Effect of Glucocorticoids on Gene Expression of Cutaneous Antimicrobial Peptides and Susceptibility to Chytridiomycosis in The Northern Leopard Frog (*Lithobates pipiens*)

by

Laetitia Jenny Tatiersky

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ABSTRACT

EFFECT OF GLUCOCORTICOIDS ON GENE EXPRESSION OF CUTANEOUS ANTIMICROBIAL PEPTIDES AND SUSCEPTIBILITY TO CHYTRIDIOMYCOSIS IN THE NORTHERN LEOPARD FROG (*LITHOBATES PIPIENS*)

Laetitia Jenny Tatiersky
University of Guelph, 2013

Advisor:
Dr. Jeff L. Caswell

This thesis is an investigation of the effect of corticosteroids on cutaneous innate immunity in frogs, in the context of infection with *Batrachochytrium dendrobatidis* (*Bd)*. Chytridiomycosis is an emerging cutaneous fungal disease that contributes to recent global declines and extinction of amphibian species, caused by infection of the skin with a fungus known as *Bd*. Many species of frogs secrete antimicrobial peptides onto their skin that are capable of killing *Bd*. The general hypothesis was that injections of glucocorticoids would impair the cutaneous synthesis of these antimicrobial peptides, thereby increasing susceptibility to *Bd* infection.

The objective of the first experiment was to measure and compare gene expression levels of cutaneous AMP's in frogs treated with glucocorticoids with sham-treated controls. Wild-caught *Lithobates pipiens* were acclimatized and administered either the corticosteroid methylprednisolone or saline every 48 hours. Norepinephrine-elicited cutaneous secretions were collected prior to the first injection of corticosteroid or saline, and then every 8 days for 40 days. Gene expression of the AMP's brevinin and ranatuerin in the cutaneous secretions was quantified relative to the reference genes EF1-α and RPL8 using reverse transcription quantitative polymerase chain reaction (RT-qPCR). Corticosteroid treatment was associated with a significant ($P<0.027$) increase in brevinin gene expression, which was most notable at 24-40 days of corticosteroid administration. Ranatuerin expression followed a similar but nonsignificant trend.

The second experiment was a pilot study intended to establish a *Bd* challenge protocol in *L. pipiens*. Frogs were immersed in water containing 0, 10^4, 10^5 or 10^6 zoospores of *Bd* strain JEL 423. Cutaneous swabs were collected prior to challenge and tested for *Bd* by qPCR; unexpectedly, some tested positive, indicating pre-challenge infection. The analysis was complicated by an identified cross-reactivity of the assay with other fungi.

The findings of the first experiment refuted the hypothesis, and suggested that corticosteroids promote rather than impair AMP gene expression in the skin of *L. pipiens*, under these experimental conditions. Further, the second study demonstrated that none of the frogs showed clinical abnormalities or died, despite exposure to *Bd* zoospores and despite molecular and histologic evidence of cutaneous *Bd* infection in some frogs.

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List of Abbreviations

AMP	Antimicrobial peptide
Bd	*Batrachochytrium dendrobatidis*
CMI	Cell-mediated immunity
Cp	Crossing point
EF1-α	Elongation factor 1-α
EPH	Endemic pathogen hypothesis
GAS	Group A *Streptococcus pyogenes*
GMS	Gomori's methanamine silver
HE	Hematoxylin and eosin
I-κB	Inhibitor of nuclear factor-κB
JEL 423	Strain of *Batrachochytrium dendrobatidis* isolated from infected frogs in Panama
NF-κB	Nuclear factor-κB
NPH	Novel pathogen hypothesis
PAS	Periodic acid Schiff
qPCR	Real-time polymerase chain reaction
RPL8	60S ribosomal protein L8
RT-qPCR	Reverse transcriptase real-time polymerase chain reaction
SES	Surface electrical stimulation
TAP	Tracheal antimicrobial peptide
TEM	Transmission electron microscopy
UV	Ultraviolet

Declaration of Work Performed

I performed the work reported in this thesis, with the following exceptions:

- Mary Ellen Clark performed the final preparations for sequencing regarding specificity of the *Bd* qPCR assay.
- Sequencing was provided by the Laboratory Services Division, University of Guelph, Canada.
- The histology division of the Animal Health Laboratory, University of Guelph, Canada prepared all of the histology slides including the additional stains.
- Dr. William Sears performed the statistical analyses that required the SAS program.

