to make (Figure 5).

Figure 5. Peptide Injection Gun (PIG). New tool addition to the peptide extraction methodology, used to force fluids through the SepPak cartridges with 50ml syringes.

Peptide content assay (all samples)

Peptides were removed from SepPak cartridges in the laboratory using a 12 port vacuum (Figure 6). Cartridges were washed with 10ml of buffer A before eluting the peptides with 10ml of buffer B at a rate of 30 drops per minute. The 10ml of peptide-buffer elution of each individual was separated into two test tubes. Using Bradykinin as standard, the peptide content per individual was quantified by Micro BCA protein assay (Pierce #23235) in flat bottom 96 well-microplates (Falcon 353912). A total of 300µl of peptide-buffer elution per individual, divided into two samples of 150µl, were used for assays. Plates were read in an automatic plate reader (Molecular Devices E-Max Precision Microplate Reader) at a wavelength of 570nm. Remaining peptide-buffer elution was dried by speed-vacuum (LABCONCO CentriVap Console) overnight at 40°C and stored at -20°C for chytrid growth inhibition analysis.

Chytrid growth inhibition assay

Peptides were reconstituted to a concentration of 500ug/ml in HPLC water. Pellet was removed from the test tube with a sterile metal probe and brought into solution using a vortex mixer. A stock solution of 600µl was needed for each assay. Stock solutions were serially diluted to seven concentrations: 500µg/ml, 250µg/ml, 100µg/ml, 50µg/ml, 25µg/ml, 12.5µg/ml and 6.12µg/ml. Five replicates at each concentration were analyzed by adding

50µl of peptide solution to 50 µl of BD zoospores in H Broth (500,000 zoospores) in sterile 96 well microplates with low evaporation lids (Costar 3370). Replicates were assigned to wells non-randomly (Figure 7). Each assay included eight replicates of three controls. Each control well included 50µl of HPLC water. Blanks included H Broth without zoospores and controlled for bacterial contamination or any physical changes in the medium. Positive controls included H Broth with zoospores and controlled for BD growth during the experiment. Negative controls included zoospores, which had been killed by immersion in a 60°C water bath for ten minutes, and controlled for degradation of zoospores as well as bacterial contamination.

Plates were read daily from day one until day seven at 490nm. Optical density (OD) of each well was recorded. All but one assay used one plate reader (Molecular Devices E-Max Precision Microplate Reader). The assay for *Plethodon yonahlossee* used a BIO-RAD Microplate Reader Model 680. Between readings plates were wrapped in aluminum foil to minimize effects of evaporation and light and stored at room temperature under a sterile hood. When individual animals produced little peptide, extracts from more than one individual of the same species were combined to provide the necessary amount of peptide needed for the assay. By combining some peptide solutions, sample sizes changed for some of the species for this assay.

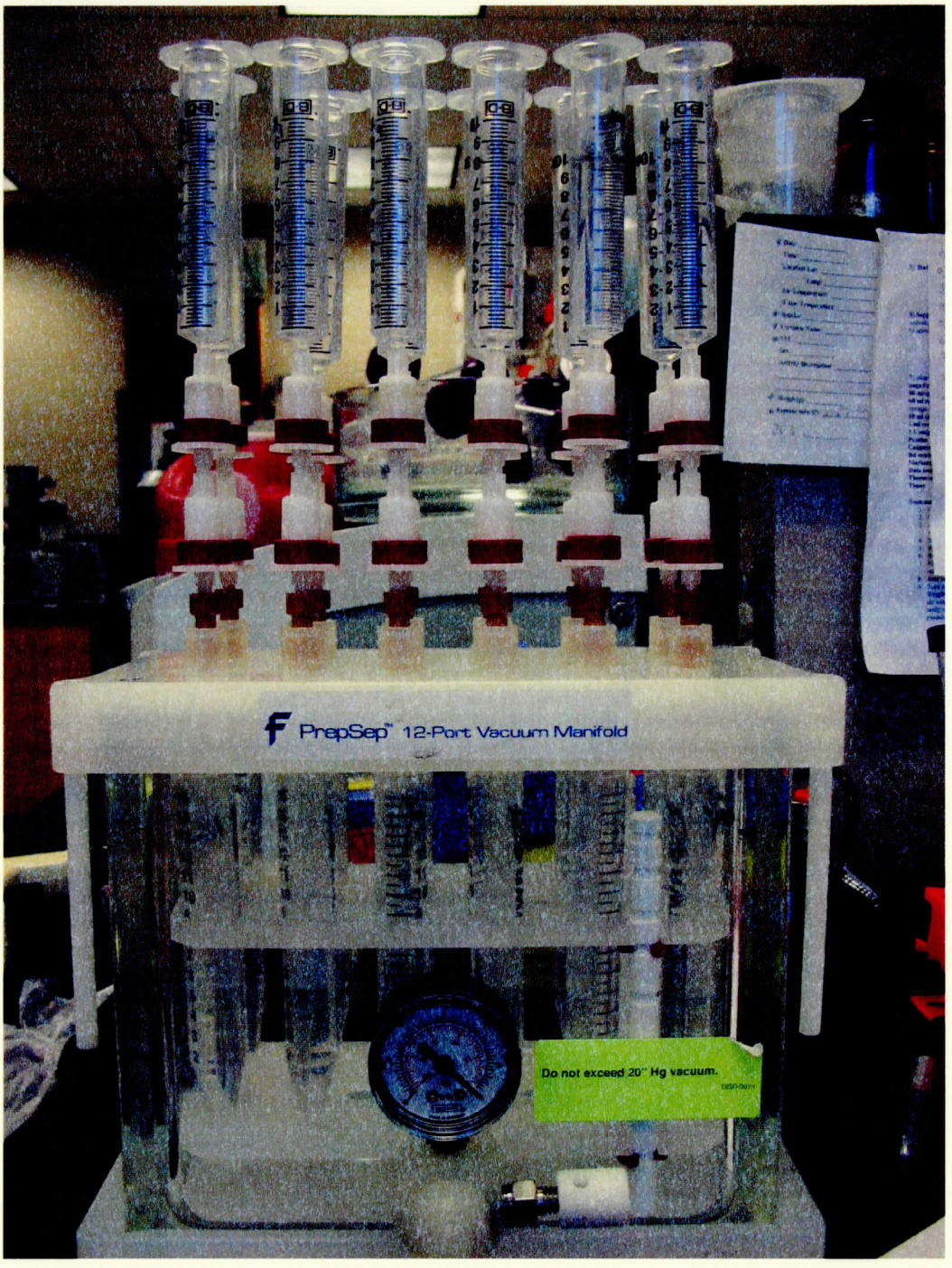

Figure 6. PrepSep 12 port Vacuum Manifold. Pressure vacuum used to extract the peptides from the SepPak cartridges and SepPak cartridge activation.

PLATE 1	1	2	3	4	5	6	7	8	9	10	11	12
A	BLANK	(+) control	500µg/ml	500µg/ml	500µg/ml	500µg/ml	500µg/ml					(-) control
B	BLANK	(+) control	250µg/ml	250µg/ml	250µg/ml	250µg/ml	250µg/ml					(-) control
C	BLANK	(+) control	100µg/ml	100µg/ml	100µg/ml	100µg/ml	100µg/ml					(-) control
D	BLANK	(+) control	50µg/ml	50µg/ml	50µg/ml	50µg/ml	50µg/ml		Start with	individual		(-) control
E	BLANK	(+) control	25µg/ml	25µg/ml	25µg/ml	25µg/ml	25µg/ml		2 same as	shown		(-) control
F	BLANK	(+) control	12.5µg/ml	12.5µg/ml	12.5µg/ml	12.5µg/ml	12.5µg/m					(-) control
G	BLANK	(+) control	6.12µg/ml	6.12µg/ml	6.12µg/ml	6.12µg/ml	6.12µg/m					(-) control
H	BLANK	(+) control	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	(-) control

Figure 7. Peptide titer allocations for BD inhibition assays in 96 well microplates. Color codes are Orange for blank wells, Blue for positive control wells, Gray for five replicates of seven peptide concentrations for one individual, Yellow for wells assigned for second individual, and Green for negative control wells.

Statistical analysis

All statistical tests were evaluated for significance at $\alpha \leq 0.05$. Peptide concentration data were transformed to their logarithm to achieve normality before analysis (Zar, 1999). Peptide content was compared for differences with single-factor ANOVAs on data sorted by phylogenetic lineage, life history or geographical origin using SAS. Post-hoc comparisons of all group means were made using Tukey Studentized Range tests using (SAS, 1999). Confidence intervals (95%) were calculated to determine whether species represented by single samples could be added to larger, phylogeneticly meaningful groups. If the value for the single sample fell inside the 95% confidence interval, the single sample was included

with the group for further comparisons. Total peptide content was analyzed by country and by developmental strategy using a nested ANOVA (SAS, 1999). Nested ANOVA complemented with a Tukey's Studentized Range test for concentrations were designed nesting peptide content per individual within each species and each species nested within development strategy (Zar, 1999). This ANOVA design compared the peptide content of the species from each separate country as well as by development strategy. Tukey tests revealed significant contrasts among all possible pair-wise comparisons between variables. The same design was used to compare peptide content of all species collected by development strategy.

For BD growth inhibition all optical densities (OD) were compared to the positive controls using *t*-tests (SAS, 1999). The lowest peptide concentration that produced significantly lower growth than the positive controls was recorded as minimal inhibitory concentration (MIC). Species with only one individual were analyzed by comparison with the 95% confidence interval for the positive control. Values outside the confidence interval were considered significant. Minimal inhibitory concentrations were compared using a Mann-Whitney test (Zar, 1999). Species were ranked from lowest to highest MIC. Rank sums for developmental strategies or by country were compared with critical values. Peptide content of species with inhibitory responses was compared with MIC's using a simple regression analysis. Species with ID were analyzed separately from DD species.

