FACTORS AFFECTING SUSCEPTIBILITY TO CHYTRIDIOMYCOSIS

by

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(Under the Direction of David Porter)

ABSTRACT

Chytridiomycosis is an emerging infectious disease of amphibians that has caused population declines in North and Central America and Australia. The disease is caused by the fungus *Batrachochytrium dendrobatidis*. Factors contributing to susceptibility of amphibians to this disease are not well known. Temperature has been suggested as a key factor since chytridiomycosis is thought to occur more frequently at cool high-elevation sites where enigmatic amphibian declines have been observed. To study the effects of temperature on susceptibility to chytridiomycosis, we experimentally exposed *Gastrotheca peruana* at 18 and 23ºC and *Plethodon metcalfi* and *Desmognathus monticola* at 8 and 16ºC to *B. dendrobatidis*. Mortality of infected *G. peruana* was higher at 18℃ than at 23℃. Mortality of *P. metcalfi* due to *B. dendrobatidis* was higher at 8°C than at 16°C. Therefore, temperatures at the low end of the range suitable to maintain the health of amphibians appear to increase susceptibility to the pathogen.

Phylogeny is also a possible factor in susceptibility, since a few studies indicate salamanders may respond differently to infection with *Batrachochytrium dendrobatidis*. We compared infection of *Plethodon metcalfi* and *Desmognathus monticola* to that of anuran species. Infected salamanders exhibited few of the clinical signs associated with *B. dendrobatidis* infection; however, they exhibited histological signs of disease similar to those previously observed in other salamanders. Some salamanders may have cleared

infection. The amphibian immune system likely plays a role in susceptibility to chytridiomycosis.

Cutaneous peptides secreted from amphibian skin are effective at inhibiting growth of *Batrachochytrium dendrobatidis in vitro*. To examine the role of cutaneous peptides *in vivo* we depleted *Rana catesbeiana* of peptides and then exposed them to about 10 million *B. dendrobatidis* zoospores. All frogs became infected with *B. dendrobatidis*, as determined by PCR; however no clinical or histological signs of disease were observed. The natural mixtures of peptides of *R. catesbeiana* had low effectiveness against *B. dendrobatidis in vitro*. Therefore antimicrobial peptides are not the sole source of resistance to chytridiomycosis in *R. catesbeiana*. Further research is required on the factors discussed here and the many others likely involved in susceptibility to chytridiomycosis.

INDEX WORDS: chytridiomycosis, *Batrachochytrium dendrobatidis*, amphibian, frog, salamander, mortality, susceptibility, temperature, antimicrobial peptides, fungus

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A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2008

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DEDICATION

This dissertation is dedicated to Myriam Migdalia Soto (1948-2001) and Angel Manuel Soto (1947-2005), loving parents who sacrificed for my education and happiness.

ACKNOWLEDGEMENTS

I thank David Porter and the rest of my committee for continued guidance. I am forever indebted to my family for support and patience. Special thanks go to Hector Vazquez, Alejandro Soto, and Nilda Santiago.

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CHAPTER 1

INTRODUCTION

In 1998 scientists reported infection of amphibian skin by *Batrachochytrium dendrobatidis*, the first vertebrate infection by any chytridiomycete fungus (Berger et al. 1998). Since that time several hypotheses have been proposed to explain the emergence of this pathogen in numerous amphibian populations (Weldon et al. 2004, Rachowicz et al. 2005, Lips et al. 2006, Pounds et al. 2006, Lips et al. 2008, Pounds & Coloma 2008). The fungus continues to be recorded in new places, reaching at least 35 countries by 2008 (www.spatialepidemiology.net). The effects of the fungus on amphibian populations has grown dire in Panama (Lips et al. 2006), where some biologists have resorted to capturing amphibians for captive breeding programs (Mendelson et al. 2006). Efforts to save frogs susceptible to chytridiomycosis, as well as to the serious threats of habitat loss, pollution, invasive species, and other diseases, resulted in 2008 being named the "Year of the Frog" by Amphibian Ark (www.amphibianark.org/yearofthefrog.htm).

Batrachochytrium dendrobatidis is the fungal pathogen that causes the disease chytridiomycosis. *B. dendrobatidis* is in the phylum Chytridiomycota and the order Chytridiales (Longcore et al. 1999). Researchers have isolated *B. dendrobatidis* from many amphibian species in different countries, and little variation has been observed between these isolates, suggesting the fungus is a recently emerged clone (Morehouse et al. 2003). This supports the hypothesis put forth by Weldon et al. (2004) that states *B. dendrobatidis* originated in South Africa, where it is still endemic in native frog populations, and has recently spread throughout the world. If it spread recently, the organism would not have had sufficient time to evolve in response to local pressures, thus resulting in almost identical genetic sequences between the isolates. The authors suggest *B. dendrobatidis* spread to other countries when the African clawed frog, *Xenopus laevis*,

was exported for use in human pregnancy tests. The genome has recently been sequenced by the Broad Institute (*Batrachochytrium dendrobatidis* Sequencing Project 2008), allowing researchers to determine more definitively if there is any difference between isolates and, more importantly, if the difference includes virulence factors.

Batrachochytrium dendrobatidis is an aquatic fungus with a simple life cycle. *B. dendrobatidis* has an asexual flagellated zoospore, which is the only motile stage of this organism. Within 24 h of release from a zoosporangium the zoospore resorbs its flagellum, encysts, and germinates to produce rhizoids, which attach to a substrate. After a period of growth by enlargement and mitosis it becomes a zoosporangium. A new population of 15-20 zoospores is produced in the zoosporangium and released through a discharge tube, starting the cycle again. Some zoosporangia are divided by a septum, a diagnostic character of *B. dendrobatidis*. Zoospores and a discharge tube are produced on both sides of the septum. The entire life cycle *in vitro* takes 5-6 days (Berger et al. 2005a).

The fungus has a wide permissible temperature range *in vitro* for survival. Piotrowski et al. (2004) found that the optimal temperature range for *B. dendrobatidis*, as determined by growth, is 17-25ºC. *B. dendrobatidis* can also grow at 10ºC, but at a slower rate. They also found that *B. dendrobatidis* survives temperatures as low as 4ºC and as high as 30ºC. Few experiments have addressed the effect of temperature on *B. dendrobatidis* infection of amphibians. When exposed to *B. dendrobatidis*, *Mixophyes fasciolatus*, an Australian frog, experienced 100% mortality at 17ºC and 23ºC and 50% at 27ºC (Berger et al. 2004). The orange-eyed tree frog, *Litoria chloris*, was cleared of infection when placed at 37ºC for 16 hours (Woodhams et al. 2003). This experiment mimicked a proposed behavioral fever in which amphibians can increase their body temperature by basking in the sun. A field study of *Litoria wilcoxii* found prevalence of *B. dendrobatidis* infection in this species peaked at air temperatures between 14ºC and 20ºC and dropped to 0 at temperatures above 20ºC (Kriger & Hero 2007). One

chytridiomycosis emergence hypothesis is based on temperature. Pounds et al. (2006) suggested the temperature at high-elevation sites has shifted towards the growth optimum of *B. dendrobatidis*, thus supporting outbreaks of chytridiomycosis in the montane tropics.

Batrachochytrium dendrobatidis infects keratinized amphibian skin, which is found in tadpole mouthparts and adult skin (Berger et al. 1998, Pessier et al. 1999). *B. dendrobatidis* only causes death in adults (Berger et al. 1998) and is thought to do so by preventing osmoregulation (Voyles et al. 2007). Infection primarily occurs on the venter and feet of adults, likely because these body parts have the most contact with water (Berger et al. 2005b). Both histology and polymerase chain reaction (PCR) are used to diagnose *B. dendrobatidis* infection (Hyatt et al. 2007). PCR has become the prominent tool used because it is noninvasive and more accurate at detecting infection (Kriger et al. 2006). Real-time PCR is required to determine the level of infection (Boyle et al. 2004), but researchers should not rely on this technique to diagnose chytridiomycosis because it does not account for the host's response to infection by the pathogen, as does histology.

Batrachochytrium dendrobatidis zoospores are transmitted between tadpole and adult life stages (Rachowicz & Vredenburg 2004) and between conspecific adults (L. Schloegel, pers. comm.) by contact with infected amphibians, water, or wet soil. The fungus is thought to persist in environmental or biological reservoirs outside the host for several reasons: 1) many other chytridiomycetes are saprobes, living on dead or dying organic matter (Barr 1990); 2) *B. dendrobatidis* can survive in sterile lake water for up to seven weeks (Johnson & Speare 2003); and 3) *B. dendrobatidis* DNA has been detected in the environment in water (Kirshtein et al. 2007) and on rocks (Lips et al. 2006). Diseases with environmental or biological reservoirs can cause extinction in a host species (deCastro & Bolker 2004), making chytridiomycosis a major threat to amphibians.

Species from two orders of amphibians have been observed infected with *Batrachochytrium dendrobatidis*: Caudata (salamanders) and Anura (frogs). No strong phylogenetic pattern of susceptibility appears to exist within the orders, with nine of 22 anuran and three of nine caudate families affected. However, many more anurans than caudates are known to be susceptible. In total at least 147 species are susceptible to infection with *B. dendrobatidis* (Table 1), and this number continues to increase as more field surveys and laboratory experiments are conducted. *B. dendrobatidis* affects amphibian populations differently. It is most notable for causing declines in Australia and Central America, where it was first discovered (Berger et al. 1998, Daszak et al. 1999). It has also caused declines in amphibian species in the western United States, such as the yellow-legged frog, *Rana muscosa* (Rachowicz et al. 2006). At least one species of frog has recovered after a decline. The Eungella day frog, *Taudactylus eungellensis*, currently persists in Australia with an endemic infection of *B. dendrobatidis* (Retallick et al. 2004). *R. muscosa* at some sites in the northern Sierra Nevadas also persist with infection, but the overall trend is towards extinction (Briggs et al. 2005). One frog species, *Taudactylus acutirostris*, is thought to have gone extinct due to chytridiomycosis (Schloegel et al. 2006). A few species are known to be resistant to chytridiomycosis, even though they can be infected by *B. dendrobatidis*, including *Rana catesbeiana* and *Xenopus laevis* (Daszak et al. 2004, Rollins-Smith et al. 2005).

The majority of research conducted on amphibian immune response to chytridiomycosis has focused on innate immunity, namely antimicrobial peptides and cutaneous microflora. Antimicrobial peptides are secreted from granular glands in amphibian skin in response to stress and inhibit the growth of some bacteria and fungi (Rollins-Smith et al. 2005). *In vitro* studies have shown some peptides to be active against *Batrachochytrium dendrobatidis*, including four that are secreted from *Rana catesbeiana* (Rollins-Smith et al. 2002). Amphibians produce various skin peptides, with the combination of peptides and total quantity produced varying by species (Woodhams

et al. 2006). Cutaneous microflora of amphibians include bacteria, which can produce antibiotics active against other microorganisms (Austin 2000). The following genera of bacteria active against *B. dendrobatidis* have been isolated from amphibian skin: *Arthrobacter*, *Bacillus*, *Kitasatospora*, *Lysobacter*, *Paenibacillus*, *Pedobacter*, *Pseudomonas*, and *Streptomyces*. Like antimicrobial peptides, the diversity and quantity of bacteria growing on amphibian skin varies by species (Harris et al. 2006).

Although much has been learned about *Batrachochytrium dendrobatidis* in the past 10 years, three major questions remain: 1) Does *B. dendrobatidis* persist long-term in the environment without an amphibian host? 2) How does *B. dendrobatidis* cause death? and 3) Which factors affect susceptibility to infection with *B. dendrobatidis* and development of chytridiomycosis? It is this last question that I addressed in my research. There are three categories of factors that affect susceptibility: pathogen, host and environment. For my research I focused on the host and the environment, and specifically on temperature (Chapters 2 and 3), phylogeny of amphibian species (Chapter 3), and antimicrobial peptides (Chapter 4).

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Table 1.1: Amphibian species susceptible to infection by *Batrachochytrium dendrobatidis*

Species	**References**
Acris crepitans	Rothermel et al. 2008
Adelotus brevis	Berger et al. 2004
Alytes obstetricans	Bosch et al. 2001
Ambystoma maculatum	Ouellet et al. 2005
Ambystoma tigrinum	Davidson et al. 2003
Atelopus chiriquiensis	Berger et al. 1998; Lips et al. 2003
Atelopus varius	Berger et al. 1998; Puschendorf, Bolanos, Chaves 2006
Atelopus zeteki	Lips et al. 2006
Bokermannohyla gouveai	Carnaval et al. 2006
Bolitoglossa colonnea	Lips et al. 2006
Bolitoglossa dofleini	Pasmans et al. 2004
Bolitoglossa schizodactyla	Lips et al. 2006
Bufo americanus	Ouellet et al. 2005; Longcore et al. 2007
Bufo boreas	Muths et al. 2003; Blaustein et al. 2005; Carey et al. 2006
Bufo bufo	Bosch & Martinez-Solano 2006
Bufo canorus	Green & Sherman 2001
Bufo haematiticus	Berger et al. 1998; Lips et al. 2006
Bufo marinus	Berger et al. 1998; Berger et al. 2004
Centrolene ilex	Lips et al. 2006
Centrolene prosoblepon/ *Centrolenella prosoblepon*	Lips et al. 2006; Picco & Collins 2007
Cochranella albomaculata	Berger et al. 1998; Lips et al. 2006
Cochranella euknemos	Lips et al. 2006
Cochranella prosoblepon	Berger et al. 1998
Colostethus flotator	Lips et al. 2006
Colostethus nubicola	Lips et al. 2006
Colostethus olfersioides	Carnaval et al. 2006
Colostethus panamensis	Lips et al. 2006
Colostethus pratti	Lips et al. 2006
Colostethus talamancae	Lips et al. 2006
Craugastor cf. azueroensis	Lips et al. 2006
Craugastor bransfordii	Puschendorf, Bolanos, Chaves 2006
Craugastor cf. bransfordii	Lips et al. 2006
Craugastor bufoniformis	Lips et al. 2006
Craugastor cerasinus	Lips et al. 2006
Craugastor crassidigitus	Lips et al. 2006
Craugastor fitzingeri	Puschendorf, Bolanos, Chaves 2006
Craugastor gollmeri	Lips et al. 2006