Chapter 1: Introduction

> "Of the diseases known from amphibians, one, chytridiomycosis, is clearly linked to population declines and extinctions. This fungal disease is appearing in new regions, causing rapid population disappearances in many amphibian species. It is the worst infectious disease ever recorded among vertebrates in terms of the number of species impacted, and its propensity to drive them to extinction." (Gascon et al 2007)

Chytridiomycosis is an emerging fungal disease of frogs, caused by the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*), and is implicated as one cause for recent global declines and extinction of amphibian species. Several theories have been proposed as to the underlying mechanisms of mortality, either implicating *Bd* as a novel pathogen with geographical spread or an endemic pathogen with localized outbreaks. There are studies that appear to support either theory, and researchers are beginning to delve into the underlying mechanisms of disease resistance and susceptibility on both the population and individual levels.

The role of the immune system in disease resistance/susceptibility to chytridiomycosis, particularly the innate immune system, has only been investigated within the last decade. The role of cutaneous antimicrobial peptides is unclear: antimicrobial peptides from some species of frogs that are susceptible to chytridiomycosis are effective in killing *Bd in vitro*. In a separate study, it has been shown that corticosteroids suppress cutaneous antimicrobial peptide expression in frogs, similar to studies with cutaneous antimicrobial peptides in mice and tracheal AMP in cattle.

Lithobates pipiens is the current name for the Northern Leopard Frog, formerly known as *Rana pipiens*. I have converted old to new taxonomy when referring to scientific names quoted from literature prior to 2006. This project consisted of two parts. First, to evaluate whether AMP gene expression within skin glands is altered by glucocorticoids in *L. pipiens,* a frog species that is known to have been moderately susceptible in the past to chytridiomycosis (Voordouw et al 2010). This was compared to the results of similar work already done in *Xenopus laevis,* a frog species thought to be a resistant "carrier", to determine any species differences with respect to this specific innate immune response. This was accomplished using norepinephrine to stimulate cutaneous gland secretions and subsequent quantitative RT-PCR for gene expression of antimicrobial peptides known to inhibit *Bd* growth *in vitro*. Second, an attempt was made to experimentally infect *L. pipiens* with *Bd*, with the intent of examining the possible effect of injectable glucocorticoid on levels of antimicrobial peptide expression in relation to susceptibility to this disease, using the techniques described above. The latter work was not completed, based on the findings in the first study and in the preliminary infection model.

Although there have been scattered and separate studies of glucocorticoids, antimicrobial peptides, and experimental infection of frogs with *Bd*, this was the first experiment to compare susceptible and resistant species in this context, and to attempt to look at all three factors within one study.

The literature review will attempt to amalgamate scattered details of the disease aspects of chytridiomycosis, including the gross and histopathologic lesions, as well as a detailed examination of the pathogenesis as understood to date. The focus of the review will then change to address the core topics involved in this project, which include the relationship between innate immunity and fungal disease in mammals and amphibia, with a large focus on the cutaneous glands in amphibians and the antimicrobial peptides produced within them, and the role of "stress" to tie all these concepts together.

Please refer to the list of abbreviations if required.

1.1: Chytridiomycosis – The Concern

> "Nearly one-third (32%) of the world's 5,743 amphibian species have been classified as threatened with extinction, representing 1,856 species. 122 species, perhaps many more, appear to have gone extinct since 1980. Further research may increase this number, since 23% of all species were classified as Data Deficient. At least 43% of all species have undergone population declines, but less than one percent is increasing in population size.
>
> Many species are declining for reasons, such as disease, climate change, invasive species, and over-harvesting, that cannot be readily addressed through traditional conservation strategies. A newly recognized fungal disease, chytridiomycosis, causes catastrophic mortality in amphibian populations, and subsequent extinctions." (Gascon et al 2007)

1.1.1 Nature of the Problem: History and Recent News

The current extinction rate of amphibians is estimated to be 211 times the background rate based on the fossil record (McCallum 2007). McCallum states that this general trend "suggests catastrophic future losses and uncertain opportunity for recovery", and that the current rate far exceeds those from previous mass extinctions, suggesting global stressors and human impact (McCallum 2007). Historic mass extinctions of amphibians have been associated with super volcanoes, extraterrestrial impacts and mass glaciation (Wake et al 2008). McCallum also suggests that this should be termed "global amphibian extinction" rather than the more subtle "amphibian declines" in order to convey the real impact of these rates (McCallum 2007).