RESULTS

A total of 134 individuals of 24 amphibian species were collected for this study (Appendix B). These included six species from the Appalachians (Table 1) and eighteen species from three sites in Costa Rica (Table 2). All ten species of salamanders belonged to the family of the lungless salamanders (Plethodontidae). Three salamanders exhibited ID and seven DD. All Appalachian amphibians were salamanders. Appalachian species with DD were *Plethodon cylindraceus* (*n*=9), *Plethodon montanus* (10) and *Plethodon yonahlossee* (*n*=10). Appalachian species with ID were *Desmognathus orestes* (*n*=10), *Desmognathus quadramaculatus* (*n*=10) and *Eurycea wilderae* (*n*=9). The 18 species collected in Costa Rica included four DD salamanders: *Bolitoglossa nigrescens* (*n*=1), *Bolitoglossa pesrubra* (*n*=9), *Bolitoglossa* species. (*n*=1) and *Oedipina uniformis* (*n*=9). All 14 frogs collected were from Costa Rica and belonged in four families: Leptodactylidae (six species, three genera), Centrolenidae (three species, three genera), Hylidae (two species, two genera) and Dendrobatidae (three genera, three species). Four of the frogs had DD: *Eleutherodactylus diastema* (*n*=2), *Eleutherodactylus fitzingeri* (*n*=5), *Eleutherodactylus stejnegerianus* (*n*=6) and *Eleutherodactylus cruentus* (*n*=1); and seven were ID frogs: *Cochranella granulosa* (*n*=1), *Centrolene prosoblepon* (*n*=1), *Hyalinobatrachium pulveratum* (*n*=3), *Hyla ebraccata* (*n*=10), *Hyla microcephala* (*n*=10), *Leptodactylus bolivianus* (*n*=4) and *Physalaemus pustulosus* (*n*=10). Dendrobatid frogs collected were: *Dendrobates auratus* (*n*=1), *Phyllobates vittatus* (*n*=1) and *Colostethus talamancae* (*n*=1) but were not used for statistical

analyses because of their sample sizes. Also, peptide extractions were not done for several species collected in Costa Rica due to lack of seppak cartridges (Appendix B).

Peptide Content

Peptide content of skin secretions varied greatly within species which resulted in large standard errors for some of the species (Table 1, 2). Species secreted statistically different amounts of peptides (ANOVA, F=3.5814, df=15, $p<0.0001$). Comparisons among salamanders also revealed significant differences in peptide contents (ANOVA, F= 3.9816, df=7, $p=0.0011$). All salamanders secreted peptide concentrations of no less than 317μg/g except *Desmognathus* salamanders that had less peptide secretions (49.18μg/g for *Desmognathus quadramaculatus* and 211.24μg/g for *Desmognathus orestes*) than the rest of the salamanders collected.

Average peptide content of Appalachian amphibian secretions ranged from 49μg/g for *Desmognathus quadramaculatus* to 428.51μg/g for *Eurycea wilderae* (Table 1). Peptide concentrations for Appalachian salamanders varied significantly depending on developmental strategy (ANOVA, F= 4.7199, df=5, $p=0.0012$). Direct development salamanders were significantly different than those with ID (Nested ANOVA, F=15.57, df=1, $p=0.0002$). *Desmognathus quadramaculatus* produced significantly less peptide (49.18μg/g) than all salamanders except *Eurycea wilderae* and *Desmognathus orestes* (Tukey's post-hoc test). These small, common, heavily preyed upon ID salamanders were the most variable of the Appalachian salamanders (Table 1). *Desmognathus quadramaculatus* had significantly less peptide content than all DD salamanders (ANOVA, F=29., df=5, $p=0.0013$). Salamander

species with DD had very uniform peptide contents (317.97µg/g-334.84µg/g) which did not vary among species (ANOVA, F=0.0196, df=2, p=0.9805). Salamanders with ID did not produce significantly different peptide concentrations (ANOVA, F=2.2812, df=2, p=0.1222), despite the low value for *Desmognathus quadramaculatus*. *Desmognathus quadramaculatus* was also the least variable species (Table 1, 2). Both *Desmognathus* species had significantly less peptide secretions than all DD salamanders (ANOVA, F=30.3033, df=1, p<0.0001). Furthermore, the two *Desmognathus* species had significantly less peptide content in their secretions than the three *Plethodon* salamanders (ANOVA, F=29.4285, df=1, p<0.0001) and also less peptide content than all Costa Rican salamanders (ANOVA, F=17.6738, df=1, p=0.0002).

Peptide content secreted by Costa Rican amphibians was also rather variable. For dendrobatid species, peptide content was higher in poisonous frogs (*Phyllobates vittatus* (180.50µg/g) and *Dendrobates auratus* (117.90µg/g)), while *Colostethus talamancae* a non poisonous dendrobatid secreted only 13.37µg/g of peptide (Table 2). Average peptide content of Costa Rican amphibians ranged from 13.37µg/g for the single *Colostethus talamancae* to 6298.33µg/g for the single *Bolitoglossa nigrescens* (Table 2). Both these values were the most extreme peptide contents from all amphibians collected (Table 1, 2). The single *Bolitoglossa* species and the average peptide content of *Eleutherodactylus stejnegerianus* had very similar peptide concentrations to the three *Plethodon* species from the Appalachians (Table 1, 2). Species with sample sizes (n=1) were not used in statistical analyses except centrolenids. Values for these two species were included to the 95% CI for *Hyalinobatrachium pulveratum* for later tests. Costa Rican species as a group showed significant differences (F= 2.9091, p=0.0065) related to phylogeny of the animals. Peptide

concentrations did not differ in groups with different developmental pattern (Nested ANOVA, F=0.19, df=1, p=0.6635). Within the larger group Costa Rican salamanders showed no significant differences among species (ANOVA, F=0.04082, df=1, p=0.8424).

Salamanders in general, had higher peptide contents than most frogs. Frogs collected produced significantly different peptide concentrations (ANOVA, F= 4.2082, df=7, p=0.0013). The two ID hylids and *Eleutherodactylus stejnegerianus* (DD) were the only frogs that secreted more than 300µg/g of peptide regardless of developmental strategy. Costa Rican salamanders had higher peptide concentrations (376.29µg/g-6298.33µg/g) than frogs (29.79µg/g-614.78µg/g) (ANOVA, F=5.089, df=1, p=0.0278). All Costa Rican amphibians with DD had similar peptide concentrations (ANOVA, F=2.5965, df=4, p=0.0596), but frogs with ID had significantly different variation between species (ANOVA, F=3.2498, df=4, p=0.0236). Phylogenetic patterns were also observed. Leptodactylids didn't vary significantly in peptide concentrations among species (ANOVA, F=2.1130, df=4, p=0.1134). Peptide concentrations of leptodactylids with only DD (all in Genus *Eleutherodactylus*) had significant variation among species (ANOVA, F=4.1288, df=2, p=0.0493), this peptide variability was not present with the two leptodactylids with ID (ANOVA, F=0.1784, df=1, p=0.6796). More phylogenetic relationships in peptide content were found in related species. Peptide contents of glass frogs *Cochranella granulosa* (n=1) and *Centrolene prosoblepon* (n=1) were inside the 95% confidence interval for *Hyalinobatrachium pulveratum* (n=3); so centrolenids seemed to represent a significant group. The two hylid frogs (*Hyla microcephala* and *Hyla ebraccata*) were not different from each other (ANOVA, F= 0.1010, df=1, p=0.7543) and exhibited the highest peptide concentrations found in ID amphibians (Table 1, 2). Hylid frogs did not differ from

centrolenids (ANOVA, F= 3.2333, df=1, p=0.0852). Furthermore, peptide contents from centrolenids, and leptodactylids with ID did not differ significantly (ANOVA, F= 1.3071, df=1, p=0.2688), but leptodactylids with ID were significantly different from peptide concentrations secreted by hylids (ANOVA, F= 14.6187, df=1, p=0.0005).

Peptide contents from all amphibians collected showed no significant differences by developmental strategy (Nested ANOVA, F=0.27, df=1, p=0.6066). It must be noted that on November 25th of 2005, the amphibian storage room at Appalachian State University Animal Care Facility suffered a flood that killed some salamanders and stressed all others before skin secretion induction. Geographical and developmental differences in peptide content were observed. Costa Rican species with ID (148.51µg-g-614.78µg/g) were significantly different from Appalachian ID species (49.18µg/g-428.51µg/g) (ANOVA, F= 9.4754, df=1, p=0.0030). On the other hand, Costa Rican and Appalachian species with DD did not produce significantly different peptide concentrations (ANOVA, F= 0.2342, df=1, p=0.6302). Furthermore, Costa Rican salamanders (all DD) and the DD *Plethodon* species from the Appalachians did not produce significantly different peptide concentrations in the skin secretions (ANOVA, F=1.3013, df=1, p=0.2600).