Craugastor megacephalus	Lips et al. 2006
Craugastor noblei	Lips et al. 2006
Craugastor podiciferus	Puschendorf, Bolanos, Chaves 2006
Craugastor cf. podiciferus	Lips et al. 2006
Craugastor punctariolus	Lips et al. 2006
Craugastor tabasarae	Lips et al. 2006
Craugastor talamancae	Lips et al. 2006; Puschendorf, Bolanos, Chaves 2006
Crossodactylus caramaschii	Carnaval et al. 2006
Cyclorana platycephala	Berger et al. 2004
Dendrobates auratus	Pessier et al. 1999; Nichols et al. 2001
Dendrobates azureus	Pessier et al. 1999
Dendrobates pumilio	Puschendorf, Bolanos, Chaves 2006
Dendrobates tinctorius	Nichols et al. 2001
Duellmanohyla rufioculus	Picco & Collins 2007
Duellmanohyla uranochroa	Puschendorf, Bolanos, Chaves 2006
Dyscophus antongilii	Oevermann et al. 2005
Eleutherodactylus aurilegulus	Puschendorf, Castaneda, McCranie 2006
Eleutherodactylus cf. caryophyllceus	Lips et al. 2006
Eleutherodactylus coqui	Burrowes et al. 2004; Beard and O'Neill 2005
Eleutherodactylus cruentus	Berger et al. 1998; Lips et al. 2006
Eleutherodactylus cf. diastema	Lips et al. 2006
Eleutherodactylus emcelae	Berger et al. 1998
Eleutherodactylus fitzingeri	Puschendorf & Bolanos 2006
Eleutherodactylus karlschmidti	Burrowes et al. 2004
Eleutherodactylus melanostictus	Lips et al. 2003
Eleutherodactylus museosus	Lips et al. 2006
Eleutherodactylus ridens	Lips et al. 2006
Eleutherodactylus saltator	Lips et al. 2004
Eleutherodactylus underwoodi	Picco & Collins 2007
Eleutherodactylus vocator	Lips et al. 2006
Gastrotheca cornuta	Lips et al. 2006
Heleioporus australiacus	Berger et al. 2004
Hyalinobatrachium colymbiphyllum	Lips et al. 2006
Hyla arenicolor	Bradley et al. 2002
Hyla chrysoscelis	Parris & Baud 2004
Hyla ebraccata	Picco & Collins 2007
Hyla pseudopuma	Picco & Collins 2007
Hyla regilla	Blaustein et al. 2005
Hyla versicolor	Ouellet et al. 2005
Hyla versicolor/chrysoscelis	Rothermel et al. 2008
Hylomantis lemur	Lips et al. 2006
Hyloscirtus colymba	Lips et al. 2006
Hyloscirtus palmeri	Lips et al. 2006
Hypsiboas freicanecae	Carnaval et al. 2006
Istmohyla pseudopuma	Puschendorf, Bolanos, Chaves 2006
Litoria pearsoniana	Berger et al. 2004
Lechriodus fletcheri	Berger et al. 2004
Leiopelma archeyi	Bell et al. 2004
Leptodactylus ocellatus	Herrera et al. 2005
Leptodactylus pentadactylus	Lips et al. 2006
Limnodynastes dumerilii	Berger et al. 1998; Berger et al. 2004
Limnodynastes peronii	Berger et al. 2004
Limnodynastes tasmaniensis	Berger et al. 1998; Berger et al. 2004

Limnodynastes terraereginae	Berger et al. 2004
Litoria aurea	Berger et al. 2004
Litoria caerulea	Berger et al. 1998; Pessier et al. 1999; Berger et al. 2004; Berger et al. 2005a; Berger et al. 2005b; Woodhams et al. 2007
Litoria chloris	Berger et al. 2004; Woodhams et al. 2007
Litoria citropa	Berger et al. 2004
Litoria ewingii	Berger et al. 2004
Litoria genimaculata	Berger et al. 2004; Woodhams & Alford 2005
Litoria gracilenta	Berger et al. 2004
Litoria infrafrenata	Berger et al. 2004
Litoria lesueuri	Berger et al. 1998; Berger et al. 2004; Woodhams & Alford 2005
Litoria nannotis	Berger et al. 1998; Berger et al. 2004; Woodhams & Alford 2005
Litoria peronii	Berger et al. 2004
Litoria raniformis	Waldman et al. 2001; Berger et al. 2004
Litoria rheocola	Berger et al. 1998; Berger et al. 2004; Woodhams & Alford 2005
Litoria spenceri	Berger et al. 1998; Berger et al. 2004
Litoria tyleri	Berger et al. 2004
Litoria verreauxii	Berger et al. 2004
Litoria wilcoxii	Kriger and Hero 2006, Kriger and Hero 2007
Mixophyes fasciolatus	Berger et al. 1998; Berger et al. 2004; Woodhams et al. 2007
Mixophyes fleayi	Berger et al. 1998; Berger et al. 2004
Mixophyes shevilli	Woodhams & Alford 2005
Nelsonophryne aterrima	Lips et al. 2006
Neobatrachus kunapalari	Berger et al. 2004
Notophthalmus viridescens	Ouellet et al. 2005; Rothermel et al. 2008
Nyctimystes dayi	Berger et al. 2004; Woodhams & Alford 2005
Oedipina collaris	Lips et al. 2006
Pleurodema marmorata	Seimon et al. 2006
Pseudacris crucifer	Rothermel et al. 2008
Pseudacris fouquettei	Rothermel et al. 2008
Pseudacris triseriata	Ouellet et al. 2005; Retallick & Miera 2007
Pseudophryne pengilleyi	Berger et al. 2004
Ptychohyla erythromma	Lips et al. 2004
Rana aurora	Pearl et al. 2007
Rana boylii	Davidson et al. 2003; Ouellet et al. 2005
Rana cascadae	Blaustein et al. 2005
Rana catesbeiana	Mazzoni et al. 2003; Daszak et al. 2004; Hanselmann et al. 2004; Blaustein et al. 2005; Cunningham et al. 2005; Daszak et al. 2005; Ouellet et al. 2005; Longcore et al. 2007; Pearl et al. 2007; Rothermel et al. 2008
Rana chiricahuensis	Bradley et al. 2002
Rana clamitans	Ouellet et al. 2005; Longcore et al. 2007; Rothermel et al. 2008
Rana luteiventris	Pearl et al. 2007
Rana maculata	Puschendorf, Castaneda, McCranie 2006
Rana muscosa	Rachowicz & Vredenburg 2004

Rana palustris	Ouellet et al. 2005; Longcore et al. 2007; Rothermel et al. 2008
Rana pretiosa	Pearl et al. 2007
Rana pipiens	Ouellet et al. 2005; Longcore et al. 2007; Parris et al. 2006
Rana septentrionalis	Ouellet et al. 2005; Longcore et al. 2007
Rana sphenocephala	Daszak et al. 2005; Rothermel et al. 2008
Rana sylvatica	Ouellet et al. 2005; Longcore et al. 2007; Rothermel et al. 2008
Rana vibicaria	Puschendorf, Bolanos, Chaves 2006
Rana warszewitschii	Lips et al. 2006
Rana yavapaiensis	Bradley et al. 2002; Davidson et al. 2003; Schlaepfer et al. 2007
Salamandra salamandra	Bosch & MartInez-Solano 2006
Taudactylus acutirostris	Berger et al. 1998; Berger et al. 2004; Schloegel et al. 2006
Taudactylus eungellensis	Berger et al. 1998; Berger et al. 2004; Retallick et al. 2004
Telmatobius marmoratus	Seimon et al. 2006
Thoropa miliaris	Carnaval et al. 2006
Uperoleia laevigata	Berger et al. 2004
Xenopus gilli	Weldon et al. 2004
Xenopus laevis	Weldon et al. 2004
Xenopus meulleri	Weldon et al. 2004

CHAPTER 2

EXPERIMENTAL INFECTION OF THE PERUVIAN MARSUPIAL FROG *GASTROTHECA PERUANA* WITH *BATRACHOCHYTRIUM DENDROBATIDIS*, THE CAUSATIVE AGENT OF AMPHIBIAN CHYTRIDIOMYCOSIS[1]

[1] Vazquez, V. M. and D. Porter. To be submitted to *Journal of Wildlife Diseases*.

ABSTRACT

Chytridiomycosis is an emerging infectious disease of amphibians that has caused population declines in North and Central America and Australia. Environmental factors contributing to susceptibility of amphibians to this disease are not well known; however temperature has been suggested as a key factor since chytridiomycosis is believed to occur more frequently at cool high-elevation sites where enigmatic amphibian declines have been observed. The purpose of this study was to test the susceptibility of *Gastrotheca peruana* to infection with the chytrid fungus *Batrachochytrium dendrobatidis* at different temperatures. Mortality of infected frogs was higher at 18°C than at 23°C. Both of these temperatures were within the *in vitro* optimal temperature range for *B. dendrobatidis*, but 18ºC was at the low end of the range of temperatures suitable to maintain the health of *Gastrotheca peruana* in the lab. More research on the effects of temperature on infection with *B. dendrobatidis* and development of chytridiomycosis is required, including experiments that test realistic temperature fluctuations.

KEYWORDS

Mortality, temperature, fungus

1. INTRODUCTION

Chytridiomycosis is an emerging infectious disease of amphibians that has caused population declines in North and Central America and Australia (Berger et al. 1998, Lips 1999, Lips et al. 2006, Rachowicz et al. 2006). The causative agent of the disease is *Batrachochytrium dendrobatidis*, an aquatic fungus with motile zoospores that infects the keratin layer of adult epidermal tissue and larval mouthparts (Longcore et al. 1999). It is hypothesized that the pathogen causes death in adult amphibians by preventing osmoregulation (Voyles et al. 2007). Environmental factors contributing to susceptibility

of amphibians are not well known; however temperature has been suggested as a key factor since chytridiomycosis is believed to occur more frequently at cool high-elevation sites where enigmatic amphibian declines have been observed (Ron 2005). The fungus grows best *in vitro* from 17-25ºC (Piotrowski et al. 2004). Pounds et al. (2006) proposed the chytrid-thermal-optimum hypothesis, which suggests that the recent decreasing daytime and increasing nighttime temperatures in Monteverde, Costa Rica have aided the development of chytridiomycosis in amphibians and the spread of the epidemic in the region.

The International Union for the Conservation of Nature (IUCN) classifies 53 of Peru's amphibian species as critically endangered or endangered and 28 species as vulnerable (IUCN et al. 2004). *Batrachochytrium dendrobatidis* has been recorded in amphibians in Peru (Seimon et al. 2006) and suggested as the cause of recent amphibian declines (Lips et al. 2005, Lips et al. 2006, Schloegel et al. 2006). The threat this pathogen poses to imperiled anurans is unknown. The Peruvian marsupial frog, *Gastrotheca peruana*, is a non-threatened species living in various habitat types with an extensive range overlapping with ranges of 10 critically endangered or endangered species in Peru (IUCN et al. 2004). *G. peruana* could therefore serve as a carrier of the pathogen between regions and habitat types, much like *Rana catesbeiana* and *Xenopus laevis* are suggested to be (Daszak et al. 2004, Weldon et al. 2004). The purpose of this study was to test the susceptibility of *G. peruana* to infection with *B. dendrobatidis* at different temperatures.

2. MATERIALS AND METHODS

2.1 Study organism

Thirty-two *Gastrotheca peruana* (Fig. 2.1) of the same cohort were obtained from the Atlanta Botanical Garden as tadpoles, reared to adults, and then maintained communally in terrariums in two incubators kept at 23ºC. Three weeks prior to initiation

of the experiment incubator temperatures were slowly adjusted above and below 23ºC to determine the maximum and minimum temperatures at which *G. peruana* maintained normal activity and feeding levels. The maximum temperature chosen was 23ºC and the minimum was 18ºC. Two weeks prior to initiation of the experiment the frogs were transferred to 19.69 cm x 29.21 cm x 20.32 cm containers (Kritter Keeper, Lee's Aquarium and Pet Products) and kept on a 12-hour light/12-hour dark cycle. Aged tap water was provided in 90 x 15 mm glass Petri dishes and changed every other day. Five to seven crickets dusted with JurassiCal™ calcium supplement powder (JurassiPet™) were fed to each frog every other day.

2.2 Fungus inoculum

Batrachochytrium dendrobatidis cultures of the type isolate (JEL 197, from a dead *Dendrobates azureus* in captivity) were maintained in 1% tryptone broth (10 g tryptone, 1 L double distilled water (ddH_2O)) at 21-23ºC and transferred to new broth every 8 wk. To harvest zoospores, 1.0 ml of *B. dendrobatidis* broth was transferred to 1% tryptone agar (10 g tryptone, 10 g agar, 1 L ddH_2O). After 7 d each plate was flooded with 20 ml of sterile tryptone broth. After 30 minutes the liquid from each plate was pooled. One hundred µl of the inoculum was added to 100 µl Lugol solution (Sigma), which kills and stains zoospores. Microscopic counts of zoospores in this combined solution were made with a hemacytometer. The inoculum volume was adjusted to a concentration of 7.2×10^5 zoospores ml^{-1}.

2.3 Experimental design

Frogs were randomly assigned to one of 4 treatment groups: control/18°C (n = 4), control/23°C (n = 4), exposed/18°C (n = 12), or exposed/23°C (n = 12). Control frogs were placed on the top shelves of the incubators to avoid cross contamination from exposed frogs. Survivorship and time-to-death were measured. Exposed frogs were

placed in 61.2 cm^3 containers, which prevented escape from inoculum. Eight ml of inoculum containing 5.76 x 10^6 zoospores was added to each container. Eight ml of sterile ddH_2O was added to control containers. Frogs were held in the inoculum or sterile water for 8 h. The prolonged exposure period and high number of zoospores were chosen to eliminate exposure duration and dosage as confounding factors of any results. All frogs were observed daily. Clinical signs of chytridiomycosis, including lethargy, excessive shedding of skin, inability to right themselves, and mortality, were recorded. The experiment was terminated after 89 d and all surviving frogs euthanized using Beuthanasia-D Special (Schering-Plough, Union, NJ). All frogs were preserved in 10% buffered formalin, either upon death due to disease or following euthanasia due to extreme morbidity or termination of the experiment.

2.4 Diagnostic Procedures

Shed skin was observed by light microscopy at 400x magnification when such skin was available. Histological analysis was performed for every frog. One whole foot per frog was fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Slides were observed by light microscopy under 400x magnification for *Batrachochytrium dendrobatidis* thalli in the stratum corneum. Polymerase chain reaction (PCR)-based assays were used when histological results were inconclusive because of tissue embedding and sectioning problems. The ventral skin of each frog was swabbed 30 times with a cotton-tipped cleaning stick (Puritan). The swabs were preserved in 70% ethanol and submitted to Pisces Molecular (Boulder, CO) for PCR assay for the presence of *B. dendrobatidis* using primers developed by Annis et al. (2004). Following swabbing and tissue collection, dead frogs were preserved in 10% neutral buffered formalin.

2.5 Data analysis

SAS Software 9.1 (SAS Institute, Inc., Cary, NC) was used to analyze all data. Fisher's Exact Test was used to compare mortality rates between frogs infected and uninfected with *Batrachochytrium dendrobatidis* (PROC FREQ). Cox regression survival analysis was used to determine if initial body weight was a predictor of mortality (PROC PHREG).