There are three main causes of amphibian declines: habitat loss, over-exploitation, and "enigmatic", the latter a broad category thought to encompass both climate change and disease (Stuart et al 2004). Chytridiomycosis is not the only infectious disease impacting amphibian populations; several others, including redleg (*Aeromonas hydrophila*) (Bradford 1991) *Ranavirus* and *Saprolegnia* water mould (Daszak et al 2003) have been implicated, although not as a proximate cause like chytridiomycosis (Skerratt et al 2007). In 2010, a study was published using a Species Distribution Model to link rapid amphibian declines to chytridiomycosis (Lotters et al 2010) in agreement

with previous statements (Wake et al 2008, Skerratt et al 2007). An interesting point from this paper was that areas with high climatic suitability for *Bd* do not have rapid declines in amphibian populations (Lotters et al 2010), supporting previous theories that there are differences in species susceptibility to *Bd* (Bielby et al 2008).

1.1.2 Geographic Distribution and Ecology

Although chytridiomycosis has been detected in amphibian specimens in museums dating as far back as 1938, drastic global declines associated with the disease first began occurring in the 1960's-70's (Houlahan et al 2000), and there were almost simultaneous outbreaks in Costa Rica and Australia in the 1990's (Berger et al 1998, Lips 1998, Lips 1999). Outbreaks were then recorded in a wave-like fashion throughout Central America toward Panama over the next decade (Lips et al 2006). Although some species are in great decline or have become extinct, *Bd* has been detected in post-decline and non-declining amphibian populations as well (Retallick et al 2004, Beard et al 2005, Woodhams et al 2005, Ouellet et al 2005, Kriger et al 2006, Puschendorf et al 2006, Sanchez et al 2008).

The literature is brimming with published reports on naturally occurring chytridiomycosis, which is now documented in different ecosystems on every continent where amphibians are known to exist (Skerratt et al 2007, Lotters et al 2010), with particularly large (up to 89%) enigmatic declines in both Australia/New Zealand and neotropical countries, areas where habitat destruction and overexploitation are not major concerns (Stuart et al 2004). Chytridiomycosis is also documented in Europe (Garner et al 2005) and has recently been reported in various regions of Asia, including an outbreak in Japan (Une et al 2008), and *Bd* has been found on amphibians without associated morbidity/mortality in South Korea (Yang et al 2009).

1.1.3 Current Theories on Chytridiomycosis and Amphibian Declines

Two main theories have been proposed as to the underlying mechanisms of mortality, either implicating *Bd* as a novel pathogen with geographical spread or an endemic pathogen with localized outbreaks, reviewed in detail by Rachowicz et al (Rachowicz et al 2005). These are two theories that apply to outbreaks of any emerging infectious disease, and Rachowicz discusses these in the context of the current evidence based on case reports of chytridiomycosis outbreaks all over the world. It is important to understand where these two hypotheses differ and overlap, in order to effectively determine strategies to manage the emerging disease (Alford et al 2001).

1.1.3.1 Novel Pathogen Hypothesis

> "The novel pathogen hypothesis (NPH) states that the pathogen (or a newly evolved virulent strain of the pathogen) has recently spread into a new geographic area, encountering naïve host individuals or species that are highly susceptible to infection." (Rachowicz et al 2005 citing Alford et al 2001)

There is support for the NPH from the aspect of the pathogen itself. *Bd* infects a wide range of host species with high depopulation rates in many cases (Daszak et al 1999). About a decade after being discovered as a distinct

pathogen, two pathogenicity genes that are known to be important in fungal infections in vertebrates were sequenced in the *Bd* genome (Rosenblum et al 2008). As well, Morehouse et al (Morehouse et al 2003) found a low level of genetic variance in the *Bd* genome.

In addition to the massive population declines discussed above, a recent study from Arizona (Savage et al 2011) supports the NPH. Two separate populations of a single frog species (the Lowland Leopard Frog, *Lithobates yavapaiensis)*, one infected with *Bd* and the other being free of infection, were found to be genetically distinct, with evidence for recent positive selection of MHC Class II genes.