Table 1. Peptide content of Appalachian amphibians. Abbreviations are DD=direct development, ID=indirect development, PC=*Plethodon cylindraceus*, PM=*Plethodon montanus*, PY=*Plethodon yonahlossee*, DO=*Desmognathus orestes*, DQ=*Desmognathus quadramaculatus* and EW=*Eurycea wilderae.*

Developmental Strategy	Species	Sample size	Mean (µg/g)	Standard error	Minimum (µg/g)	Maximum (µg/g)
DD	PC	9	334.84	65.50	67.36	745.16
	PM	10	334.96	73.19	62.96	780.45
	PY	10	317.97	55.31	97.91	580.89
ID	DO	10	211.24	107.89	2.64	1156.50
	DQ	10	49.18	7.20	20.05	86.51
	EW	9	428.51	150.30	15.04	1155.00

Table 2. Peptide content of Costa Rican amphibians. Abbreviations are DD=direct development, ID=indirect development, BoSp=*Bolitoglossa* species, BoNi=*Bolitoglossa nigrescens*, BoPe= *Bolitoglossa pesrubra*, ElDi=*Eleutherodactylus diastema*, ElFi=*Eleutherodactylus fitzingeri*, ElSt=*Eleutherodactylus stejnegerianus*, ElCr=*Eleutherodactylus cruentus*, OU= *Oedipina uniformis*, CoGr= *Cochranella granulosa*, CePr=*Centrolenella prosoblepon*, HM= *Hyla microcephala*, HyEb= *Hyla ebraccata*, HyPu= *Hyalinobatrachium pulveratum*, LeBo=*Leptodactylus bolivianus*, PhPu=*Physalaemus pustulosus*, PhVi= *Phyllobates vittatus*, CoTa= *Colostethus talamancae* and DeAu=*Dendrobates auratus*.

Developmental Strategy	Species	Sample size	Mean (µg/g)	Standard error	Minimum (µg/g)	Maximum (µg/g)
DD	BoSp.	1	376.29	NA	NA	NA
	BoNi	1	6298.33	NA	NA	NA
	BoPe	9	1262.85	646.49	21.09	6195.65
	OU	9	671.62	244.03	82.86	1795.00
	ElDi	2	68.34	27.76	40.59	96.10
	ElFi	5	84.99	32.09	9.04	170.75
	ElSt	6	341.75	120.28	135.00	876.00
	ElCr	1	29.79	NA	NA	NA
ID	CoGr	1	182.14	NA	NA	NA
	CePr	1	225.11	NA	NA	NA
	HyPu	3	170.54	19.57	134.02	201.00
	HyEb	10	614.78	173.99	105.91	1567.78
	HM	10	447.31	104.93	104.29	116.84
	LeBo	4	163.71	81.86	5.75	485.98
	PhPu	10	148.51	28.85	47.54	339.19
Not used (dendrobatids)	PhVi	1	180.50	NA	NA	NA
	CoTa	1	13.37	NA	NA	NA
	DeAu	1	117.90	NA	NA	NA

Chytrid Growth Inhibition Assay

Sixteen species provided enough peptides to perform BD inhibition assays. Both developmental patterns and regions were represented. Ten amphibian species assayed for BD inhibition were from Costa Rica (two DD frogs, five ID frogs and three DD salamanders) and six species were from the Appalachians (three DD and three ID salamanders) (Table 3). Chytrid inhibition assays were complicated by unexpected results and high inherent variability. No consistent patterns of inhibition were evident until day seven when most species exhibited inhibition of BD growth at least at one peptide concentration. Secretions of three Costa Rican species (*Eleutherodactylus stejnegerianus, Hyalinobatrachium pulveratum* and *Bolitoglossa nigrescens*) did not inhibit BD growth at any concentration. The single specimen of *Bolitoglossa nigrescens* was damaged during collection and produced huge quantities of peptide but no inhibition. Five of seven frogs showed some inhibition of BD.

One pattern of inhibition was best illustrated by data for *Hyla microcephala*, a Costa Rican frog with ID. While all concentrations of peptide produced significant inhibition (OD significantly lower than positive controls) magnitude of this effect increased with peptide concentration (Figure 8). The lowest possible peptide concentration (3.06μg/ml) in this case was also the MIC. *Leptodactylus bolivianus*, a larger ID leptodactylid did not inhibit BD growth until its MIC of 6.25μg/ml, but the inhibition persisted over five concentrations to 125μg/ml (Figure 9). For this species the highest concentration of peptide stimulated growth of BD. This pattern of enhanced growth of BD at higher concentrations of peptides was observed in seven additional species (*Plethodon cylindraceus*, *Plethodon montanus,*

Desmognathus quadramaculatus, Oedipina uniformis, Eleutherodactylus stejnegerianus, Hyla ebraccata, and *Hyalinobatrachium pulveratum*) (Table 3).

All Appalachian species inhibited growth of BD in at least one peptide concentration. All three *Plethodon* species inhibited growth of BD at peptide concentration of 50μg/ml, but only *Plethodon montanus* inhibited the fungus at the lower concentrations of 3.06μg/ml, and 6.25μg/ml. *Plethodon montanus* was also the only *Plethodon* that inhibited BD growth at more than one concentration of peptides (3.06, 6.25, and 50μg/ml). *Plethodon yonahlossee* inhibited BD at only 50μg/ml of peptide and *P. cylindraceus* inhibited BD growth at 50μg/ml and 125μg/ml. All three ID salamanders assayed also inhibited growth of BD. *Desmognathus quadramaculatus* had the highest MIC (12.5μg/ml) and it was also the only inhibitory peptide concentration for this species. *Desmognathus orestes* inhibited BD growth at two concentrations only, one being the lowest possible (3.06μg/ml) and the other 125μg/ml. *Eurycea wilderae* was the Appalachian amphibian with the strongest inhibitory response with a MIC of 3.06μg/ml but also with inhibitory responses at peptide concentrations of 25, 50 and 125μg/ml (Table 3).

Costa Rican amphibians with inhibitory responses against BD, MIC's ranged from 3.06μg/ml for the ID frog *Hyla microcephala* to 25μg/ml for the DD frog *Eleutherodactylus fitzingeri*. Two salamanders (*Oedipina uniformis* and *Bolitoglossa pesrubra*) inhibited BD growth at the lowest possible peptide concentration (3.06μg/ml). Besides inhibiting at the lowest peptide concentrations, *Oedipina uniformis* also inhibited at 6.25μg/ml while *Bolitoglossa pesrubra* also inhibited at125μg/ml. *Eleutherodactylus fitzingeri* was the only DD frog that presented significant inhibitory responses to BD growth with only two inhibitory peptide concentrations 25 and 125μg/ml. Inhibition at no more than three peptide

concentrations seems to be a pattern shared by all DD amphibians assayed from both countries. *Hyla ebraccata* had the MIC at the lowest peptide concentration (3.06µg/ml), but BD growth was stimulated from 50µg/ml to 250µg/ml. This inhibition pattern by *Hyla ebraccata* was not shared by the other hylid assayed (*Hyla microcephala*), which inhibited BD growth at all concentrations. *Physalaemus pustulosus*, the other ID leptodactylid assayed, had inhibitory responses against BD at three concentrations (12.5, 25, 50µg/ml). Both ID leptodactylids (*Physalaemus pustulosus* and *Leptodactylus bolivianus*) showed inhibitory responses against BD growth with MIC's of 12.5µg/ml and 6.12µg/ml respectively (Table 3).

Table 3. Results of *Batrachochytrium dendrobatidis* inhibition assays. Symbols are (+) for significant inhibition (t-test), (0) for no difference between treatment and positive control, and (-) for enhanced BD growth. Minimum inhibitory concentration (MIC) is the lowest concentration with inhibition for each species. Abbreviations: DD=direct development; ID=indirect development; PC=*Plethodon cylindraceus*; PM=*Plethodon montanus*; PY=*Plethodon yonahlossee*; DO=*Desmognathus orestes*; DQ=*Desmognathus quadramaculatus* and EW=*Eurycea wilderae*; BoSp= *Bolitoglossa* species; BoNi=*Bolitoglossa nigrescens*; BoPe=*Bolitoglossa pesrubra*; ElDi=*Eleutherodactylus diastema*; ElFi=*Eleutherodactylus fitzingeri*; ElSt=*Eleutherodactylus stejnegerianus*; ElCr=*Eleutherodactylus cruentus*; OU=*Oedipina uniformis*; CoGr= *Cochranella granulosa*; CePr=*Centrolene prosoblepon*; HM= *Hyla microcephala*; HyEb= *Hyla ebraccata*; HyPu=*Hyalinobatrachium pulveratum*; LeBo=*Leptodactylus bolivianus*; PhPu=*Physalaemus pustulosus*.

Species	Origin	Development	Peptide concentrations in µg/ml						
			3.06	6.25	12.5	25	50	125	250
PC	USA	DD	0	0	0	0	+	+	-
PM	USA	DD	+	+	0	0	+	0	-
PY	USA	DD	-	0	0	0	+	0	0
DO	USA	ID	+	0	0	0	0	+	0
DQ	USA	ID	0	0	+	0	0	-	-
EW	USA	ID	+	0	0	+	+	+	0
OU	CR	DD	+	+	0	0	-	-	-
BoNi	CR	DD	0	0	0	0	0	0	0
BoPe	CR	DD	+	0	-	0	-	+	0
ElFi	CR	DD	0	0	0	+	0	+	0
ElSt	CR	DD	0	0	0	0	-	0	-
HyMi	CR	ID	+	+	+	+	+	+	+
HyEb	CR	ID	+	0	0	0	-	-	-
LeBo	CR	ID	0	+	+	+	+	+	-
PhPu	CR	ID	-	0	+	+	+	0	0
HyPu	CR	ID	0	0	0	0	0	0	-