3. RESULTS

The chytridiomycosis-caused mortality rate was 45.8% (n = 11) for frogs exposed to *Batrachochytrium dendrobatidis*. No control frogs were infected with *B. dendrobatidis*. Overall mortality was significantly higher for infected frogs (100%) than for uninfected ones (14.29%; includes exposed frogs that did not become infected plus control frogs; $\chi^2 = 21.551$, Fisher's exact test two-sided, $p = 2.821 \times 10^{6}$). Mortality of infected frogs was higher at 18°C (83.3%) than at 23°C (8.33%). The mean time-to-death of frogs infected with *B. dendrobatidis* was 46 d (Fig. 2), and it was higher at 18°C (48 d) than at 23°C (24 d). Initial body weight did not affect time to death (Cox regression analysis, hazard ratio = 0.724, p = 0.0521).

Gastrotheca peruana infected with *Batrachochytrium dendrobatidis* had excessive sloughing skin with a yellow tint. Histological evaluation of preserved epidermal tissue stained with hematoxylin and eosin revealed focal hyperkeratosis with *B. dendrobatidis* thalli in the stratum corneum (Fig. 2.3). Two exposed frogs that died during the experiment, one at each temperature, were not infected with *B. dendrobatidis* as determined by negative PCR results. However, a few fungal sporangia were observed in the unstained freshly shed skin of the frog at 18ºC that died on day 48. This frog was found dead and its body was dry, indicating it had been dead for many hours.

Histological evaluation of preserved epidermal tissue of this frog did not reveal any sporangia.

4. DISCUSSION

This study tested the susceptibility of a non-threatened Peruvian tree frog to chytridiomycosis. The chytridiomycosis-caused mortality rate (45.8%) was not as high as some species that have experienced chytridiomycosis-induced declines in the wild (100% mortality of *Bufo boreas*, (Carey et al. 2006). However, it was similar to other experimentally infected frogs (eg. 40-79% of *Pseudacris triseriata*, Retallick and Miera 2007; up to 50% of *Rana yavapaiensis*, Davidson et al. 2003). Time to death (46 d) is difficult to compare to that of other studies due to differences in exposure time and zoospore dose. One frog that died at day 48 was negative for *Batrachochytrium dendrobatidis* infection by histology of preserved tissue and PCR. However, a few sporangia were observed on a wet mount of shed skin collected from the body. This frog could have died from chytridiomycosis. The swab may not have been able to collect shedding skin, and thus *B. dendrobatidis*, from the dry animal. Also, histology is known to be produce false negative results (Kriger et al. 2006).

The effect of temperature on the rate of mortality due to *Batrachochytrium dendrobatidis* observed in this study is noteworthy. Both temperatures tested (18ºC and 23ºC) were within the *in vitro* optimal temperature range for *B. dendrobatidis* (Piotrowski et al. 2004), but 18ºC was at the low end of the range of temperatures suitable to maintain the health of *Gastrotheca peruana* in the lab. Similar results were observed in *Plethodon metcalfi* exposed to *B. dendrobatidis* (see Chapter 3) and *Ambystoma tigrinum* exposed to *Ambystoma tigrinum* virus (Rojas et al. 2005). Some scientists have observed greater mortality due to chytridiomycosis in the winter (Berger et al. 1998, Berger et al. 2004), and attribute this observation to *B. dendrobatidis* growing better at lower temperatures (Berger et al. 2004) . But the higher mortality in winter also

may be due to effects of low relative temperatures on the amphibian hosts. Components of the immune system of some anurans are reduced at low temperatures (Green and Cohen 1977, Wang and Herman 1996, Maniero and Carey 1997). However, as Fisher (2007) noted, the effects of realistic daily and seasonal changes in temperature on amphibian immune systems must be determined.

Gastrotheca peruana lives in permanent and seasonal wetlands, agricultural lands, and urban areas from 2,300 m to 4,600 m (IUCN et al. 2004). It is a species of Least Concern according to the IUCN (2004). *G. peruana* has not been observed infected with *Batrachochytrium dendrobatidis* in the wild, even though *B. dendrobatidis* is present in amphibian populations in Peru (Seimon et al. 2006). *G. peruana* in the wild may not have been exposed to the pathogen yet, which thus far just has been observed above 5000 m in the Cordillera Vilcanota in southern Peru in *Pleuroderma marmorata* and *Telmatobius marmoratus* (Seimon et al. 2006). Alternatively, environmental temperatures to which *G. peruana* has been exposed may not have been adequate to allow the species to become susceptible to *B. dendrobatidis* infection. *G. peruana* should be considered at risk for developing chytridiomycosis in the wild since the climate in Peru is changing, indicated by glacial retreat at the sites of *B. dendrobatidis* occurrence (Seimon et al. 2006). The range of *G. peruana* overlaps the range of *P. marmorata* and of several critically endangered and endangered amphibians (Fig. 2.4), thus providing the opportunity for *G. peruana* to transmit the disease to vulnerable species.

Susceptibility of *Gastrotheca peruana* to chytridiomycosis necessitates monitoring of this species in the pet trade to prevent the spread of *Batrachochytrium dendrobatidis* in Peru and abroad. Native populations in Peru should also be monitored since this species could spread the pathogen to vulnerable amphibian species. More research on the effects of temperature on infection with *B. dendrobatidis* and development of chytridiomycosis is required. Emphasis should be placed on experiments that include realistic temperature fluctuations. There are numerous hypotheses as to why

some amphibian species are succumbing to chytridiomycosis while others remain unaffected. Sorting out host-, pathogen-, and environment-driven hypotheses requires continued extensive field and laboratory research.

ACKNOWLEDGEMENTS

We thank R. Gagliardo for providing the frogs, J. Longcore for supplying the fungal isolate, H. Vazquez for laboratory set-up, and J. Marlow, B. Lammers, and C. Norman for animal husbandry. Funding for this project was provided by the Consortium for Conservation Medicine. Animal protocols were approved by the Institutional Animal Care and Use Committee (Animal Welfare Assurance #A3437-01).

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Figure 2.1. Two male *Gastrotheca peruana* sitting in water.

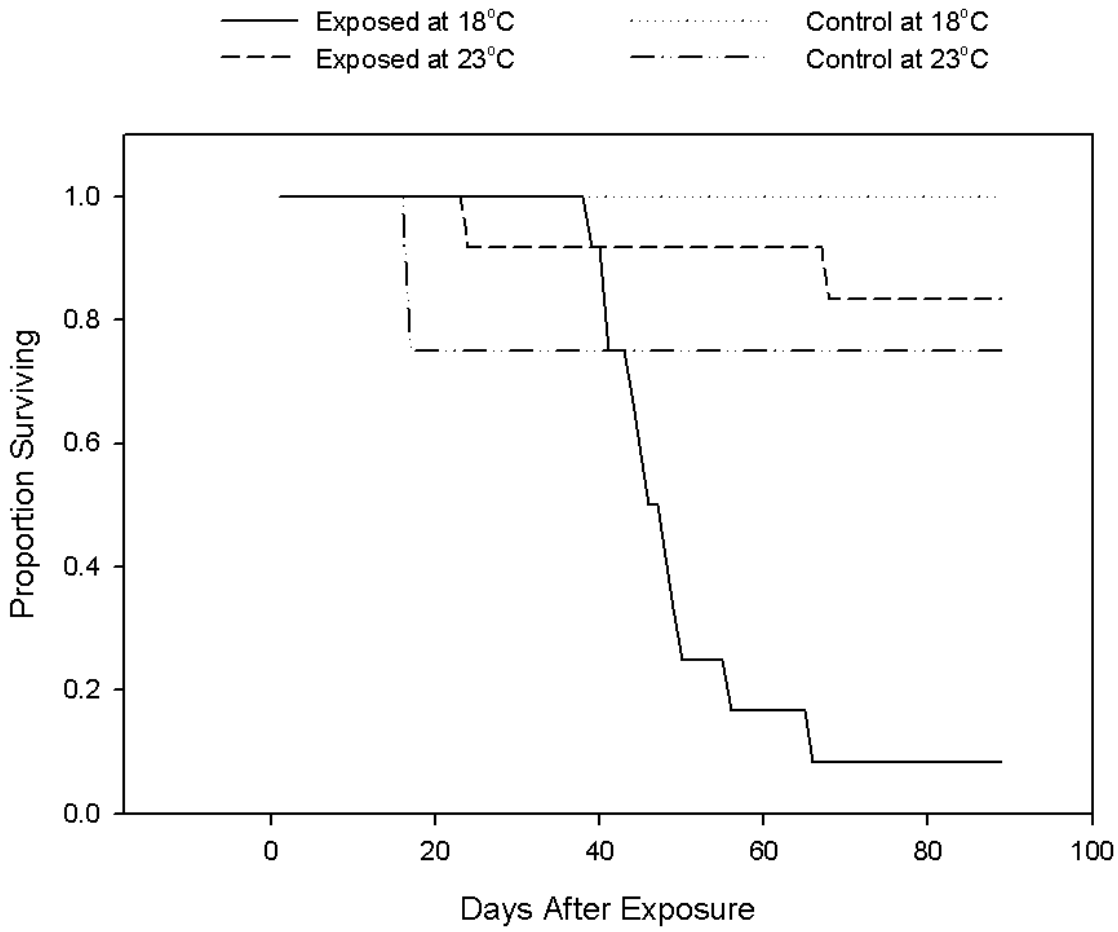

Figure 2.2. Survival curves for *Gastrotheca peruana* exposed to *Batrachochytrium dendrobatidis* and for controls at 18ºC and 23ºC.

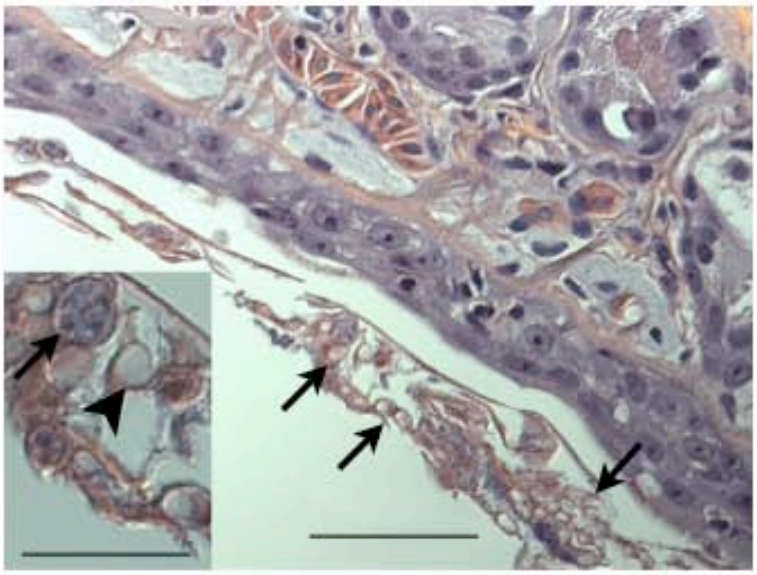

Figure 2.3. Histologic section of skin from the foot of a *Gastrotheca peruana*. There is moderate hyperkeratosis with intracellular thalli of *B. dendrobatidis* (arrows). Scale bar = 50 µm. Inset shows detail of thalli including a thallus full of zoospores (arrow) and an empty thallus (arrowhead) that has released its zoospores. Scale bar = 20 µm. (H&E)

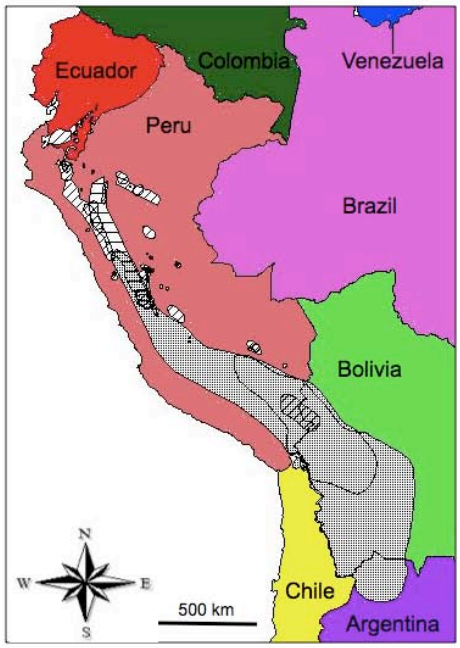

Figure 2.4. Ranges of endangered Peruvian frogs and frogs susceptible to chytridiomycosis. Diagonal hash marks indicate ranges of 51 critically endangered or endangered amphibian species in Peru (Species status data from IUCN et al. 2004; Range data from NatureServe 2005; range data not available for *Cochranella saxiscandens* or *Gastrotheca zeugocystis*). Horizontal hash marks indicate range of *Gastrothecea peruana*. Dotted marks indicate combined range of *Pleuroderma marmorata* and *Telmatobius marmoratus* recorded infected with *Batrachochytrium dendrobatidis* (Seimon et al. 2006).

CHAPTER 3

EXPERIMENTAL INFECTION OF NORTH AMERICAN PLETHODONTID SALAMANDERS WITH THE FUNGUS *BATRACHOCHYTRIUM DENDROBATIDIS*[2]

[2] Vazquez, V. M., B. B. Rothermel, A. P. Pessier. Submitted to *Diseases of Aquatic Organisms* 05/01/08.

ABSTRACT

In the southeastern United States the presence of *Batrachochytrium dendrobatidis*, a fungal pathogen of amphibians, is a potential threat to the diverse salamander assemblages in the region. In this study, we tested the susceptibility of plethodontid salamanders to infection with *B. dendrobatidis*. We experimentally infected one terrestrial species (*Plethodon metcalfi*) and one stream-dwelling species (*Desmognathus monticola*). Mortality of *P. metcalfi* due to *B. dendrobatidis* was 41.7% and was higher at 8°C (75%) than at 16°C (8.3%). *B. dendrobatidis* did not cause any mortality in *D. monticola*. Infected salamanders exhibited few of the clinical signs associated with *B. dendrobatidis* infection; however, they exhibited histologic signs of disease. Our results suggest that *Plethodon* species in the southeastern United States are at risk of becoming infected with *B. dendrobatidis* and developing chytridiomycosis. However, some animals may have survived with or cleared infection. Additional studies are required to determine if chytridiomycosis is a significant factor in declines of plethodontids.