If the NPH holds true, there are some questions to be answered. Why do some frogs die of acute chytridiomycosis within days or weeks after exposure to *Bd* (Berger et al 1998, Nichols et al 2001, Woodhams et al 2003, Berger et al 2004, Rachowicz et al 2004, Garcia et al 2006), while others develop no clinical signs (Daszak et al 2004)? It seems that *Bd* can persist with stable, endemic infection where it has previously presented as an outbreak, as seen in the population of *Taudactylus eungellensis* in the rainforests of Queensland, Australia (Retallick et al 2004).

There are cases on both sides of the argument, suggesting that the pathogen-host interaction is more complex than simply a newly evolved pathogen affecting naïve populations.

1.1.3.2 Endemic Pathogen Hypothesis

The endemic pathogen hypothesis (EPH) suggests the pathogen "has been present in the environment but has entered new host species or increased in pathogenicity because of environmental changes, or possibly, simply escaped previous human notice"(Rachowicz et al 2005).

One of the most interesting papers supporting the endemic pathogen hypothesis is that of Ouellet et al (Ouellet et al 2005) who examined museum specimens of frogs collected between 1895-2001 from 25 different countries for chytridiomycosis. Specifically in populations of North American frogs, the earliest detected case of chytridiomycosis (detected by histology and electron microscopy) was in 1961 in Quebec. The prevalence of the disease in the 1960-1969 period was not significantly different from the 1990-2001 period, and no morbidity or mortality associated with chytridiomycosis was observed despite a 17.8% prevalence of chytrid infection. Therefore, the authors suggest that the disease is enzootic in these populations that appear healthy, and therefore lethal outbreaks may be the result of underlying predisposing factors. The authors later go on to suggest that a difference in infection severity among species may be due to alterations in immune status that is species-specific.

The paper most pertinent to my current project finds that species that produce antimicrobial peptides lethal to *Bd* in culture continue to experience major die-offs (Rollins-Smith et al 2006). Another factor that may be important is skin-associated microbial species that secrete anti-fungal metabolites (Harris et al 2009, Harris et al 2006, Woodhams et al 2007, Lauer et al 2008). Also inconsistent with the NPH is the observation of different mortality rates from *Bd* in closely related amphibian species within a single habitat (Rachowicz et al 2005).

However, criticism of the EPH is valid: opportunistic pathogens that typically signify immune suppression have *not* been found in chytrid-infected amphibians (Bradley et al 2002), except for one recent report of *Bd* diagnosed along with *Aeromonas hydrophila* and *Mycobacterium* species in a single laboratory-maintained *Xenopus laevis* (African clawed frog) (Hill et al 2010). There were numerous tests performed to rule out other pathogens, including hematology, various viral tests, bacterial culture, histology and electron microscopy. However, one must consider that perhaps *Bd* is just the first opportunistic pathogen in an immune-compromised host, and is able to cause death before other opportunistic pathogens are able to colonize.

1.1.3.3 Other Hypotheses

There are subsets of the EPH that focus on the role of altitude and environmental temperature as being predisposing factors to *Bd* infection in certain populations of frogs. Several studies are published on *Bd* infecting populations of frogs at high altitudes of montane regions (Bradford 1991, Lips 1999, Rachowicz et al 2006, LaMarca et al 2005, Muths et al 2003, Bosch et al 2001); however, other authors have discovered no significant difference in infection parameters between differing altitudes (Kriger et al 2008). As well, some authors believed global warming (increasing environmental temperatures) was contributing (Bosch et al 2007), while others found that *Bd* actually prefers to survive at lower temperatures (Woodhams et al 2003), and experimentally infected frogs clear the infection when housed at higher temperatures (Andre et al 2008).

1.2: Chytridiomycosis – The Disease

1.2.1 Introduction and Life Cycle

Chytridiomycosis is a cutaneous fungal disease of amphibians, caused by a chytrid fungus in the genus *Batrachochytrium*, and given the species name *dendrobatidis* after being first characterized in frogs of the *Dendrobates* species (Pessier et al 1999). *Bd* is unusual because it is the only chytrid fungus that parasitizes vertebrates, specifically amphibians (Gascon et al 2007).