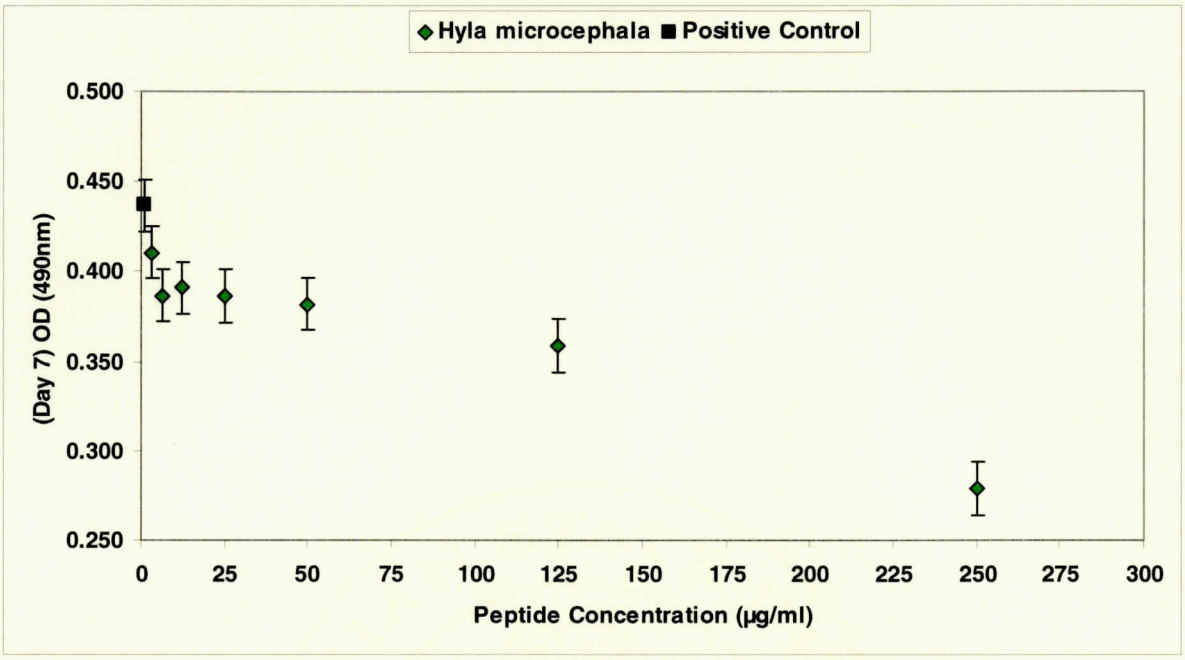

Figure 8. Chytrid growth inhibition for *Hyla microcephala*. Green color indicates significant inhibition (*t*-test). Standard errors of the means are indicated with error bars. Minimal inhibitory concentration (MIC) is the lowest concentration with significant inhibition.

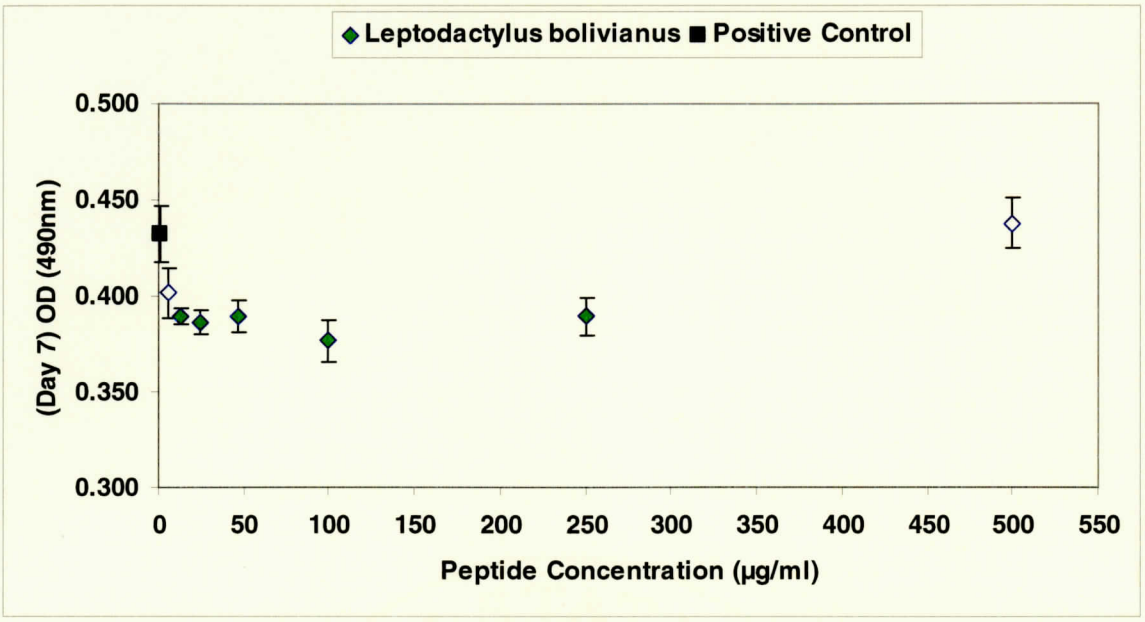

Figure 9. Chytrid growth inhibition for *Leptodactylus bolivianus*. Green color indicates significant inhibition (*t*-test). Standard errors of the means are indicated with error bars. Minimal inhibitory concentration (MIC) is the lowest concentration with significant inhibition.

Minimal inhibitory concentrations for the peptide secretions of all amphibians used for inhibition assays did not vary by country (Mann Whitney *U* critical=27<*U*a=16 and <*U*b=14) or development strategy (Mann Whitney *U* critical=27<*U*a=8 and <*U*b=22). Peptide content per species showed a negative correlation with MIC's for the ID amphibians (r^2=0.7025, p=0.0185, n=7) (Figure 12). Peptide content of DD amphibians was not significantly correlated with MIC (r^2=0.1803, p=0.7186, n=6). Peptide concentrations and MIC's by species are summarized in Figure 13. *Bolitoglossa nigrescens* was not included in this figure because no inhibition occurred and the only peptide concentration available (6298.33µg/g) was associated with exceptional injury during collection.

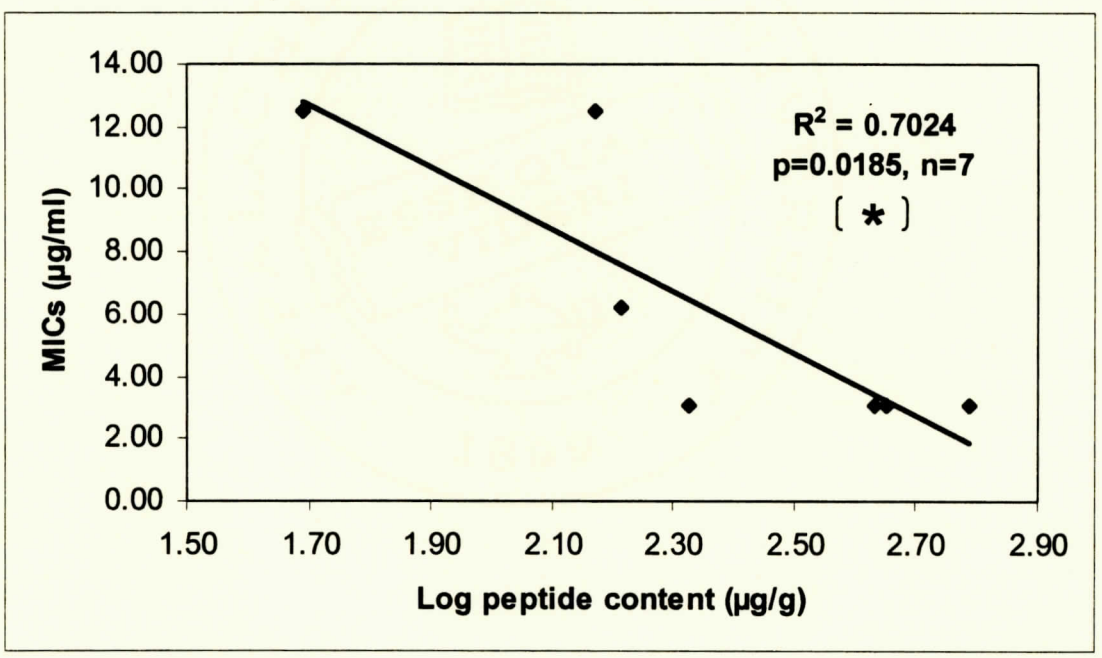

Figure 10. Regression analysis for minimal inhibitory concentrations (MIC) and log of peptide content for ID species.