KEYWORDS

Chytridiomycosis, amphibian, mortality

1. INTRODUCTION

The Appalachian Highlands region of eastern North America hosts 38 species of endemic plethodontid salamanders, more than any other region in the world (Duellman and Sweet 1999). Many of these species have restricted ranges, often on mountain tops, in essence rendering them island populations surrounded by inhospitable lowlands (MacArthur & Wilson 1967). Endemic species on islands are vulnerable to population declines resulting from disease introduction (Warner 1968). The presence in the southeastern United States of *Batrachochytrium dendrobatidis*, a fungal pathogen of

amphibians, is a potential threat to the region's diverse salamander assemblages (Rothermel et al. 2008). *B. dendrobatidis* has been detected in wild populations of amphibians in at least two sites in the southern Appalachian Mountains, one in northeast Georgia and one in western North Carolina (Rothermel et al. 2008). Investigations of disease susceptibility and population trends of plethodontid salamanders in the Appalachian Highlands are urgently needed, given recent reports of declines in this region (Highton 2005) and in the Mesoamerican Highlands (Lips & Donnelly 2005).

Batrachochytrium dendrobatidis causes chytridiomycosis, an often fatal disease that has caused population declines in Panama, Costa Rica, Australia, and western North America (Berger et al. 1998, Lips 1999, Muths et al. 2003, Lips et al. 2006, Rachowicz et al. 2006) and a species extinction in Australia (Schloegel et al. 2006). Infection with *B. dendrobatidis* is limited to keratinized epithelium in the skin of postmetamorphic animals and the oral disc of anuran larvae (Berger et al. 1998). Death has been attributed to disruption of cutaneous physiologic functions, such as osmoregulation and ion transport (Voyles et al. 2007). Although a small number of wild-caught plethodontid salamanders have been found infected with *B. dendrobatidis* (Pasmans et al. 2004, Cummer et al. 2005), their susceptibility to infection has not been thoroughly investigated. In this study, we tested the susceptibility of plethodontid salamanders to infection with *B. dendrobatidis*. We experimentally infected one terrestrial species, the southern gray-cheeked salamander (*Plethodon metcalfi*), and one stream-dwelling species, the seal salamander (*Desmognathus monticola*). *P. metcalfi* inhabits the forest floor, seeking refuge in leaf litter, under logs, or in small underground tunnels during the day. *D. monticola* typically inhabits rocky, cool-water streams and sometimes frequents crevices in wet rock faces (Petranka 1998). Both species are locally common and therefore were suitable model species for our initial investigation of the disease risk to plethodontid salamanders.

2. MATERIALS AND METHODS

2.1 Salamander collection and maintenance

Adult *Plethodon metcalfi* and *Desmognathus monticola* were captured by hand during diurnal and nocturnal searches of stream and forest sites within 6 km of Highlands, NC from April 20-23, 2006. Salamanders were housed individually in 415 ml GladWare® (The Clorox Company) plastic containers with perforated lids in two environmental chambers at 12°C for 7 wk. Prior to the experiment, each salamander was screened for *Batrachochytrium dendrobatidis* infection by polymerase chain reaction (PCR)-based assay (see below section 2.4). Salamanders were fed pinhead crickets dusted with JurassiCal™ calcium supplement powder (JurassiPet™) every five days, provided with wet, unbleached paper towels for moisture and cover, and maintained on a 12-hour light/12-hour dark schedule.

2.2 Preparation of the fungus inoculum

Zoospores of the type isolate of *Batrachochytrium dendrobatidis* (JEL 197) (Longcore et al. 1999) were used for the inoculum. This isolate was first isolated from a dead *Dendrobates azureus* infected with *Batrachochytrium dendrobatidis* and held in captivity. Since its isolation in 1997, JEL 197 had been serially passed *in vitro* 28 times. The fungus was maintained at 23°C in tryptone broth (10 g tryptone, 1 L double distilled water (ddH_2O). To harvest zoospores, cultures were transferred to tryptone agar (10 g tryptone, 10 g agar, 1 L ddH_2O). After 7 d of growth, when the maximum number of fungal zoospores was being produced, the agar plates were flooded with sterile ddH_2O and allowed to sit for 30 min. The ddH_2O in which the zoospores had collected was combined to form the inoculum. To determine zoospore concentration in the inoculum, 100 µl of the inoculum was added to 100 µl Lugol solution (Sigma-Aldrich) in a microcentrifuge tube. Lugol solution kills and stains zoospores. Microscopic counts were made with a hemacytometer. The inoculum volume was adjusted to a concentration of

3.56×10^5 zoospores ml^{-1}. Each salamander was exposed to 1.07×10^7 zoospores in 30 ml, a volume sufficient to submerge the venter (see below section 2.3).

2.3 Experimental design

Salamanders were randomly assigned to one of the following treatment groups: 8°C/exposed, 8°C/unexposed, 16°C/exposed, 16°C/unexposed (n = 12 for each species per treatment). The experiment was conducted at two temperatures because temperature is known to affect growth of *Batrachochytrium dendrobatidis*. The low temperature (8°C) is below the suitable growth range for isolate JEL 197 of *B. dendrobatidis* of 10-25°C on TghL agar culture and the high temperature (16°C) is within this range. Temperatures were below the maximal growth range of 17-25°C (Piotrowski et al. 2004). The two temperatures chosen are representative of the relatively cool microhabitats *Plethodon metcalfi* and *Desmognathus monticola* inhabit and thus well within the thermal tolerance range for these species (Spotila 1972). The salamanders were arranged in two incubators according to a randomized complete block design with incubator shelf as the block. Each block contained an equal number of replicates of each species-by-disease treatment.

Thirty ml of inoculum was added to the exposed treatment containers and 30 ml of sterile water to the unexposed treatment containers. After 8 h the inoculum and water were drained from the containers and replaced with wet paper towels. Salamanders in the exposed treatment were only exposed to *Batrachochytrium dendrobatidis* once. Treatment groups were not indicated on the containers, which ensured unbiased observation of salamanders for clinical signs of disease, such as lethargy, skin sloughing, inappetence (Nichols et al. 2001), and other abnormal behavioral and physical changes. Severely moribund animals were euthanized with MS-222 (tricaine methanesulfonate, Sigma-Aldrich). Prior to the start of the experiment it was decided that the experiment

would be ended under two circumstances: (1) if no clinical signs of disease or mortality were observed in the first 30 d or (2) if, after observing clinical signs of disease or mortality, no additional signs of disease or mortality were observed for at least 60 d. The experiment was terminated at Day 189 and all surviving salamanders were euthanized with MS-222.

2.4 Diagnostic procedures

Histologic examination of a single foot was performed on every salamander after natural death or euthanasia to look for evidence of *Batrachochytrium dendrobatidis* infection. The foot was fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Slides were observed by light microscopy under 400x magnification for characteristic *B. dendrobatidis* sporangia in the epidermis (Longcore et al., 1999; Berger et al., 2005). In addition, 8 salamanders representing a range of experimental outcomes (Table 3.1) were selected for histologic evaluation of whole body sections to include skin and internal organs in order to better define the distribution of *B. dendrobatidis* infection and to search for evidence of other underlying disease that could contribute to mortality. Histologic evaluation was performed blind to infection status and outcome. After fixation in 10% neutral buffered formalin, the carcasses were immersed for 6 h in a hydrochloric acid solution for demineralization. After demineralization, the body and tail were serially cross-sectioned at 3 mm intervals in the transverse plane and the head was bisected in the median plane with the entirety of the resultant sections processed for histology as described for the feet. Tissues represented histologically in these sections included skin, brain, spinal cord, heart, skeletal muscle, bone and bone marrow, liver (including subcapsular hematopoietic tissue), spleen, kidney, gonad, stomach, small and large intestines and pancreas.

PCR assays were used for infection screening prior to the experiment and when histologic results were inconclusive. For the pre-experiment PCR assays, the ventral skin

of each salamander was swabbed 30 times with a cotton-tipped cleaning stick (Puritan Medical Products Company). For the post-experiment PCR assays, the whole right hind foot was removed. Skin swabs and feet were preserved in 70% ethanol and submitted to Pisces Molecular (Boulder, CO) for standard PCR assay for the presence of *Batrachochytrium dendrobatidis* using DNA primers developed by Annis et al. (2004). DNA from *Rhizophydium haynaldii*, a chytrid fungus, was used as a negative control and water was used to detect sample contamination. Each sample was run only once. Swabs in ethanol were prepared by vortexing the sample tube, transferring the ethanol to a clean tube, spinning down for 3 min, and removing the supernatant. Feet were prepared by cutting off the toes and placing them in a clean tube. QIAGEN® ATL+PK tissue lysis buffer was added to prepared swabs and feet and incubated at 55ºC for 60 min. The DNeasy® Blood and Tissue Kit (QIAGEN) protocol was used for the rest of the procedure.

2.5 Data analysis

All data were analyzed using SAS Software 9.1 (SAS Institute, Cary, NC). Mortality rates were compared between exposure groups using Fisher's Exact Test (PROC FREQ). The effect of salamander experimental location on mortality was determined using Cox regression analysis (PROC PHREG). The effect of initial body weight on susceptibility to *Batrachochytrium dendrobatidis* infection was analyzed also using Cox regression analysis (PROC PHREG).

3. RESULTS

3.1 Mortality

All salamanders tested negative by PCR for *Batrachochytrium dendrobatidis* infection at the onset of the study. Overall, mortality was significantly higher for salamanders exposed to *B. dendrobatidis* zoospores (31.3%) than for unexposed

salamanders (4.2%) ($\chi^2 = 12.0804$, p = 0.0005). Of the *Plethodon metcalfi* exposed to *B. dendrobatidis*, 41.7% died due to *B. dendrobatidis* infection and 16.7% died due to unknown causes. No unexposed *P. metcalfi* died. Mortality of *P. metcalfi* due to *B. dendrobatidis* was higher at 8°C (75%, n = 9) than at 16°C (8.3%, n = 1). Mortality of *P. metcalfi* due to unknown causes was also higher at 8ºC (25%, n = 3) than at 16ºC (8.3%, n = 1), but not substantially.

No *Desmognathus monticola* that died before termination of the experiment had PCR evidence of *Batrachochytrium dendrobatidis* infection. Only one surviving *D. monticola* in the exposed treatment group was infected, according to PCR assay (DM11, Table 1). A low level of mortality was observed in *D. monticola* due to unknown causes (unexposed group: 8.33%, n = 2; exposed group: 4.17%, n = 1), and only at 8ºC.

The mean time-to-death of salamanders infected with *Batrachochytrium dendrobatidis* was 36.0 d (Fig. 1). Incubator shelf had no effect on time-to-death (Cox regression analysis, hazard ratio = 0.85, p = 0.4436). Initial salamander body weight did not affect susceptibility to *B. dendrobatidis* infection in *Plethodon metcalfi* (t = 0.64, p = 0.53).

3.2 Signs of disease

The most common physical abnormality observed in the course of the study was redness of the ventral surface of the digits, feet, and occasionally chin and tail. However, this type of abnormality was observed in both exposed (n = 29) and unexposed (n = 24) salamanders. There was no evidence of red leg disease. Infected salamanders exhibited few of the clinical signs associated with *Batrachochytrium dendrobatidis* infection in other studies of salamanders (Davidson et al. 2003, Pasmans et al. 2004, Cummer et al. 2005) or anurans (Nichols et al. 2001, Berger et al. 2004, Rachowicz & Vredenburg 2004, Carey et al. 2006) such as excessively shedding (sloughing) skin or changes in skin

pigmentation. Only one infected *Plethodon metcalfi* was noted to have excessive shedding skin at the time of death. Two infected *P. metcalfi* autotomized part or all of their tails 1-2 d before death with no other signs of disease.

In contrast, histologic examination of the feet of *Plethodon metcalfi* exposed to *Batrachochytrium dendrobatidis* revealed multifocal moderate parakeratotic hyperkeratosis with myriad intracellular chytrid thalli consistent with *B. dendrobatidis* infection (Fig. 3.2). The feet of unexposed *P. metcalfi* and all *Desmognathus monticola* appeared normal. Three *P. metcalfi* (PM1, PM5, PM6; Table 3.1) exposed to *B. dendrobatidis* and examined comprehensively by histology had similar lesions consistent with *B. dendrobatidis* infection. Lesions in these animals were most evident in the skin of the ventral and lateral body. In portions of the body cranial to the pelvic region, lesions were minimal to mild and chytrid thalli were present multifocally in small numbers (Fig. 3.3). In the body caudal to the pelvis, lesions were mild to moderate with higher numbers of chytrid thalli (Fig. 3.4) similar to the pattern observed in the feet. Examination of other organs did not reveal evidence of significant disease processes. A single *P. metcalfi* (PM1; Table 3.1) had small numbers of encysted nematode parasites in the submucosa of the stomach that were interpreted as an incidental finding. In five additional animals exposed to *B. dendrobatidis* (PM10, PM19, DM13, DM11, DM1; Table 3.1), no *B. dendrobatidis* thalli could be identified by histologic examination. In one of these animals (DM1; Table 3.1), there was moderate parakeratotic hyperkeratosis and epidermal hyperplasia in the skin of the body caudal to the pelvis similar to that observed in animals with identifiable *B. dendrobatidis* thalli. A second animal (PM10; Table 3.1) had minimal to mild parakeratotic hyperkeratosis in multiple sections from skin from throughout the body. These two salamanders (DM1, PM10; Table 1) died before the termination of the experiment and were considered negative for *B. dendrobatidis* infection due to a negative PCR result and the absence of *B. dendrobatidis* thalli in the histologic samples observed. The remaining exposed animals (PM19, DM13, DM11;

Table 1) had histologically normal skin. No evidence of significant underlying disease processes was observed in these animals.

4. DISCUSSION

This is the first study to test experimentally the susceptibility of plethodontid salamanders to infection with *Batrachochytrium dendrobatidis* and to the development of chytridiomycosis. The mortality rate of *Plethodon metcalfi* due to chytridiomycosis was low compared to the mortality rate of this disease in dendrobatids (Nichols et al. 2001) and bufonids (Carey et al. 2006), but was substantially higher than the mortality rates due to unknown causes. One *Desmognathus monticola* (DM11) survived with *B. dendrobatidis* infection for 189 d and remained asymptomatic throughout this time. The infection was detected by PCR, but no *B. dendrobatidis* thalli were observed by histologic examination. This animal may have been clearing the infection, as Davidson et al. (2003) observed in *Ambystoma tigrinum*. It also may have been persisting with a low-level infection, as do *Rana catesbeiana* (Daszak et al. 2004). The remaining *D. monticola* exposed to *B. dendrobatidis* may have become infected with *B. dendrobatidis* but cleared the infection, a phenomenon that could not be observed in the study because salamanders were tested only at the beginning and end of the experiment. Alternatively, these *D. monticola* may have been resistant to infection altogether. Both species experienced low levels of mortality unrelated to *B. dendrobatidis* infection, which might be attributed to stress from captivity.