In 1999, Pessier was the first to determine that this cutaneous organism in two species of frogs was a chytridiomycete (Chytridiomycota division of the fungal kingdom) (Pessier et al 1999), which are generally saprobes in aquatic environments, growing on a variety of substrates, including pollen, vegetable matter, chitinous materials and keratin (Gleason et al 2009). In the same year, Longcore isolated the organism to describe the morphology and ultrastructural features, and named it *Batrachochytrium dendrobatidis* gen. et sp. nov (Longcore et al 1999).

There are two main life stages for *Bd*: the motile and water-borne zoospore and the encysted thallus in the cytoplasm of epidermal cells, that develops into a zoosporangium for asexual amplification (Berger et al 2005). Living *Bd* zoospores are 3-5 um in diameter, spherical to elongate, have no cell wall (Berger et al 2005) and have a single, posteriorly directed flagellum that, as in other chytrids, is used for swimming based on the amount of endogenous

energy reserves (Pessier et al 1999, Gleason et al 2009, Longcore et al 1999). Some zoospores of *Bd* can remain motile for up to 24 h in either 1% tryptone or water before they encyst. Other chytrids have both ameboid movement (along wet surfaces) and passive movement (currents, water column) (Gleason et al 2009). *Bd* zoospores are chemotactically attracted to sugar (glucose, lactose), proteins and amino acids (glycine and cysteine) and keratin in its immediate environment *in vitro,* therefore suggesting it has the ability to sense nutritional cues of susceptible hosts (Moss et al 2008). Whether this is important in transmission of infection is not yet determined.

The zoospore then encysts on a substrate and is known as a germling (Berger et al 2005). Transmission electron microscopy (TEM) has provided further insight into the microstructure of the *Bd* zoosporangia within tissue sections. Perhaps most interesting are long, thin tubular extensions (rhizoids) from zoosporangia that exit the basal aspect of keratinocytes and enter the deeper portions of the epidermis (Pessier et al 1999). These are postulated to absorb nutrients from the host substrate (Berger et al 2005). The germling undergoes mitotic divisions, becoming multinucleate, and eventually cleaves into rounded flagellated zoospores that do not discharge if the environment is too dry (Berger et al 2005). The mature zoosporangia have small discharge tubes that merge with and dissolve epithelial cell membranes in order to facilitate the release of motile zoospores onto the surface of the skin (Berger et al 2005). The structure that remains is empty and the walls eventually collapse, or bacteria may enter through the open discharge tube to colonize the inside (Berger et al 2005).

1.2.2 Clinical Signs

For a disease that is so widely investigated from the population level down to the molecular level, there is remarkably little in the peer-reviewed literature on the clinical signs and gross lesions. Most authors quote work by Berger, Speare and Hyatt in 1999 (Berger et al 1999) which quotes unpublished work from Berger, Speare and Marantelli that "clinical signs in Australian frogs with chytridiomycosis include lethargy, inappetance, skin discoloration, presence of excessive sloughed skin, and sitting unprotected during the day with hind legs held loosely to the body… moribund in terminal stages with loss of righting reflex". Berger (Berger et al 1999) also quotes unpublished work from Speare in 1994 stating that frogs (*Litoria* and *Taudacylus* sp.) infected in northern Queensland predominantly showed neurological signs, including an abnormal sitting posture with hind legs adducted and a slow response to tactile stimuli; as well, when handled, these frogs became rigid and trembled with extension of the hindlimbs and flexion of the forelimbs.

Severe neurological signs prior to death have been reported by other authors as well (Bishop et al 2009), and Voyles et al (Voyles et al 2007) quote Berger (Berger 2001) describing neurological signs, including muscle tetany, preceding death. In Australian frogs, behavioral changes (lack of burrowing or hiding during the day) were noted three days prior to death, and then other clinical signs were observable 2-3 days prior to death (Berger et al 2004). Clinical signs mentioned by other authors include decreased respiration rate (Voyles et al 2007), anorexia one day prior to death (Pessier et al 1999), and excessive buoyancy and emaciation (Parker et al 2002). However, Parker et al (Parker et al 2002) mention emaciation, which conflicts with the findings of Berger et al (Berger et al 1999), that most diseased frogs were in reasonable body condition, with 65% having moderate to large fat bodies.