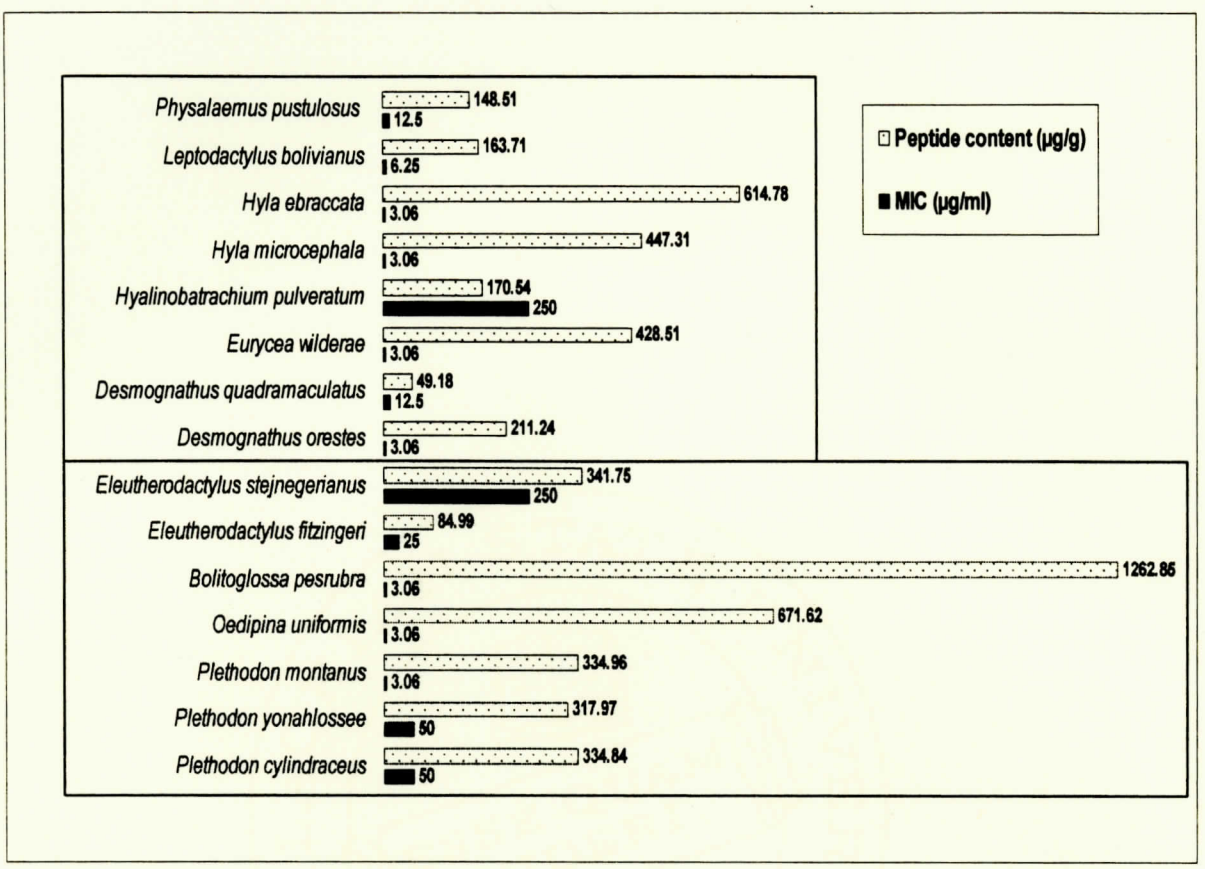

Figure 11. Peptide content and minimal inhibitory concentration (MIC) against *Batrachochytrium dendrobatidis* for 15 species of amphibians. Gray box encloses species with direct development (DD) and white box represent species with indirect development (ID). Black columns are MIC (µg/ml) to BD and white dotted columns represent the peptide content.

DISCUSSION

Aquatic environments are used by the huge numbers of potentially pathogenic organisms like bacteria, fungi and parasites (Todd, 2007). Escape from aquatic predators has been a classical explanation for why some amphibians became more terrestrial (Todd, 2007). However, pathogens may produce selective forces as strong as those related to predators (Todd, 2007). Selective forces related to pathogens can contribute to the evolution of the diversity of reproductive strategies of amphibians by increasing terrestrial habits (Todd, 2007). Modern amphibians display many attributes related to the transition between fully aquatic and fully terrestrial animals with direct development. The amphibian chytrid fungus *Batrachochytrium dendrobatidis* (BD) is one of many parasites of amphibians producing selective pressures on these animals in aquatic environments. Evolutionary responses to the pressures include the whole immune system and peptide secretions; but peptide contents in amphibian skin secretions also show evidence of selection independent from BD. Since predators and pathogens are selective agents, examination of poison gland secretions, quantity of peptides and specific activity of those peptides against pathogens among amphibians from different regions and with different life history strategies allow evaluation of the importance of pathogens in the history of amphibians.

Distribution of skin peptides was quite variable in the amphibians studied in the present study, but the values were not random and the null hypothesis of selective neutrality was not supported. Developmental strategy was an important factor in determining peptide

content for Appalachian salamanders. Surprisingly, salamanders with ID had less peptide than did DD salamanders. This life history difference supports the importance of developmental strategy in peptide content differences of amphibian skin secretions of the Appalachians and does not support the aquatic predator or parasite hypothesis. *Desmognathus quadramaculatus* (ID) had sufficiently low and constant quantity of peptide that it differed from the DD *Plethodon* species. The other two Appalachian species with ID (*Desmognathus orestes* and *Eurycea wilderae*) had individuals with very low and very high peptide content, so they did not actually differ from the uniform peptide content of the DD *Plethodon* species. The high and constant peptide concentration in skin secretions of the *Plethodon* species with DD are consistent with several evolutionary hypotheses involving either phylogenetic constraint or life history but not history of exposure to pathogens similar to BD. Peptide contents of *Plethodon* suggest a developmental pattern constraint on DD salamanders in the Appalachians.

Concentrations of skin peptides in Appalachian salamanders are consistent with strong selection related to the presence of other defensive mechanisms (Brodie, 1983). The *Plethodon* species with high concentrations of skin secretions also have noxious secretions as a defense mechanism against predators. The *Desmognathus* species with lower amounts of skin secretions is associated with a lack of noxious or lethal secretions as defense mechanisms (Brodie, 1983). *D. quadramaculatus* is a robust and common stream dwelling salamander. This salamander was often parasitized by leeches which may produce injury that stimulate release of gland contents. Replacement of gland contents is slow (Rollins-Smith *et al.*, 2005a,b). So injury by leeches could produce the low peptide amounts in this salamander. Skin secretions of *D. quadramaculatus* were easily diluted in the laboratory (Personal

observation). So a similar dilution of peptide in the natural habitat would greatly reduce effectiveness of peptides (Rollins-Smith *et al.*, 2005a,b). If secretions by *D. quadramaculatus* are easily diluted in the environment as well as in the laboratory, low quantities of peptide content probably reflect overall effectiveness of skin secretions in this species. *Desmognathus orestes* spends more time on land and might have fewer problems with dilution of peptides. Higher peptide concentrations secreted by this species are in agreement with the time the animal spends inland.

Peptide content in all the salamanders seems to support the phylogenetic constraint hypothesis. The high and similar peptide content of species with DD would be expected if peptide content is a trait inherited from a common ancestor and maintained by selection. Regional differences do not seem to be important for the salamanders. As a recently derived family (Chippindale *et al.*, 2004), less peptide content variability should be expected among species. Evolution of these salamanders suggests a selective advantage in the DD members of the family with high peptide content. This constraint seemed to extend to plethodontids with ID as well. As a group, ID species produced less peptide than DD salamanders and more than ID frogs from Costa Rica. In general, salamanders secreted more peptide than did frogs; so the comparison of life history types between the two regions confounded two sources of variation. Salamanders from Genus *Desmognathus* seem to be producing both the geographical and phylogenetic differences in ID amphibians as they did for the comparison of reproductive types in the Appalachians. *Desmognathus* is usually placed in a separate subfamily from the rest of the plethodontids (Chippindale *et al.*, 2004), but the genus represents a rather divergent and early split in the family.

In Family Plethodontidae, salamanders from Genus *Desmognathus* had a reversal in evolution of their developmental pattern. These salamanders were previously DD amphibians that retained their larval hyobranchial apparatus in the egg. This apparatus is a key feature for aquatic respiration and feeding. Retention of this trait made it possible for these salamanders to re-evolve the indirect developmental pattern. Other members of the family have very reduced hyobranchial apparatus structures which would require re-evolution of these structures before a change in ID pattern. This reversal in life history for *Desmognathus* was probably driven by selective pressure from high diversity of sympatric DD members of the family in the Appalachians (Chippindale *et al.*, 2004). Both *Desmognathus* species secreted significantly less peptide than did Costa Rican salamanders (all DD) or DD Appalachian *Plethodon*. Perhaps the difference is related to this reversal in evolution for *Desmognathus*. Once again, phylogenetic constraints in peptide concentrations seem to be supported by these data. The biological role of peptide content in skin secretions of salamanders from Genus *Desmognathus* is unknown, but the wide ecological range of species in this genus suggests that further study of this genus should provide valuable data and insights into the evolutionary history of this group.

When the amphibians were injected with norepinephrine, the needle penetrating the skin was sometimes enough stimulus to produce skin secretions. Peptide secretions are regulated by the sympathetic nervous system. Thus, alarm responses are necessary triggers for secretion of poison gland contents. Also, magnitude of the alarm stimulus results in more peptide content in the skin secretions (Rollins-Smith *et al.*, 2005b). In nature, predator attacks prior to collection can cause the glands to secrete their contents, which can translate into less peptide collected by the researcher and high intraspecific variability. Storage of

Appalachian amphibians was done to allow poison gland recovery, as indicated for *Xenopus laevis* (Rollins-Smith *et al.*, 2005b). The smallest Appalachian salamanders *Eurycea wilderae* and *Desmognathus orestes* were the most variable in peptide content. This variability in peptide content of these two salamanders could be the result of difficult handling for NE injection and more stress caused to the animal. Smaller animals were harder to keep still for clean injection, making the process more invasive and some of the individuals more defensive (alarm/injury response). When this happened copious secretions were observed which supports the proportional relationship between intensity of alarm responses and quantity of peptide secreted (Rollins-Smith *et al.*, 2005b). The flood of November of 2005 killed some salamanders and flooded the rest for at least a day. A container full of water for a day might be enough stress to cause an alarm response in some animals. In the present study, storage of the animals did not control for gland recovery between collection of the animal in the field and treatment in the lab. However, storage of animals should be a necessary addition to the protocol whenever feasible. If recovery time of glands is controlled, skin secretions might have more realistic peptide concentrations and less intraspecific variability.