The histologic lesions observed in the feet and body of *Plethodon metcalfi* were very similar to those observed in *Ambystoma tigrinum* (Davidson et al. 2003). These lesions differed from the lethal diffuse lesions seen in many anurans because there was less hyperkeratosis and more multifocal distribution of thalli. The distribution of heavier levels of infection to the caudal body and tail in the experimental cases was similar to that described for a single naturally infected *Plethodon neomexicanus* (Cummer et al. 2005).

Future research should address differences between caudates and anurans that could contribute to different disease responses in these two amphibian groups.

Multiple factors can influence the susceptibility of individual species to development of chytridiomycosis, including differences in host behavior; innate defenses, such as skin peptides; and skin bacterial flora. Time spent in water, basking, or in contact with other amphibians could affect the probability of transmission of *Batrachochytrium dendrobatidis* (Rowley & Alford 2007); however, it is unlikely host behavior caused the difference in infection susceptibility observed in this study since both species were maintained under the same conditions, which inhibited behavioral response. Skin peptide profiles differ substantially among amphibian species (Woodhams et al. 2006), which could account for the difference in susceptibility between *Plethodon metcalfi* and *Desmognathus monticola*. In addition, Harris et al. (2006) observed variation in the species of cutaneous bacteria isolated from *P. cinereus* and *Hemidactylium scutatum* and their effectiveness in inhibiting *B. dendrobatidis* growth in. Differences in abundance of *B. dendrobatidis*-inhibiting bacteria likely exist between individual hosts (Woodhams et al. 2007a) and between host populations (Woodhams et al. 2007b).

Disease models would be more robust if they incorporated accurate removal rates. Infection period, or time-to-death from pathogen exposure, is a critical element of disease dynamics. According to the SIR (susceptible/infected/recovered) model for epidemics, the removal rate (γ) is inversely proportional to the infection period and affects the turnover of infected organisms due to death or recovery (Anderson and May 1991). Mean time-to-death of exposed *Plethodon metcalfi* (36.0 d) was similar to that of other experimentally infected amphibians (mean time to death = 41.8 d in *Rana muscosa* (Rachowicz & Vredenburg 2004) and 40.0 d in *Mixophyes fasciolatus* (Berger et al. 2004). However, it is difficult to compare infection period and mortality rates between experiments that use different isolates of *Batrachochytrium dendrobatidis*, exposure times, doses, inoculum concentrations, and temperatures.

Plethodon metcalfi exposed to *Batrachochytrium dendrobatidis* experienced higher mortality at 8°C than at 16°C. However, Piotrowski et al. (2004) observed highest growth of *B. dendrobatidis* in cultures at 17°C, with growth slowing at 10°C. Species-specific differences in expression of cutaneous antimicrobial peptides could account for the discrepancy between our results and the *in vitro* study of *B. dendrobatidis*. For instance, *Rana sylvatica* held at 5°C do not secrete antimicrobial peptides on their skin, but do so at higher temperatures (Matutte et al. 2000). However, peptides isolated from *R. pipiens* were more effective at 10°C than at 22°C in an *in vitro B. dendrobatidis*-growth inhibition assay (Rollins-Smith et al. 2002). Salamanders could experience seasonal variation in cutaneous microflora, which would translate to susceptibility varying according to temperature. More studies should test the effects of temperature on susceptibility, including realistic fluctuating temperature regimes.

The terrestrial nature of *Plethodon* salamanders does not eliminate their risk of *Batrachochytrium dendrobatidis* infection. *B. dendrobatidis* is an aquatic fungus; however, like other Chytridiomycetes, it likely can survive in moist substrate (Johnson and Speare 2005). Aquatic salamanders traveling away from streams could spread *B. dendrobatidis* to terrestrial habitats of *Plethodon* spp. In the Nantahala National Forest, adult *Desmognathus monticola* travel up to 8.6 m away from streams during nocturnal movements (Crawford & Semlitsch 2007). As we have demonstrated, *D. monticola* can be infected with *B. dendrobatidis* and thus could serve as carriers of the pathogen.

Our results suggest that *Plethodon* species in the southeastern United States are at risk of becoming infected with *Batrachochytrium dendrobatidis* and developing chytridiomycosis. However, some animals may survive with or clear infection if given enough time, similar to *Ambystoma tigrinum* (Davidson et al. 2003). Additional studies are required to determine if chytridiomycosis is likely to be a significant factor in declines of plethodontids.

ACKNOWLEDGEMENTS

We thank S. Rothermel for help with animal collection, L. Hardeman for animal care, and H. Vazquez for lab development. This research was funded by the Amphibian Research and Monitoring Initiative of U.S. Geological Survey, through a Declining Amphibian Population Task Force Seed Grant to B. B. R. and V. M. V, and by a Consortium for Conservation Medicine grant to V. M. V. Proposal preparation and fieldwork were supported by Savannah River Ecology Laboratory (under Financial Assistance Award DE-FC09-96SR18-546 between University of Georgia and U.S. Department of Energy). Salamander collection was conducted under a U.S. Forest Service permit (authorization HIG651801) and a North Carolina Wildlife Resources Commission 2006 Wildlife Collecting License (permit #964). Laboratory procedures were approved by the University of Georgia's Animal Care and Use Committee (Animal Welfare Assurance # A3437-01).

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Table 3.1: *Desmognathus monticola* and *Plethodon metcalfii*. Salamanders examined comprehensively by histology. Euthanized salamanders survived the duration of experiment. All salamanders listed were exposed to *Batrachochytrium dendrobatidis* (Bd). N/A: not applicable; PCR not conducted because initial histology of feet (not shown here) was conclusive.

Desmognathus monticola

ID	Temperature (°C)	Histologic examination	PCR Result	Outcome	Days Post-Infection
DM1	8	Moderate lesions consistent w/ Bd; Bd thalli absent	–	Died	40
DM11	8	No lesions	+	Euthanized	189
DM13	16	No lesions	N/A	Euthanized	189

Plethodon metcalfi

ID	Temperature (°C)	Histologic examination	PCR Result	Outcome	Days Post-Infection
PM1	8	Lesions consistent w/ Bd; Bd thalli present	N/A	Died	18
PM5	8	Lesions consistent w/ Bd; Bd thalli present	N/A	Died	31
PM6	8	Lesions consistent w/ Bd; Bd thalli present	+	Died	32
PM10	8	Mild lesions consistent w/ Bd; Bd thalli absent	N/A	Died	43
PM19	16	No lesions; Bd thalli absent	–	Euthanized	18

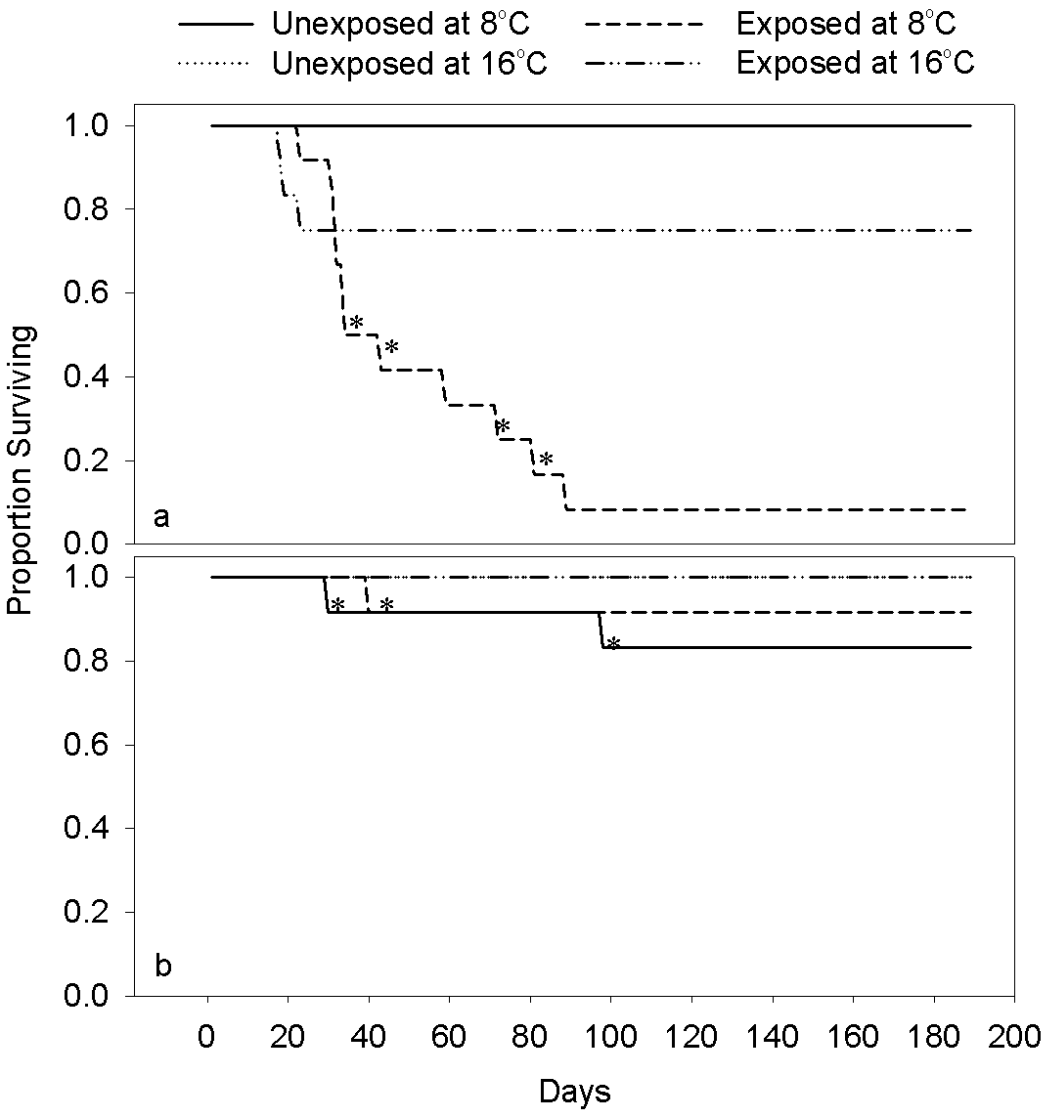

Fig. 3.1: Survival curves for (a) *Plethodon metcalfi* and (b) *Desmognathus monticola*. Unexposed *P. metcalfi* 8ºC and 16ºC lines are overlaid because both groups experienced complete survival. Unexposed and exposed *D. monticola* 16ºC lines are overlaid because both groups experienced complete survival. Asterisks indicate salamander deaths due to unknown causes.

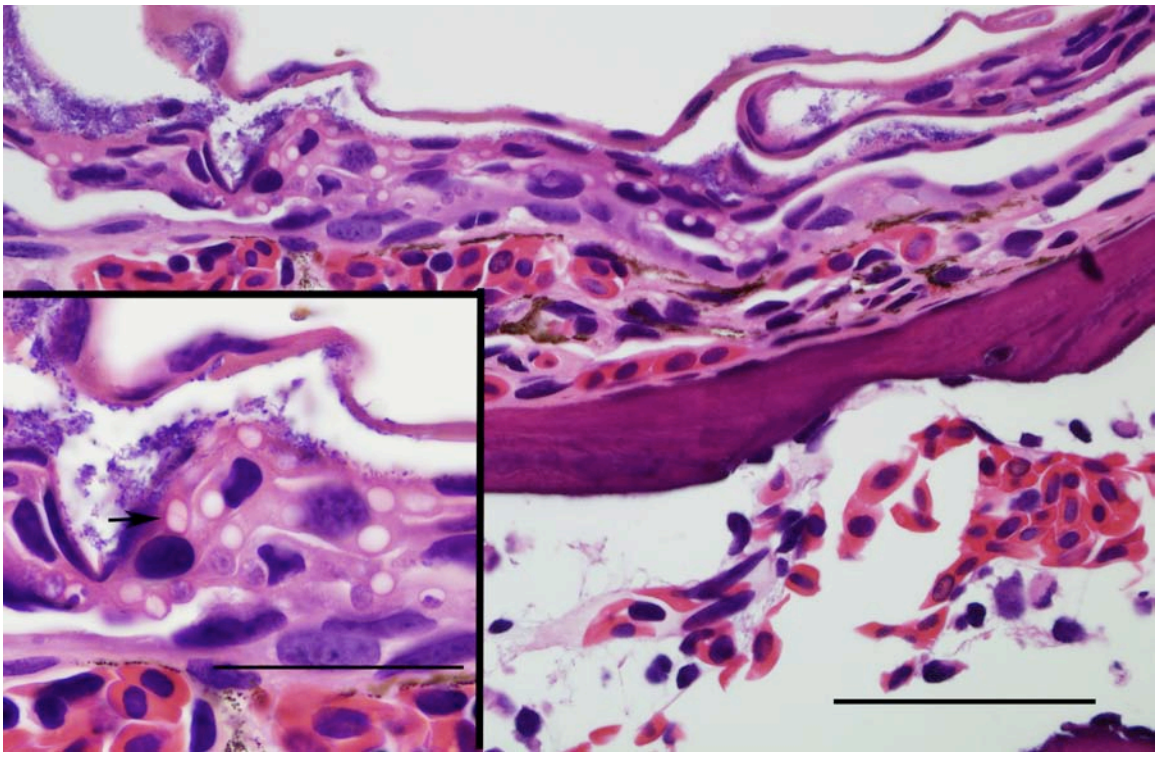

Fig. 3.2: *Batrachochytrium dendrobatidis* infecting *Plethodon metcalfi*. Histologic section of skin from the foot. There is moderate parakeratotic hyperkeratosis with intracellular thalli of *B. dendrobatidis* (arrows). Scale bar = 100 µm. Inset shows detail of thalli including an internally septate thallus (arrow; colonial thallus characteristic of *B. dendrobatidis*). Thalli in this image are empty, having discharged their zoospores. Scale bar = 50 µm. (H&E)

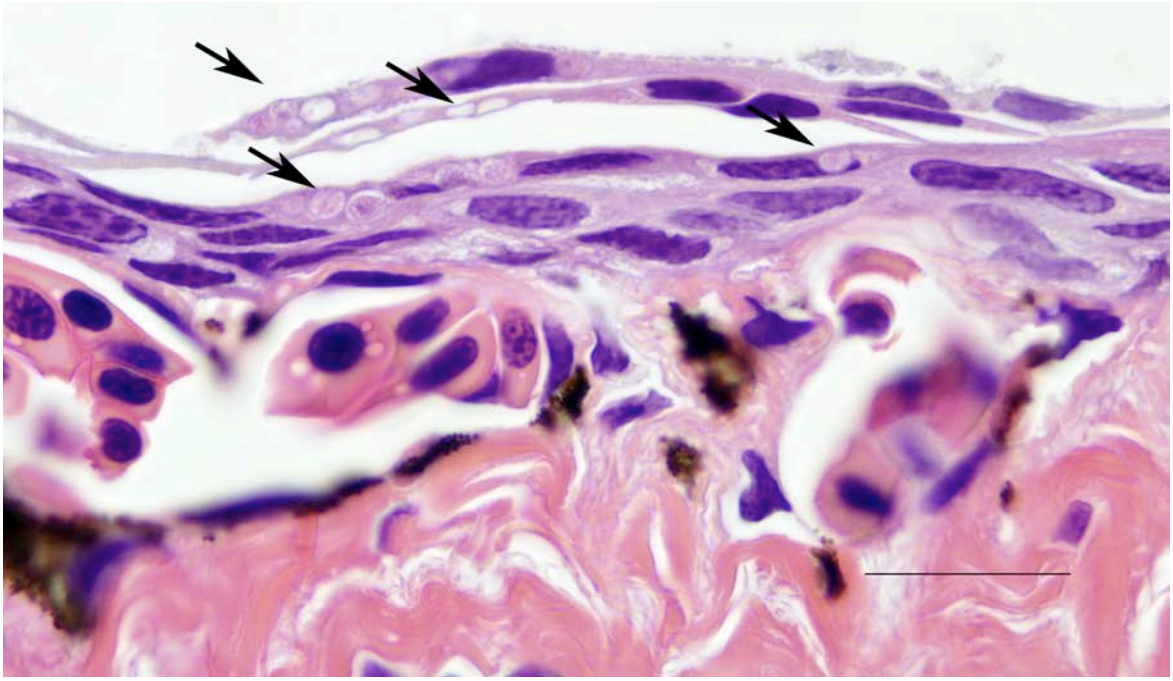

Fig. 3.3: *Batrachochytrium dendrobatidis* infecting *Plethodon metcalfi*. Histologic section of skin from the lateral body. There is mild focal hyperkeratosis with small numbers of intracellular chytrid thalli (arrows). Scale bar = 200 µm. (H&E)

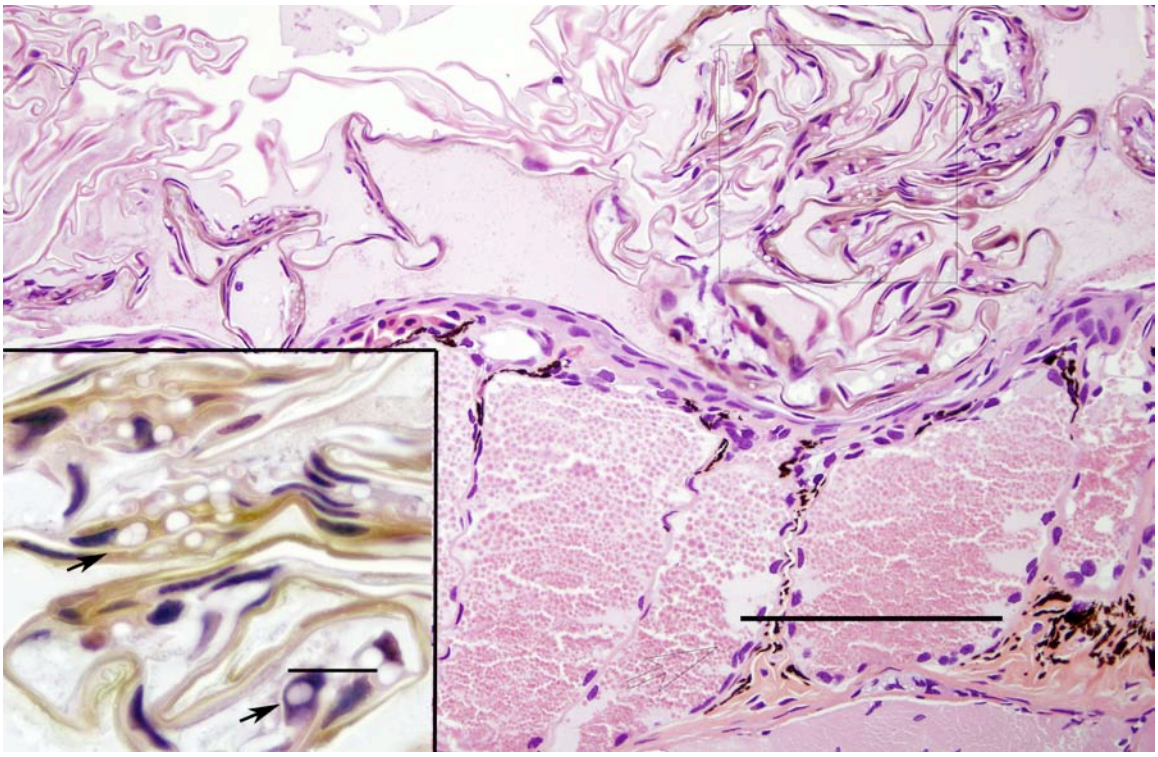

Fig. 3.4: *Batrachochytrium dendrobatidis* infecting *Plethodon metcalfi*. Histologic section of skin from the tail. There is moderate parakeratotic hyperkeratosis with intracellular *B. dendrobatidis* thalli. Scale bar = 200 µm. Higher magnification of the area within the box shows detail of the thalli, including internally septate colonial thallus (arrows). Scale bar = 20 µm. (H&E)

CHAPTER 4

ROLE OF CUTANEOUS PEPTIDES IN PROTECTION AGAINST *BATRACHOCHYTRIUM DENDROBATIDIS* INFECTION IN *RANA CATESBEIANA*[3]

[3] Vazquez, V. M., L. K. Reinert, L. Rollins-Smith, M. J. Yabsley, D. Woodhams, L. Subraveti, K. Smith. To be submitted to *Developmental and Comparative Immunology*.

ABSTRACT

Chytridiomycosis is a deadly amphibian disease that has devastated many populations throughout the world. Yet many species appear resistant to the disease, including the American bullfrog (*Rana catesbeiana*). Cutaneous peptides secreted from amphibian skin are known to be effective *in vitro* at inhibiting growth of *Batrachochytrium dendrobatidis*, the causative agent of the disease. In this study we examined *in vivo* the role of cutaneous peptides of *R. catesbeiana* in preventing or minimizing infections with *B. dendrobatidis*. We depleted frogs of peptides and then exposed them to approximately 10 million *B. dendrobatidis* zoospores. All frogs became infected with *B. dendrobatidis* but no clinical or histological signs of disease were observed in any frog. Also, the natural mixtures of peptides of *R. catesbeiana* had low effectiveness against *B. dendrobatidis in vitro*. Therefore antimicrobial peptides are not the sole source of resistance to chytridiomycosis in *R. catesbeiana*.

KEYWORDS

Antimicrobial peptides, chytridiomycosis, fungus

1. INTRODUCTION

Chytridiomycosis is a deadly fungal disease that has devastated amphibian populations in Australia, Costa Rica, and Panama (Berger et al. 1998, Daszak et al. 1999, Lips et al. 2006) and has been discovered throughout North and South America (Muths et al. 2003, Briggs et al. 2005, Longcore et al. 2007, Pearl et al. 2007, Schlaepfer et al. 2007, Rothermel et al. 2008) and Europe (Bosch et al. 2001, Cunningham et al. 2005, Bosch & Martinez-Solano 2006) and in parts of Africa (Weldon et al. 2004) and Asia (L. Schloegel, pers. comm.). The disease is caused by the chytridiomycete *Batrachochytrium dendrobatidis*, a microscopic aquatic fungus with a motile zoospore and a sessile sporangium (Longcore et al. 1999). *B. dendrobatidis* infects the keratinized epidermal tissue of amphibians (Berger et al. 2005a) and is hypothesized to cause death by

preventing osmoregulation (Voyles et al. 2007). It causes mortality only in adults, in which it primarily infects the ventral side of the organism (Berger et al. 2005b). *B. dendrobatidis* is transmitted via water (Rachowicz & Vredenburg 2004) or contact with an infected host (L. Schloegel, pers. comm.) and is thought to persist in biological or environmental reservoirs (Rachowicz & Vredenburg 2004, Lips et al. 2006, Kirshtein et al. 2007).

Some amphibian species persist throughout chytridiomycosis-induced mortality events (Retallick et al. 2004, Lips et al. 2006). Antimicrobial peptides have been suggested to provide protection in such species (Rollins-Smith et al. 2002a, Rollins-Smith et al. 2002b). Antimicrobial peptides are polypeptide chains of 20-46 amino acids (Carey et al. 1999) secreted from granular glands in amphibian skin in response to stress, including injury (Conlon et al. 2004). Norepinephrine injected subcutaneously can induce this stress response in amphibians, causing them to secrete their peptides (Conlon et al. 2004). Some peptides are active against *Batrachochytrium dendrobatidis in vitro* (Rollins-Smith et al. 2002a, Rollins-Smith et al. 2002b, Woodhams et al. 2007). The role of peptides in protection against *B. dendrobatidis in vivo* has been tested in the African clawed frog, *Xenopus laevis*, which shed higher levels of zoospores in frogs depleted of their peptide stores than in those with normal peptide stores (L. Rollins-Smith, pers. comm.).

Rana catesbeiana, the American bullfrog is another amphibian species resistant to developing chytridiomycosis (Daszak et al. 2004). *R. catesbeiana* is native to the eastern United States, Canada, and parts of Mexico. It is an introduced species in the western United States and in 25 other countries (IUCN et al. 2004). *R. catesbeiana* has been observed infected with *B. dendrobatidis* in the wild (Hanselmann et al. 2004, Rothermel et al. 2008) and in farm raised-frogs (Mazzoni et al. 2003). Experimental exposure of *R. catesbeiana* to high doses of *B. dendrobatidis* resulted in infection, but no frogs developed chytridiomycosis (Daszak et al. 2004). The global distribution of *R.*

catesbeiana combined with its susceptibility to asymptomatic infections with *B. dendrobatidis* makes it a threat to native amphibian species. In this study we examined *in vivo* the role of cutaneous peptides of *R. catesbeiana* in preventing or minimizing infections with *B. dendrobatidis.*

2. MATERIALS AND METHODS

2.1 Maintenance of host and pathogen

Captive bred recent metamorphs of *Rana catesbeiana* were obtained from Rana Ranch (Twin Falls, Idaho) and acclimated to laboratory conditions for 14 wk. *R. catesbeiana* were housed individually in five-gallon buckets with a 1.27 cm diameter hole stopped with a 1.75 cm x 1.27 cm or similarly sized rubber stopper for drainage. Frogs were provided 3.0 L of dechlorinated H_2O, 32.39 cm long x 5.08 cm diameter PVC pipe for perching, and two sheets of unbleached paper towel for cover. Buckets were secured with plastic lids with a 10.16 cm hole covered in mesh. Frogs were maintained on a 14-hour day/10-hour night light cycle. Water and paper towel were changed biweekly and the inside of the bucket cleaned as needed. Frogs were fed 10 adult crickets weekly. Paper towels were removed at the onset of the infection experiment (see below section 2.3).

Batrachochytridum dendrobatidis isolate JEL 197 was obtained form Joyce Longcore (U. Maine, Orono, ME, USA) who originally isolated it from a dead *Dendrobates azureus* that had been kept in captivity (Longcore 1999). Cultures were maintained in 1% tryptone broth (10 g tryptone, 1 L H_2O). To harvest zoospores, 1 ml of *B. dendrobatidis* in broth was transferred to a 1% tryptone agar (10 g tryptone, 10 g agar, 1 L H_2O) plate and incubated at 23ºC for 7-8 days or until zoospores were abundant. Each plate was flooded with 20 ml ddH_2O for 30 min. The liquid was then pooled and the zoospores in the liquid counted using a hemacytometer.

2.2 Determination of norepinephrine dose for peptide depletion

Peptides were collected from *Rana catesbeiana* as described in Rollins-Smith et al. (2002). Six doses of norepinephrine and a control (Fig. 4.1) were injected in the dorsal lymph sac of *R. catesbeiana* (n = 10 per dose and control). Individuals were placed in 50-100ml of collecting buffer **(**2.92 g NaCl, 2.05 g Na Acetate, 1.0 L HPLC-grade H_20**)** for 15 minutes. Frogs were removed from buffer, rinsed with dechlorinated H_2O, measured, and swabbed for polymerase chain reaction (PCR) assay of *Batrachochytrium dendrobatidis* presence (see below section 2.4). One ml of 50% HCl was immediately added to the collecting buffer containing secreted peptides. Sep-pak Plus C-18 cartridges (sep-paks; Waters Corporation) were prepared by passing through 10 ml of methanol and 10 ml of buffer A (1.0 ml HCl, 1.0 L HPLC-grade H_2O) for every 2 sep-paks. The liquid containing collecting buffer, HCL, and peptides was then passed through 2-6 sep-paks for cationic and hydrophobic peptide enrichment (Woodhams et al. 2007). The sep-paks were placed in 50 ml conical tubes (Corning) with 2 ml of buffer A to preserve samples during short-term storage. The peptides were washed with 10 ml/sep-pak of 0.1% trifluoroacetic acid, 99.9% HPLC-grade H_2O (v/v/v) and then eluted with 10 ml/Sep-pak 70% acetonitrile, 29.9% water, and 0.1% trifluoroacetic acid (v/v/v). Peptides were dried by vacuum centrifugation. The Pierce Micro BCA Assay (Pierce, Rockford) was used according to instructions to quantify the peptides, except Bradykinin (Sigma) was used as the standard (Woodhams et al. 2006a). The MRX Microplate Reader (Dynex Technologies) was used at 570 nm to measure the samples.

2.3 Depletion of peptides and exposure to *Batrachochytrium dendrobatidis*

Frogs in the depletion treatment group (n = 10) were injected with 80 nmol/g body weight norepinephrine. Control frogs (n = 10) were injected with an equivalent volume of amphibian phosphate-buffered saline (6.6 g NaCl, 2.17 g $Na_2HPO_4 \cdot 7H_2O$, 0.2 g KH_2PO_4, 1 L ddH_2O, pH = 7.4). The secreted peptides of five depletion frogs were

collected as described above (section 2.2) to verify depletion of peptides. Two days later all frogs were exposed to 8.89 x 10^7 zoospores of *B. dendrobatidis* in 137.5 ml of sterile dechlorinated H_2O in 708 ml Gladware containers (Chlorox Company) for 24 h. Frogs were then returned to normal housing and observed daily. The experiment was terminated after 72 d with no signs of disease.

2.4 Detection of *Batrachochytrium dendrobatidis* infection

All frogs were tested for the presence of *B. dendrobatidis* by PCR assay. Frogs were swabbed with a cotton-tipped cleaning stick (Puritan) 40 times each on the ventral side of both thighs and rear feet, the area of the frog most likely to be infected with the fungus (Berger et al. 2005b). All frogs were tested by conventional PCR before the start of the study and frogs exposed to *B. dendrobatidis* were tested five times during the experiment. At the end of the experiment all frogs were swabbed for real-time PCR assay and then euthanized. One foot per frog was removed for histological analysis.