Peptide concentrations in the skin secretions of Costa Rican amphibians are also variable. Once again, distribution of peptides is not random or uniform. The developmental pattern is not related to peptide content unless amphibians are separated into smaller phylogenetic groups. All Costa Rican salamanders belong to the same subfamily and tribe of Plethodontidae which is a successful of salamanders with a relatively recent origin in the Cretaceous Period about 100 million years ago (Chippindale *et al.*, 2004). Recent evolution of Plethodontidae suggests that all members should share similar peptide content. Peptide

concentrations from Costa Rican salamanders are consistent with presence of phylogenetic constraint in peptide content. All Costa Rican salamanders exhibit DD and have massive quantities of peptide secretions, as seems to be the tendency in all DD salamanders of this family. Costa Rican salamanders also produce significantly higher peptide concentrations than did frogs of either developmental type. These differences support presence of a phylogenetic constraint of increased peptide content in DD salamanders in Family Plethodontidae.

Peptide concentrations of the skin secretions of frogs vary significantly among three phylogenetic families. Three of eleven frog species have secretions with more peptide than 300μg/g. These three include both hylid frogs with ID and *Eleutherodactylus stejnegerianus* with DD. These values may show a phylogenetic increase in peptide concentrations for the two hylids. Frogs with ID differ among species and belong to three different families, so tests of phylogenetic hypotheses are possible. All six leptodactylids have similar peptide concentrations even though the family included four DD frogs and also two ID frogs. However, if leptodactylids are subdivided by developmental strategy, four species of *Eleutherodactylus* (DD) vary significantly in peptide contents while the two ID species did not. Three *Eleutherodactylus* secreted no more than 90μg/g of peptide, while *Eleutherodactylus stejnegerianus* produced more than 300μg/g. *Eleutherodactylus stejnegerianus* seems to be explaining most of the variation in the data for this family. This difference in peptide content demonstrates that any constraint of peptide distribution in the lineage ca be overcome. Some other factor affecting the DD frogs makes them produce low peptide content in some species and high in others. Different skin defense mechanisms against predators and pathogens could produce these differences in this lineage of frogs.

The peptide concentrations secreted by the three glass frogs (Family Centrolenidae) were uniform and similar. Also, both hylid frogs had uniform peptide contents with no significant differences. The three families of ID frogs (Centrolenidae, Hylidae and ID Leptodactylidae) had no significant differences among species within families. These similarities within species of the same families support the phylogenetic constraint hypothesis in peptide concentrations of the skin secretions. Only the leptodactylids with DD were inconsistent with expectations of this hypothesis. The significant difference between the two hylids and the two ID leptodactylids support the phylogenetic constraint hypothesis so peptide contents could offer a tool for phylogenetic analysis. Peptide secretions are highly conserved (Apponyi *et al.*, 2004) and results for these three families of amphibians support phylogenetic constraint for this trait.

Three poison dart frogs (Family Dendrobatidae) had sample sizes of one but varied dramatically in peptide content. The non-poisonous *Colostethus talamancae* secreted only 13 µg/g peptide compared to 181µg/g for *Phyllobates vittatus* and 118µg/g for *Dendrobates auratus* (Table 2). It is interesting to note that level of skin secretion toxicity (*Phyllobates* > *Dendrobates* > *Colostethus*) is the same as order of quantity of peptide released. It would be both interesting and important to determine whether this correlation between toxicity and peptide content of the skin secretions holds for other species in this family.

Costa Rican ID amphibians differed from Appalachian ID species by varying significantly among species. In the Appalachians all species were salamanders in the same family. For Costa Rica, frogs were included but also more species and phylogenetic groups were assayed that increased peptide content variability and the chances of observing phylogenetic differences. Costa Rican DD species had more peptides than did Appalachian

DD species, but the difference was not statistically significant. Lack of significance was probably related to the high variability in the Costar Rican DD amphibians (include *Bolitoglossa nigrescens* (n=1) with highest peptide concentration obtained, *Eleutherodactylus stejnegerianus* with the highest peptide concentration obtained from DD frogs and three *Eleutherodactylus* frogs with very low peptide concentrations). *Bolitoglossa nigrescens* was seriously injured during collection. The high peptide concentration excreted by this individual strongly suggests a relationship with a strong alarm response caused by injury. Magnitude of alarm responses are related to high peptide concentrations in the skin secretions (Rollins-Smith *et al.*, 2005b). This relationship could also be the answer for *Eleutherodactylus stejnegerianus* high peptide concentration. This was the smallest amphibian used, and as discussed for *Eurycea wilderae* and *D. orestes*, process of induction of peptide secretions for small amphibians was very invasive. Induction of peptide secretions for *Eleutherodactylus stejnegerianus* was done in the field, and several individuals were injured during the process. When injury occurred, copious skin secretions were observed similar to other small amphibians used in this study. However, during NE injection, as soon as the skin was first crossed by the needle copious secretions were observed in most amphibians. Stronger injury stimulus in small amphibians in addition to NE seems to be the cause for increased peptide concentration in *Eleutherodactylus stejnegerianus.*

The anti-chytrid activity of the peptides is more complicated but supports some types of phylogenetic constraints of the peptide content. Eight salamanders and five frogs inhibited BD growth at least at one peptide concentration. All Appalachian species had some inhibition to chytrids while three Costa Rican species did not produce inhibitory response. For Appalachian salamanders, the inhibitory effect was not supportive of the phylogenetic

constraint in peptide contents for members of Family Plethodontidae. While the peptide contents for the three *Plethodon* species were very uniform, inhibition was strong only in *Plethodon montanus.* For ID plethodontids the more aquatic *Desmognathus quadramaculatus* had only one inhibitory concentration which was also the species MIC (12.5μg/ml). For ID species *Desmognathus orestes* and *Eurycea wilderae,* MIC's were at the lowest inhibitory concentration possible (3.06μg/ml). Difference in inhibition for species with ID and DD of family Plethodontidae seems to be related to each species life history of exposure to pathogens. No real pattern in inhibition was observed for plethodontids in the Appalachians, which argues the phylogenetic constraint hypothesis for antimicrobial activity in Family Plethodontidae.

Low inhibition in *Desmognathus quadramaculatus*, a species without noxious secretions (Brodie, 1983), agrees with the low concentration of peptides found in this species. In contrast, *D. orestes* had higher peptide concentrations than *D. quadramaculatus* and a low MIC. This suggests that life history in *Desmognathus* has a great influence on antimicrobial activity and concentration of peptides. Thus peptide content and antimicrobial activity could be species dependent in these salamanders which could be related to the reversal in life history in *Desmognathus.* Reversal in life history of members of this genus may reflect an escape from interspecific pressure in terrestrial environments (Brodie, 1983). More terrestrial *Desmognathus orestes* seems to reflect progression in peptide content related to proximity to terrestrial habitats. By retaining secretions that have potential to be noxious to more predators in a terrestrial environment, species like *Desmognathus orestes* can explore inland habitats more often. On the other hand, the more aquatic *D. quadramaculatus* has decreased terrestrial predator pressure suggesting that also skin secretions were lost for defense. This

loss in skin secretions is supported by low peptide contents and high MIC for *D. quadramaculatus*. If the peptide content and antimicrobial activity of the skin secretions of *Desmognathus* have a relationship with toxicity of the secretions to predators, then maybe some members of the genus do posses noxious secretions.

Costa Rican plethodontids had similar variation in inhibitory capacity of BD to Appalachian plethodontids. Two species (*Oedipina uniformis* and *Bolitoglossa pesrubra*) had strong inhibition of BD at 3.06μg/ml. However, *Bolitoglossa nigrescens* did not inhibit growth of BD. The inhibitory action of the Appalachian salamanders and the inhibition results for these three Costa Rican salamanders support the phylogenetic constraint hypothesis for antimicrobial activity in Family Plethodontidae. Peptide content seems to be constrained in phylogenetic groups but inhibition seems to depend on other factors. One factor of variation in inhibitory capacity of peptide secretions of closely related species could be related to different histories of exposure to pathogens and predators by each species. However, different peptide families are present in the skin secretions of amphibians which seem to make peptide mixtures unique for each source species. Peptide inhibitory capacity can change with the peptide structure. Changes in peptide structure also produce differences in inhibition that can include or exclude some pathogens. Species with low inhibitory action to BD may reflect a history without exposure to pathogens similar to BD.

Peptide secreted by frogs with DD (Genus *Eleutherodactylus*) had different responses to BD. *Eleutherodactylus stejnegerianus* did not inhibit BD growth and *E. fitzingeri* had a high MIC of 25μg/ml. Inhibition again seems to be an effect of specific history of exposure to pathogens similar to BD. *Eleutherodactylus stejnegerianus* is a species restricted to terrestrial leaf litter habitats that decreases exposure to aquatic pathogens like BD. High

peptide concentrations and no inhibitory response for this species suggest no history of exposure to this sort of pathogen and loss of antimicrobial peptide defenses. *E. fitzingeri* can be found on rocks of small streams which increases exposure to aquatic pathogens like BD. This frog's behavior could be the reason for this retention of antimicrobial peptides in this species.

Phylogenetic constraints in peptide content were supported by the anti-chytrid response of the peptides in hylids. The two hylid frogs (*Hyla microcephala* and *H. ebraccata*) had similar peptide contents and both species MIC's were at the lowest peptide concentration (3.06µg/ml) (Table 3). However the pattern of inhibition was different for both species. *Hyla ebraccata* inhibited BD growth only at its MIC of 3.06µg/ml while *H. microcephala* inhibited BD growth at all peptide concentrations. Why these two closely related, often sympatric frogs have such different inhibitory patterns is uncertain. The two have similar peptide concentrations but different inhibition profiles, so they probably have different arrays of peptide. Even if peptide characterizations were not made for the skin secretions collected for this study, over 100 different peptides have been characterized from hylid frogs. Results of inhibition to BD for *Hyla microcephala* (Figure 8), suggests that novel peptide families could be present in this species. Also some of the peptides already characterized with strong inhibitory action might be present in this species suggesting some phylogenetic constraint in antimicrobial peptides for this frog family.