For conventional PCR, DNA was extracted from swabs using the DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's instructions for animal tissues. PCR to detect *B. dendrobatidis* was conducted as previously described (Boyle et al. 2004) with the following modifications: PCR reactions (25µl) included 11 µl molecular biology grade water, 2.5 µl 25mM $MgCl_2$, 5 µl 5X green buffer (Promega), 0.25 µl 20 mM dNTPs (Promega), 0.5 µl of each primer (50µM), 0.25 µl GoTaq Flexi DNA Polymerase (Promega), and 5 µl of sample DNA. Cycling was conducted in a DNA Engine Thermal Cycler (Bio-Rad) and conditions were 50 cycles of 94°C for 30s, 50°C for 30s, and 72°C for 30s. DNA from a culture of *B. dendrobatidis* was used as a positive control. Each sample was run in triplicate and was considered positive if two or three samples were positive. Samples that were only positive once were considered indeterminate. For real-time PCR, DNA extraction and PCR were conducted according to

the protocol in Boyle et al. (2004) except for the following changes: 60 µl Prepman DNA extraction buffer instead of 40 µl; 0.1µM of probe; the default Stratagene cycles and Stratagene thermocycler; and DNA at either 1/10 or 1/100 dilution in water. Feet for histology were preserved in 10% buffered formalin for at least 24 h. The feet were then decalcified, embedded in paraffin, sectioned, placed on a slide, and stained with hematoxylin and eosin. Slides were observed uder 400x magnification for the presence of *B. dendrobatidis* sporangia in the stratum corneum.

2.5 MALDI mass spectrometry of collected peptides

Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry was conducted to determine the peptides secreted from *Rana catesbeiana* (Conlon et al. 2004). Carbon-filled polyethylene film (Goodfellow) was activated with 100% HPLC methanol and then rubbed on frog skin to obtain peptide samples. A 0.6µl peptide matrix was spotted onto the blots, the blots were allowed to dry, and then the blots were run in the Voyager-DE STR mass spectrometer (Perspective Biosystems). The machine was calibrated using 0.6 µl of peptide standards and 0.6 µl matrix spotted on an activated blot. The following peptide standards were used: Bradykinin fragment 1-7 (757.3997 MW, Sigma), Angiotensin II, human (1046.5423 MW, Sigma), P14R, synthetic peptide (1533.8582 MW, Sigma), ACTH fragment 18-39, human (2465.1989 MW, Sigma). Alpha-Cyano-4-hydroxycinnamic acid (CHCA; Sigma) was used for the matrix.

2.6 *Batrachochytrium dendrobatidis* inhibition assay

Batrachochytrium dendrobatidis was prepared for the assay by growing in a flask with 1% tryptone broth at 21-22ºC for a week and then subculturing to a tryptone agar plate at 21-22ºC for 6-7 d. On the day of the assay, the plate was flooded with 1 ml 1% tryptone broth and the liquid immediately collected. Then an additional 3 ml of broth was added and left on the plate for 20 min and then collected. An additional 1.5 ml was

added to the plate and immediately collected. All 3 fractions were pooled, and then vacuum filtered over a 20 µm sterile nylon Spectra/Mesh Macroporous Filter (Spectrum Laboratories) to remove sporangia. Lugol's solution (Sigma) was added to a small sample of the pooled liquid to kill and stain zoospores for counting with a hemacytometer. The pooled liquid was diluted to a final concentration of $1x10^6$ zoospores ml^{-1}.

Collected peptides (see above section 2.2) were reususpended in HPLC water and stored at –20ºC. The following peptide concentrations were cultured with *Batrachochytrium dendrobatidis* zoospores at 21-22ºC: 1.56, 3.13, 6.12, 6.25, 12.5, 25, 50, 100, 200, 250, 500, and 1000 µg/ml. Positive controls (n=235) were comprised of live zoospores and negative controls (n = 182) were comprised of heat-killed zoospores. Growth of *B. dendrobatidis* was measured with a MRX Microplate Reader (Dynex Technologies) at 490 nm after 7 d. The minimal inhibitory concentration (MIC) was determined for the natural mixture of peptides secreted by *Rana catesbeiana*. The minimal inhibitory concentration of peptides is defined as the lowest concentration of peptides that completely inhibits the growth of a microorganism.

2.7 Statistics

SAS Software 9.1 (SAS Institute, Inc., Cary, NC) was used for statistical analyses. The PROC TTEST program was used to determine if there was a difference in weight change between treatment groups. This program was also used to determine if the mean number of zoospores shed differed between frogs depleted of their peptide stores and control frogs. The PROC ANOVA program was used to determine if there was a difference in concentration of peptides secreted by frogs injected with different doses of norepinephrine (section 2.2). The PROC GLM program was used to run a two-way analysis of variance (ANOVA) of unbalanced data to determine if the dose of norepinephrine injected into frogs and/or the peptide concentration used in the

Batrachochytrium dendrobatidis inhibition assay affected the results of the assay (section 2.6). After determining the dose did not have an effect (F = 1.85, P = 0.1036), data from the different norepinephrine doses was compiled and the PROC ANOVA program used to determine any differences in growth of *B. dendrobatidis* in the presence of different concentrations of peptides.

3. RESULTS

3.1 Infection status of frogs throughout experiment

Neither clinical nor histological signs of disease were observed in any frog (Fig. 4.1). There was no significant difference in weight change over the course of the experiment between depleted and undepleted frogs (t-test, t = 1.09, p = 0.293; Fig. 4.2). Two *Rana catesbeiana* in each treatment group tested positive by conventional PCR for *Batrachochytrium dendrobatidis* infection before the start of the experiment. Infection status fluctuated in five undepleted and two depleted frogs (Table 4.1), not considering the indeterminate results. Undepleted frogs shed an average of 3915 zoospore equivalents compared to 945 zoospore equivalents for depleted frogs, a statistically insignificant difference (t-test, t = 0.88, p = 0.3945). The second run yielded similar results, although *R. catesbeiana* 29 and 32, which were previously found negative, had 11 and 425 zoospore equivalents, respectively. Based on the second run of real-time PCR, the depleted frogs secreted an average11, 502 zoospore equivalents each compared to 2,966 zoospore equivalents for undepleted frogs. This difference also was not significant (t-test, t = 1.18, p = 0.2638).

3.2. Concentration and classification of peptides secreted from *Rana catesbeiana*

The concentration of peptides secreted from control *Rana catesbeiana* was significantly different from that secreted from *R. catesbeiana* injected with 30, 40, or 80 nmol/g body weight norepinephrine; there was no significant difference between

concentrations of peptides secreted from *R. catesbeiana* injected with 10, 20, 30, 40, 80, or 160 nmol/g body weight norepinephrine (One-way ANOVA, $F = 4.11$, $p = 0.0015$; Fig. 4.3). The concentration of peptides secreted from frogs injected with the moderate dose of 10 nmol/g body weight norepinephrine (Woodhams et al. 2006b) was 186.925 ± 61.3839 µg/g body weight. The following peptides were collected noninvasively (see above section 2.5) from nine R. *catesbeiana*, with the number of frogs secreting them in parentheses: Ranatuerin 1 (5), Ranatuerin 2 (5), Ranatuerin 3 (4), Ranatuerin 4 (2), Ranatuerin 6/7 (8), Ranatuerin 8 (6), Ranatuerin 9 + Na (1) (www.uniprot.org). One frog did not secrete any previously described peptides. Figure 4.4 is a representative MALDI mass spectrometry profile of skin peptides secreted from an individual *R. catesbeiana*.

3.3 *Batrachochytrium dendrobatidis* inhibition assays

Peptide mixtures obtained from *Rana catesbeiana* reduced the growth of *Batrachochytrium dendrobatidis*, with greater reductions observed at higher concentrations of peptides (Fig. 4.5). However, even at the highest concentration of peptides studied (1000 µg/ml), *B. dendrobatidis* growth was not completely inhibited. The growth of *B. dendrobatidis* was not statistically the same for all peptide concentrations (One-way ANOVA, $F = 100.22$, $p < 0.0001$; Fig. 4.5). The following peptide concentrations significantly decreased the growth of *B. dendrobatidis*: 50-1000 µg/ml. Peptide concentrations of 500 and 1000 µg/ml were not significantly different from the negative control (Tukey's Studentized Range Adhoc Test); therefore, the MIC of a natural mixture of peptides from *R. catesbeiana* is 500 µg/ml. The peptide effectiveness at 50µg/ml of peptides secreted from frogs injected with 10 nmol/g body weight (Woodhams et al. 2006b) was 13.3 ± 12.9.

4. DISCUSSION

All *Rana catesbeiana* in both treatment groups were infected with *Batrachochytrium dendrobatidis* by the end of the experiment, demonstrating the high susceptibility of this species. However, all frogs remained free of clinical or histological signs of chytridiomycosis, suggesting antimicrobial peptides are not responsible for this species' resistance to this disease. Furthermore, the lack of observation of *B. dendrobatidis* sporangia in histological sections indicates these frogs had light infections, since histology has been demonstrated to be less sensitive than PCR at detecting the presence of the fungus (Kriger et al. 2006). The infection status of seven frogs, as indicated by conventional PCR assay, switched more than once throughout the 72 d study. This result suggests that conventional PCR assay is not reliable in determining the infection status of amphibians with mild *B. dendrobatidis* infections. The infection severity, measured in shed zoospores, was not statistically different between depleted and undepleted frogs. However, the lack of a significant difference could be due to the low sample size. Also, swabbing frogs for real-time PCR may not be reliable because pressure could be applied differently on each frog, thus resulting in differences in zoospore equivalents due to sampling error. The great variation in shed zoospores observed (11 to 1059 for undepleted frogs and 573 to 48,192 for depleted frogs) supports this suggestion. The preferred method is to place a frog in a water bath to collect shed zoospores over a period of time and then filter the water to concentrate the zoospores for real-time PCR assay (Hyatt et al. 2007). This method was attempted, but problems arose likely due to the large quantity of water used in this study compared to that in the protocol (Hyatt et al. 2007). The lack of statistical difference in weight change of frogs between treatment groups and, more importantly, the greater weight gain observed in the depleted frogs indicates infection with *B. dendrobatidis* does not inhibit growth of *R. catesbeiana.*

The experiment to determine the dose of norepinephrine required to deplete *Rana catesbeiana* of their peptides yielded unexpected results. The lack of a significant difference between all doses of norepinephrine could be due to the low sample size per dose. The cause of the sharp decrease in peptides secreted from frogs injected with 160 nmol/g body weight compared to 80 nmol/g body weight needs to be explored. Because there was no statistical difference in the concentration of peptides secreted from frogs injected with 10-160 nmol/g body weight norepinephrine, it was reasonable to choose 80 nmol/g body weight as the experimental norepinephrine dose, a dose that was successful in depleting *Xenopus laevis* of its peptides (Rollins-Smith et al. 2005).

The concentration of peptides secreted by *Rana catesbeiana* compared to other amphibians is also of interest. *Rana catesbeiana* injected with 10 nmol/g body weight norepinephrine secreted substantially less peptides (186.9 ± 61.4 µg/g body weight) than *Xenopus laevis* (1895.4 ± 182.1 µg/g body weight), *Centrolene prosoblepon* (766.5 ± 451.0 µg/g body weight), *Phyllomedusa lemur* (2248.5 ± 351.2 µg/g body weight), *Rana pipiens* (816.7 ± 74.5 µg/g body weight), *Colostethus panamensis* (529.2 ± 86.5µg/g body weight), and *Centrolene ilex* (611.8 ± 267.9 µg/g body weight) (Woodhams et al. 2006b). *X. laevis*, a species resistant to chytridiomycosis, has a peptide effectiveness of over 60 (Woodhams et al. 2006b). However, the peptide effectiveness of the natural mixture of peptides from *R. catesbeiana* is 13.2, considered weak resistance (Woodhams et al. 2006b). This lends doubt to the hypothesis put forward by Woodhams et al. (2006b) that a species' resistance can be predicted by the *in vitro* efficacy of its skin peptides in preventing *Batrachochytrium dendrobatidis* growth.

The peptides secreted from *Rana catesbeiana* in this study have all previously been isolated from this species (Goraya et al. 1998). Ranatuerin-2 is active against *Batrachochytrium dendrobatidis* (Rollins-Smith et al. 2002b), but the other peptides have not been tested individually. The low peptide effectiveness of the natural mixture of

peptides secreted by *R. catesbeiana* could be due to low activity of specific antimicrobial peptides. More research is necessary to determine the effectiveness of individual *R. catesbeiana* peptides against *B. dendrobatidis*.

Rana catesbeiana peptides inhibit the growth of *Batrachochytrium dendrobatidis in vitro*; yet removal of these peptides does not result in development of chytridiomycosis or even an increase in the level of infection with *B. dendrobatidis*. Another layer of defense against *B. dendrobatidis* may be present in *R. catesbeiana*, but possibly not in species susceptible to developing chytridiomycosis. Cutaneous microflora have been suggested as a protective trait of amphibians (Harris et al. 2006), but they have yet to be tested in *R. catesbeiana*. Further research is necessary to determine the source of resistance to chytridiomycosis in *R. catesbeiana*. This information could be vital in predicting which species are at greatest risk from chytridiomycosis.

ACKNOWLEDGEMENTS

We thank A. Page, T. Lugo, and W. Hanna for laboratory work and caring for the frogs. Funding was received from the Consortium for Conservation Medicine (V. V.). Experiments were conducted with approval from the University of Georgia Institutional Animal Care and Use Committee (Animal Welfare Assurance #A3437-01.).

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Table 4.1: *Batrachochytrium dendrobatidis* infection status of *Rana catesbeiana* throughout experiment as tested by conventional PCR. —: negative result in all three runs; +: positive result in two or more runs; IND: one positive and two negative results in three runs; NR: no results due to error in processing. Day 72 results in zoospore equivalents as determined by real-time PCR. †Second run of real-time PCR with the same samples. *Sample from day 41 when frog died. Real-time PCR was not run on this frog.