For ID leptodactylids, the inhibitory response of the peptide secretions did not support the phylogenetic constraint showed in peptide contents. *Physalaemus pustulosus* inhibitory reaction of the peptide mixture to BD was low with a high MIC of 12.5µg/ml. Inhibition of BD growth by peptide secreted by *Leptodactylus bolivianus* was strong with a

MIC of 6.25µg/ml. This inhibitory capacity for leptodactylids with ID suggests once again a difference in life history of exposure to pathogens similar to BD by each species. As in hylids, novel peptides could be present in these two species.

Hyalinobatrachium pulveratum was the only glass frog assayed and was the only ID species without inhibition. This result contrasts with those of Woodhams *et al.*, (2006b), who found inhibition in two species of glass frogs. This contrast in glass frogs could be showing a species dependent susceptibility to infection from pathogens like BD. Selective pressure in by exposure to this kind of pathogens might increase survival of glass frogs with strong inhibition in the near future. Also phylogenetic differences might be present in this lineage of frogs. The two glass frogs assayed by Woodhams *et al.*, (2006b) were from Genus *Centrolene* while the glass frog assayed from Costa Rica is in Genus *Hyalinobatrachium.* This difference between the present study and Woodhams *et al.*, (2006b) suggests that phylogenetic constraint explains skin secretions of all Centrolenidae. It would be interesting to assay more glass frogs to see if phylogenetic differences are present in the group.

Most species (fifteen of sixteen) did not inhibit BD growth at all peptide concentrations. For eleven of sixteen species some concentrations of peptides actually enhanced growth of the pathogen. I suggest that some peptides in the mixture secreted could be present for enhancing growth of skin flora (bacteria) that can inhibit pathogen growth. Harris *et al.*, (2006) isolated skin bacteria from two salamanders, one with ID and another with DD, which inhibited growth of BD. If this is true in most amphibians, when the skin bacteria are not present the secretions may increase growth of pathogens. This could explain why enhanced growth of BD was present in several peptide concentrations and why in nature

some amphibians seem to be more susceptible to the infection of BD even if antimicrobial peptides are present.

Amphibians with ID exhibited different patterns of inhibition than did those with DD. Inhibition is strong in five of the six species with ID with low MIC's (3.06μg/ml and 6.25μg/ml). In four of these species when inhibition occurred, the response was persistent over more than two peptide concentrations. Selective pressure from pathogens like BD produces retention over time of these attributes in these amphibians that have a higher risk of infection by exposure to waterborne pathogens in their habitats. However, strong inhibition was observed in DD and ID amphibians of both regions. Inhibitory responses by amphibians from different phylogenetic groups with different developmental strategies suggest that antimicrobial peptide distribution is a product of selection related to the species history of exposure to pathogens similar to BD.

The MIC's of the species from Costa Rica were not significantly different than those of the Appalachian species. Also, MIC's were not significantly different for amphibians with different developmental strategies. However, if pathogens similar to BD favor amphibians with ID, then antimicrobial activity and peptide content should be related attributes of the innate immune system that should increase together in order to clear the skin of infection. The MIC's for the ID species had a significant relationship with the peptide concentration in the skin secretions (Figure 10). When the peptide concentration increased the MIC decreased, producing more inhibitory response against BD (Figure 10). This result supports the hypothesis that ID species have strong antimicrobial peptides as a product of exposure to aquatic pathogens similar to BD. However, higher amounts of peptides with strong inhibitory capacity were observed in DD species. It is uncertain if the sympathetic nervous system

recognizes the infection of the skin by BD and triggers an alarm response. Nevertheless, evidence shown in this study supports the hypothesis that for ID species the amount of peptide secreted can predict the magnitude of the inhibitory response against pathogens like BD.

In conclusion, selective pressure from waterborne pathogens like BD seems to be shaping the skin peptide defenses of ID species over time. This peptide content-inhibition relationship was not significant for DD species, supporting the idea that peptide secretions are not a product of selective pressures for waterborne pathogens like BD. Significant regression between MIC's and peptide contents of the skin secretions of ID amphibians suggest different roles of peptides associated with developmental strategy. These relationships have to be the result of evolutionary history of exposure to waterborne pathogens like BD. Phylogenetic patterns in peptide content were evident but not supported in most cases by the inhibitory capacity of the peptide mixtures. Patterns of inhibition to BD and peptide contents seem to reflect different selective pressures on each species. Some inhibition patterns where BD growth was enhanced suggest that peptides could have a role of enhancing growth of skin flora which can inhibit skin pathogens like BD. However this relationship of peptides with skin flora is just a speculation that needs research attention. Peptide mixtures with strong antimicrobial activity against BD are present in ID and DD species of both regions which suggests that the amphibian chytrid fungus is indeed an emerging disease. We do not know if amphibians use these peptides in nature against pathogens like BD. Further study is needed to clarify the function of the innate immune system in nature. If amphibians use their peptide secretions against BD infection, then further research of these peptide will help us understand amphibian population declines.

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APPENDIX A

Recipes for solutions used in the study

H Broth liquid media:

1g tryptone (Sigma-Aldrich T9410)
0.32g of D-(+)-glucose (Sigma-Aldrich G7021)
100ml of distilled water

TGHL agar media:

16g tryptone (source ?)
4g gelatin hydrolysate enzymatic (Sigma-Aldrich G0262)
2g α-lactose monohydrate (Sigma-Aldrich L2643)
15g agar (Sigma Aldrich A1296)
100ml distilled water

Norepinephrine solution:

13.5mg of DL-Norepinephrine Hydrochloride (Sigma Aldrich A7256)
40ml of Amphibian Phosphate-Buffered Saline (APBS)

Amphibian Phosphate-Buffered Saline (APBS):

6.6g NaCl (Sigma-Aldrich S-3014)
1.15g Na_2HPO_4 anhydrous (JT Baker 4062-01) or 2.17g $NaHPO_4 \cdot 7\ H_2O$ (EM Science 8290) 02g KH_2PO_4 (Sigma-Aldrich P-5379)
1L DD H_20)

Collecting Buffer:

2.92g NaCl (Sigma-Aldrich S-3014)
2.05g Na Acetate (Sigma-Aldrich S-2889)
1L of HPLC water

Buffer A:

1ml HCl (%)
1L water Chromasol V for HPLC (Sigma-Aldrich 270733)??

Buffer B:

350ml Acetonitrile
150ml HPLC-grade water
1ml HCl 50%

APPENDIX B

Costa Rican Field Collections

August 2006

Family	Genus	Species	Province	Location	Elevation (m)	Code
Centrolenidae	*Centrolenella*	*prosoblepon*	Cartago	Tapanti 1, near headquarters		CP2
Centrolenidae	*Cochranella*	*granulosa*	Puntarenas	Rio Piro	81	CoGr
Centrolenidae	*Cochranella*	*granulosa*	Puntarenas	Rio Piro	81	CoGr
Centrolenidae	*Hyalinobatrachium*	*pulveratum*	Puntarenas	Rio Piro	81	HyPu
Centrolenidae	*Hyalinobatrachium*	*pulveratum*	Puntarenas	Rio Piro	81	HyPu
Centrolenidae	*Hyalinobatrachium*	*pulveratum*	Puntarenas	Rio Piro	81	HyPu
Dendrobatidae	*Colostethus*	*talamancae*	Puntarenas	Rio Piro	81	CoTa
Dendrobatidae	*Colostethus*	*talamancae*	Puntarenas	Rio Piro	81	Cosp
Dendrobatidae	*Dendrobates*	*auratus*	Puntarenas	Rio Piro	81	DeAu
Dendrobatidae	*Phyllobates*	*vittatus*	Puntarenas	Rio Piro	81	PhVi
Hylidae	*Hyla*	*ebraccata*	Puntarenas	Rio Piro	81	HyEb
Hylidae	*Hyla*	*ebraccata*	Puntarenas	Rio Piro	81	HyEb
Hylidae	*Hyla*	*ebraccata*	Puntarenas	Rio Piro	81	HyEb
Hylidae	*Hyla*	*ebraccata*	Puntarenas	Rio Piro	81	HyEb
Hylidae	*Hyla*	*ebraccata*	Puntarenas	Rio Piro	81	HyEb
Hylidae	*Hyla*	*ebraccata*	Puntarenas	Rio Piro	81	HyEb
Hylidae	*Hyla*	*ebraccata*	Puntarenas	Rio Piro	81	HyEb
Hylidae	*Hyla*	*ebraccata*	Puntarenas	Rio Piro	81	HyEb
Hylidae	*Hyla*	*ebraccata*	Puntarenas	Rio Piro	81	HyEb
Hylidae	*Hyla*	*ebraccata*	Puntarenas	Rio Piro	81	HyEb
Hylidae	*Hyla*	*ebraccata*	Puntarenas	Rio Piro	81	HyEb
Hylidae	*Hyla*	*microcephala*	San Jose	Santa Ana	2856	HM1
Hylidae	*Hyla*	*microcephala*	San Jose	Santa Ana	2856	HM1
Hylidae	*Hyla*	*microcephala*	San Jose	Santa Ana	2856	HM1
Hylidae	*Hyla*	*microcephala*	San Jose	Santa Ana	2856	HM1
Hylidae	*Hyla*	*microcephala*	San Jose	Santa Ana	2856	HM1
Hylidae	*Hyla*	*microcephala*	San Jose	Santa Ana	2856	HM1
Hylidae	*Hyla*	*microcephala*	San Jose	Santa Ana	2856	HM1
Hylidae	*Hyla*	*microcephala*	San Jose	Santa Ana	2856	HM1
Hylidae	*Hyla*	*microcephala*	San Jose	Santa Ana	2856	HM1
Hylidae	*Hyla*	*microcephala*	San Jose	Santa Ana	2856	HM1
Hylidae	*Scinax*	*boulengeri*	Puntarenas	Rio Piro	81	ScBo
Hylidae	*Smilisca*	*phaeota*	Puntarenas	Rio Piro	81	SmPh
Leptodactylidae	*Eleutherodactylus*	*diastema*	Cartago	Tapanti 2, near Mirador		ED1
Leptodactylidae	*Eleutherodactylus*	*diastema*	Cartago	Tapanti 3, Junction of rios		ED2
Leptodactylidae	*Eleutherodactylus*	*fitzingeri*	Puntarenas	Rio Piro	81	ElFi
Leptodactylidae	*Eleutherodactylus*	*fitzingeri*	Puntarenas	Rio Piro	81	ElFi
Leptodactylidae	*Eleutherodactylus*	*fitzingeri*	Puntarenas	Rio Piro	81	ElFi
Leptodactylidae	*Eleutherodactylus*	*fitzingeri*	Puntarenas	Rio Piro	81	ElFi
Leptodactylidae	*Eleutherodactylus*	*melanostictus*	Cartago	Tapanti 4, Oropendμla Trail		ESP2
Leptodactylidae	*Eleutherodactylus*	*stejnegerianus*	Puntarenas	Rio Piro	81	Elst
Leptodactylidae	*Eleutherodactylus*	*stejnegerianus*	Puntarenas	Rio Piro	81	Elst
Leptodactylidae	*Eleutherodactylus*	*stejnegerianus*	Puntarenas	Rio Piro	81	Elst
Leptodactylidae	*Eleutherodactylus*	*stejnegerianus*	Puntarenas	Rio Piro	81	Elst
Leptodactylidae	*Eleutherodactylus*	*stejnegerianus*	Puntarenas	Rio Piro	81	Elst
Leptodactylidae	*Eleutherodactylus*	*stejnegerianus*	Puntarenas	Rio Piro	81	Elst