		Days After Exposure to Bd							
	Frog Id	Pre	7	14	28	35	42	72	72†
Undepleted	6	—	+	—	+	+	+	2.08	122.16
	14	—	+	—	+	—	+	147.6	2,796
	29	—	+	IND	+	IND	+	0	10.77
	31	IND	+	+	+	+	+	0	425.04
	41	+	+	+	+	+	+	41.5	333.72
	42	—	—	—	—	—	+	1.56	NR
	43	—	+	—	+	+	+	0.009	NR
	54	—	IND	—	+	+	+	7299.6	13,380
	62	+	—	—	—	—	+	327.6	825.72
	63	—	—	+	+	—	+	111.43	1059.12
Depleted	13	—	+	+	+	+	IND	24,684	48,192
	22	—	+	+	+	+	+	134.4	5,136
	23	—	+	+	+	NR	+	141.6	572.52
	28	—	+	+	+	NR	—	71.24	6,492
	36	—	+	+	+	+	+	1893.6	15,888
	38	—	+	+	+	NR	+	111.66	439.8
	39	+	+	+	+	+	+	1100.4	4,704
	40		—	+	IND	IND	IND	369.6	3,792
	67	+	+	+	+	NR	—	88.26	1,308
	70	—	—	+	+	+	+*	N/A	N/A

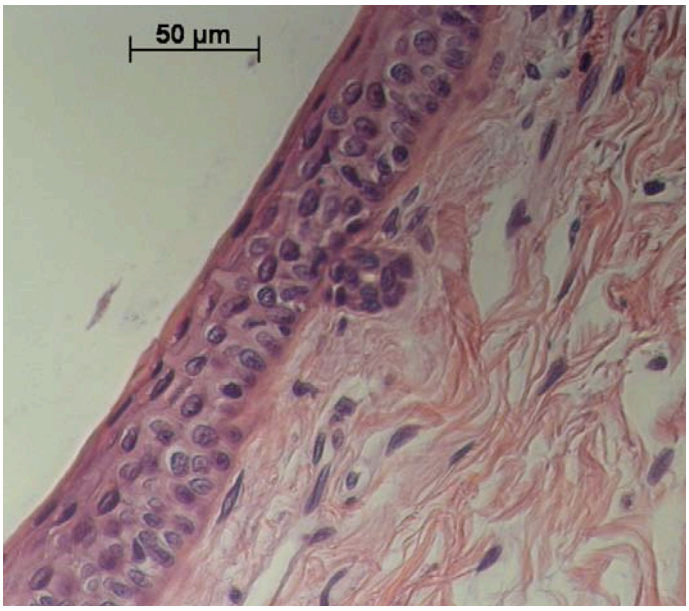

Fig. 4.1: Histologic section of skin from the foot of a *Rana catesbeiana* depleted of antimicrobial peptides and then exposed to *Batrachochytrium dendrobatidis*. No hyperkeratosis or sporangia seen. Scale bar = 50 µm.

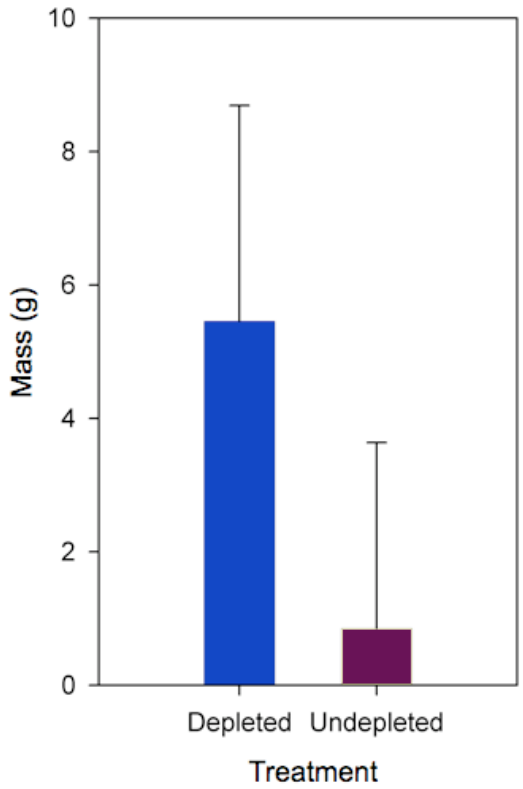

Fig 4.2: Change in body mass of *Rana catesbeiana* depleted and undepleted of their peptides.

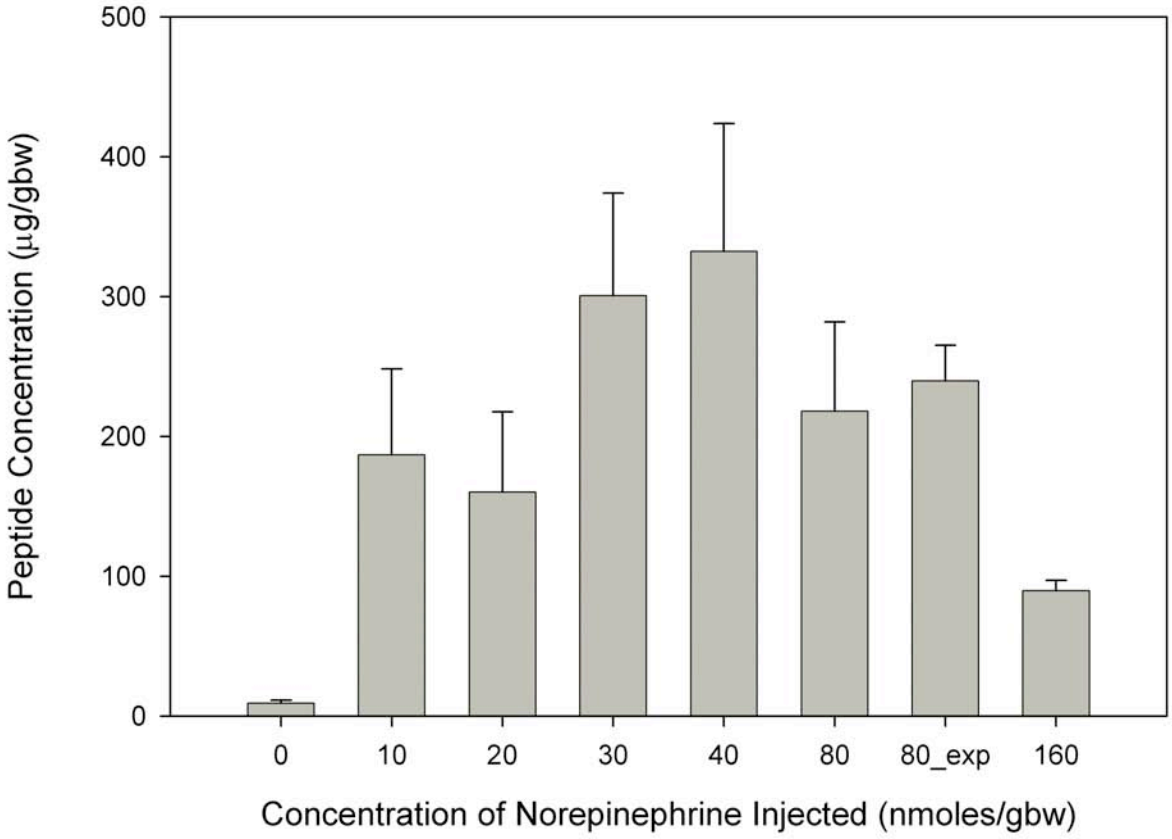

Fig. 4.3: Concentration of peptides (µg/g body weight frog) secreted from *Rana catesbeiana* when injected with six concentrations of norepinephrine (nmoles/g body weight frog) and a control solution of amphibian phosphate buffered saline (n = 10 per norepinephrine concentration and control). 80_exp = frogs in depletion experiment (see above section 2.3; n = 5).

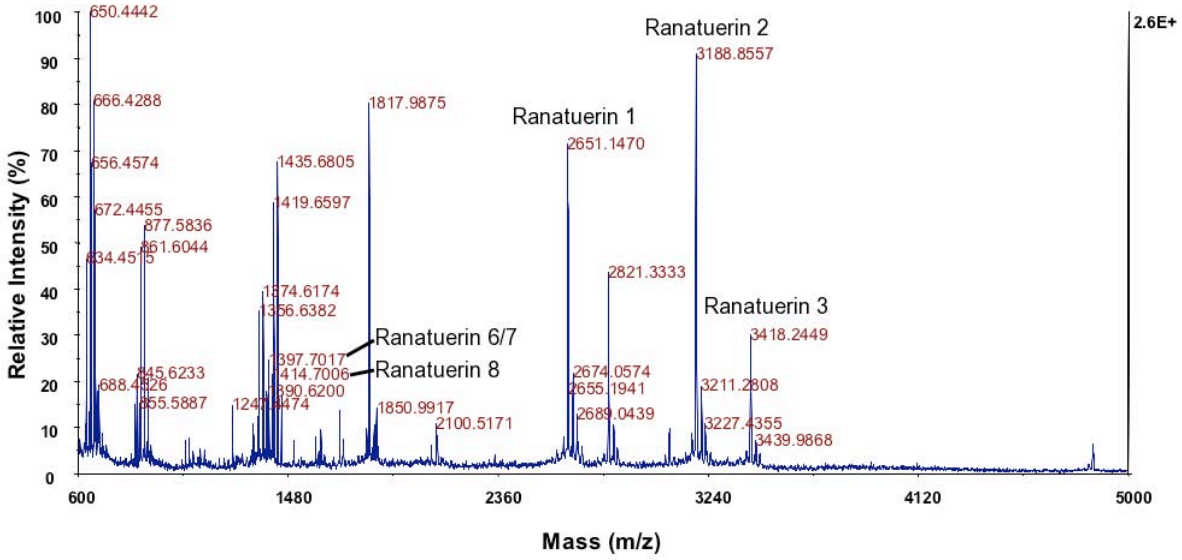

Fig. 4.4: Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry profile of skin peptides from *Rana catesbeiana*. Previously described antimicrobial peptides are labeled.

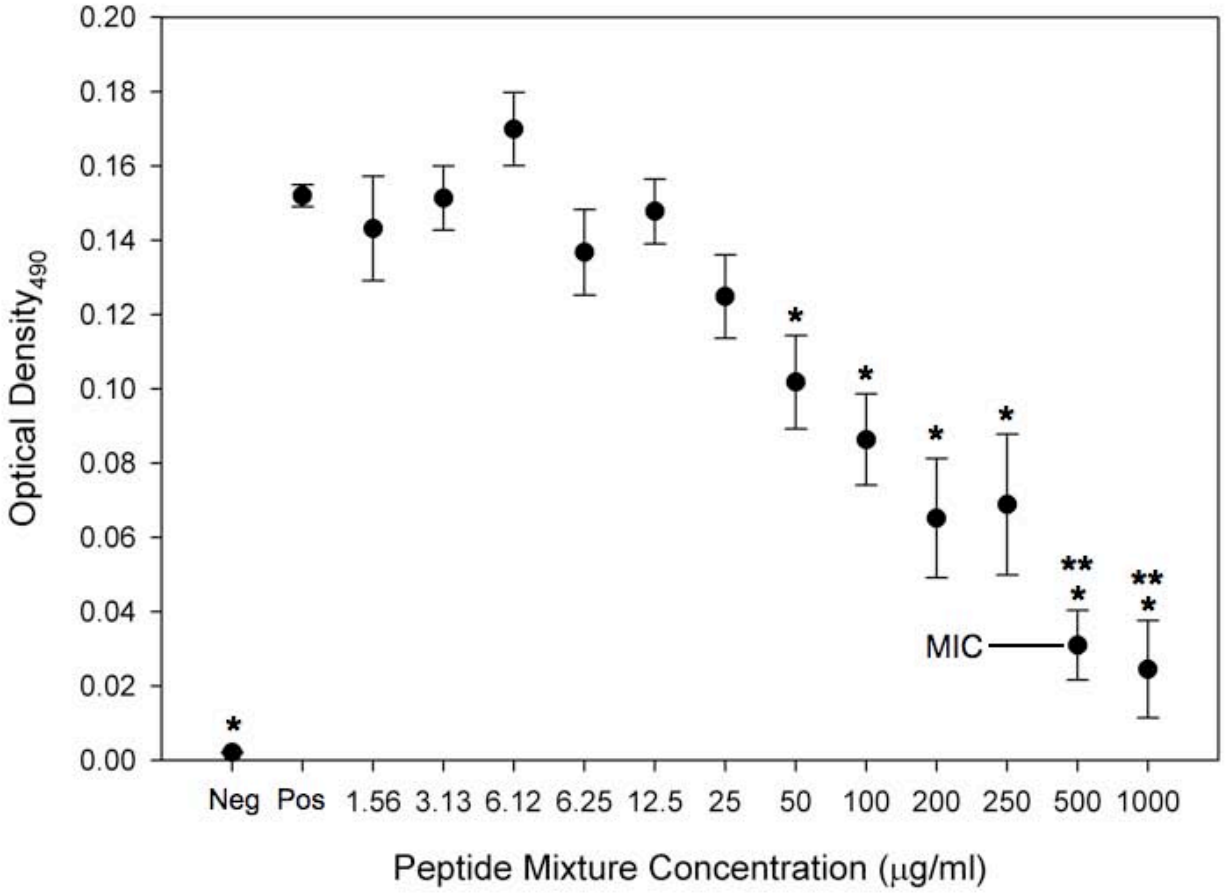

Fig. 4.5: Growth inhibition of *Batrachochytrium dendrobatidis* by natural mixtures of peptides secreted from *Rana catesbeiana*. *Significantly lower than positive control. **Not significantly different from negative control. MIC is the minimal inhibitory concentration of peptides at which no significant growth was observed.

CHAPTER 5

CONCLUSION

The factors determining susceptibility to chytridiomycosis are varied and likely numerous. Temperature (Chapters 2 and 3) and host phylogeny (Chapter 3) appear to be involved in susceptibility, but much is still unknown. Temperatures at the low end of the suitable range for the host appear to be important, but more studies must be conducted both in the lab and the field to ascertain if a pattern is truly present. Laboratory experiments with realistic fluctuating temperatures mimicking daily and seasonal changes should be conducted. Water temperature in host habitats should be measured routinely, since this value may be more relevant to the disease process.

Phylogeny of amphibian species may be linked to susceptibility to chytridiomycosis, but there is a suite of traits shared by related organisms that may be involved. More research is necessary on the susceptibility of various salamander species to chytridiomycosis to determine if the perceived pattern of difference in susceptibility between caudates and anurans is valid. If the pattern persists after rigorous research, then specific traits that may be different depending on phylogeny can be explored. Support or invalidation of the suggestion that amphibian phylogeny is relevant to susceptibility to chytridiomycosis is a useful tool to begin to tackle the long list of possible factors affecting susceptibility, such as innate and acquired immune responses, behavioral adaptations, and rates of skin shedding.

The immune system is an obvious possible source of resistance to chytridiomycosis. But immune system characteristics naturally vary across an entire class of organisms, so an experimental finding with one species of amphibians may not be relevant to all amphibians. Antimicrobial peptides are likely not the source of resistance to chytridiomycosis in *Rana catesbeiana*, but they still could be vital for protecting against this disease in other amphibian species.