Leptodactylidae	*Eleutherodactylus*	*stejnegerianus*	Puntarenas	Rio Piro	81	Elst
Leptodactylidae	*Eleutherodactylus*	*stejnegerianus*	Puntarenas	Rio Piro	81	Elst
Leptodactylidae	*Eleutherodactylus*	*stejnegerianus*	Puntarenas	Rio Piro	81	Elst
Leptodactylidae	*Eleutherodactylus*	*stejnegerianus*	Puntarenas	Rio Piro	81	Elst
Leptodactylidae	*Leptodactylus*	*labialis*	San Jose	Santa Ana	2856	LeLa
Leptodactylidae	*Leptodactylus*	*pentadactylus*	Puntarenas	Rio Piro	81	LeBo
Leptodactylidae	*Leptodactylus*	*bolivianus*	Puntarenas	Rio Piro	81	PhPu
Leptodactylidae	*Physalaemus*	*pustulosus*	Puntarenas	Rio Piro	81	PhPu
Leptodactylidae	*Physalaemus*	*pustulosus*	Puntarenas	Rio Piro	81	PhPu
Leptodactylidae	*Physalaemus*	*pustulosus*	Puntarenas	Rio Piro	81	PhPu
Leptodactylidae	*Physalaemus*	*pustulosus*	Puntarenas	Rio Piro	81	PhPu
Leptodactylidae	*Physalaemus*	*pustulosus*	Puntarenas	Rio Piro	81	PhPu
Leptodactylidae	*Physalaemus*	*pustulosus*	Puntarenas	Rio Piro	81	PhPu
Leptodactylidae	*Physalaemus*	*pustulosus*	Puntarenas	Rio Piro	81	PhPu
Leptodactylidae	*Physalaemus*	*pustulosus*	Puntarenas	Rio Piro	81	PhPu
Leptodactylidae	*Physalaemus*	*pustulosus*	Puntarenas	Rio Piro	81	PhPu
Leptodactylidae	*Physalaemus*	*pustulosus*	Puntarenas	Rio Piro	81	PhPu
Plethodontidae	*Bolitoglossa*	*species*	Cartago	Tapanti 5, Esperanza Station		BoSp3
Plethodontidae	*Bolitoglossa*	*species*	Cartago	Tapanti 5, Esperanza Station		BoSp4
Plethodontidae	*Bolitoglossa*	*species*	Cartago	Tapanti 6, Los Santos		Bp1
Plethodontidae	*Bolitoglossa*	*species*	Cartago	Tapanti 6, Los Santos		Bp2
Plethodontidae	*Bolitoglossa*	*species*	Cartago	Tapanti 6, Los Santos		Bp3
Plethodontidae	*Bolitoglossa*	*species*	Cartago	Tapanti 6, Los Santos		Bp4
Plethodontidae	*Bolitoglossa*	*species*	Cartago	Tapanti 6, Los Santos		Bp5
Plethodontidae	*Bolitoglossa*	*species*	Cartago	Tapanti 6, Los Santos		Bp6
Plethodontidae	*Bolitoglossa*	*species*	Cartago	Tapanti 6, Los Santos		Bp7
Plethodontidae	*Bolitoglossa*	*species*	Cartago	Tapanti 6, Los Santos		Bp8
Plethodontidae	*Bolitoglossa*	*species*	Cartago	Tapanti 6, Los Santos		Bp9
Plethodontidae	*Oedipina*	*uniformis*	San Jose	Santa Ana	2856	OU1
Plethodontidae	*Oedipina*	*uniformis*	San Jose	Santa Ana	2856	OU2
Plethodontidae	*Oedipina*	*uniformis*	San Jose	Santa Ana	2856	OU3
Plethodontidae	*Oedipina*	*uniformis*	San Jose	Santa Ana	2856	OU4
Plethodontidae	*Oedipina*	*uniformis*	San Jose	Santa Ana	2856	OU5
Plethodontidae	*Oedipina*	*uniformis*	San Jose	Santa Ana	2856	OU6
Plethodontidae	*Oedipina*	*uniformis*	San Jose	Santa Ana	2856	OU7
Plethodontidae	*Oedipina*	*uniformis*	San Jose	Santa Ana	2856	OU8
Plethodontidae	*Oedipina*	*uniformis*	San Jose	Santa Ana	2856	OU9
Plethodontidae	*Oedipina*	*uniformis*	San Jose	Santa Ana	2856	OU10

APPENDIX C

Mann Whitney nonparametric test ranks and results for the minimal inhibitory concentrations for Costa Rican and Appalachian amphibians with DD and ID

Mann Whitney 2 sample, 2 way test for MIC by Country

Species	Ranks	MIC (µg/ml)	Country	Development
Plethodon cylindraceus 2 (*)	10.5	100	USA	DD
Plethodon montanus (*)	3.5	6.12	USA	DD
Plethodon yonahlossee (*)	10.5	100	USA	DD
Desmognathus orestes (*)	3.5	6.12	USA	ID
Desmognathus quadramaculatus (*)	3.5	6.12	USA	ID
Eurycea wilderae (*)	3.5	6.12	USA	ID
SUM of Ranks	**35**			

Species	Ranks	MIC (µg/ml)	Country	Development
Eleutherodactylus fitzingeri (*)	9	50	CR	DD
Oedipina uniformis (*)	3.5	6.12	CR	DD
Hyla microcephala (*)	3.5	6.12	CR	ID
Physalaemus pustulosus (*)	8	25	CR	ID
Leptodactylus bolivianus (*)	7	12.5	CR	ID
SUM of Ranks	**31**			

Ua	16
Ub	14
Ucritical	27

Mann Whitney 2 sample, 2 way test for MIC by Development

Species	Ranks	MIC (µg/ml)	Country	Development
Hyla microcephala (*)	6.12	3.5	CR	ID
Physalaemus pustulosus (*)	25	8	CR	ID
Leptodactylus bolivianus (*)	12.5	7	CR	ID
Desmognathus orestes (*)	6.12	3.5	USA	ID
Desmognathus quadramaculatus (*)	6.12	3.5	USA	ID
Eurycea wilderae (*)	6.12	3.5	USA	ID
SUM of Ranks	**29**			

Species	Ranks	MIC (µg/ml)	Country	Development
Eleutherodactylus fitzingeri (*)	50	9	CR	DD
Oedipina uniformis (*)	6.12	3.5	CR	DD
Plethodon cylindraceus 2 (*)	100	10.5	USA	DD
Plethodon montanus (*)	6.12	3.5	USA	DD
Plethodon yonahlossee (*)	100	10.5	USA	DD
SUM of Ranks	**37**			

Ua	8
Ub	22
Ucritical	27

VITA

Jorge Esquivel was born and raised in San Jose, Costa Rica in Central America. In 2003 he obtained a B.S. in Tropical Biology from Universidad Nacional Autonoma (UNA) in Costa Rica and a B.S. in Natural Resources Management and Protection from Universidad Estatal a Distancia (UNED), also in Costa Rica. He came to Boone, NC, where he started his M.S. graduate program in Biology at Appalachian State in the fall of 2004 under the direction of Dr. Robert Wayne Van Devender. Jorge's major area of study is amphibian population decline, and his research project involves the study of the amphibian's antimicrobial peptides secreted by their skin granular glands and how these can affect the growth of the cosmopolitan amphibian Chytrid fungus *Batrachochytrium dendrobatidis*.

After the M.S. degree is awarded in the fall of 2007, he and his wife Fern Perkins will begin working towards the goal of building a station in Costa Rica for conservation, management and research. His current residence is 328 Oak St, Boone, North Carolina, 28607. His parents are Rodrigo Alberto Esquivel Mora and Patricia Sibaja Adams of San Jose, Costa Rica.