1.2.3 Gross Pathologic Findings

Gross lesions are not always observed in frogs that have histopathological lesions of chytridiomycosis, as only 8/27 Native Arizona frogs showed evidence of diffuse erythema of the skin of the abdomen, pelvis and legs (Bradley et al 2002), though erythema of the feet was common (Berger et al 1999). A light brown discoloration and granular texture in these areas has been described in *Dendrobates* spp. (poison dart frogs) and *Litoria caerulea* (White's tree frogs), with multifocal patches of soft, brown, loosely adherent material interpreted as excessive shedding (Pessier et al 1999). Other less common gross lesions that have been documented include minute skin ulcers and hemorrhage in the skin, muscle or eye, and pale muscles and internal organs, although Berger et al (Berger et al 1999) refer to unpublished work by Berger and Hines that some *Litoria caerulea* had marked congestion and reddening of internal organs rather than pallor.

The only keratinized tissues in tadpoles are the toothrows and jawsheaths; therefore lesions of chytridiomycosis, visible with a hand-held lens (10x), are restricted to the mouthparts. In *Lithobates muscosa*, they include asymmetrical, depigmented gaps in the toothrows, sometimes with a raised ridge of white to pinkish-white tissue (Fellers et al 2001). The depigmentation or misshapen appearance is variably intense, affecting 10-100% of the oral disc (Fellers et al 2001).

1.2.4 Histopathologic Findings

Histopathological findings include irregular, moderate thickening of the stratum corneum of the epidermis, from the normal 2-5 μm to 60 μm, with moderate to marked orthokeratotic hyperkeratosis and intracellular life stages of *Bd* in the stratum corneum and stratum granulosum (Berger et al 1998, Pessier et al 1999). Other authors report severe spongiosis of the epidermis (Parker et al 2002). The sporangia are round to oval, with refractile walls that are 0.5-2.0 μm thick, a diameter of 6-20 μm, and occasionally have visible short tubular extensions (discharge tubes/discharge papillae) up to 10 μm in length, oriented toward the outer surface, giving it a flask-like appearance (Berger et al 1998, Pessier et al 1999, Berger et al 2005, Parker et al 2002).

In hematoxylin and eosin (HE)-stained sections, three to four forms of the encysted thallus form of *Bd* are noted (the motile zoospores only live in water) (Pessier et al 1999, Berger et al 1999). The immature stages are rarely uninucleate, with homogenous basophilic cytoplasm (rare), or more commonly multinucleate, with numerous fine, densely basophilic nuclei separated by lightly stippled to microvacuolated pale basophilic cytoplasm (Pessier et al 1999, Berger et al 1999). The third form, the zoosporangium, is cyst-like, containing multiple (4-10) discrete round to oval basophilic 1-3 μm zoospores, or empty, but retaining the spherical shape. The fourth form is the collapsed, irregularly-shaped empty zoosporangium, occasionally containing numerous basophilic bacterial rods and cocci (Pessier et al 1999, Berger et al 1999). The immature stages are typically present in the superficial layer of viable epidermal cells, while the older, empty stages that have released the zoospores are commonly seen in the outer dead layers of keratin (Longcore et al 1999, Berger et al 1999). *Bd* does not produce a hyphal form (Berger et al 1998, Berger et al 1999), which is useful for distinguishing it from other cutaneous fungal infections such as saprolegniasis

or mucormycosis (Berger et al 1999).

Bacteria are typically noted within the excess surface keratin, but dermal or epidermal inflammation is inconsistent, and usually absent (Berger et al 1998). When present, it consists of mild dermal edema and low numbers of neutrophils, lymphocytes, and fewer macrophages within the superficial dermis (Pessier et al 1999) and, occasionally, the epidermis (Parker et al 2002). There are rare foci of epidermal degeneration and/or necrosis, and there are no histologic lesions of septicemia (Pessier et al 1999).

The walls of the various life stages of *Bd* stain positively with the periodic acid-Schiff reaction and Gomori's methanamine silver stain, but are negative with Gridley's fungal stain as well as Kinyoun acid fast and Gram stains, although zoospores within the zoosporangia are Gram positive and weakly positive with Giemsa (Pessier et al 1999). Congo Red stains zoospores (including the flagellum) orange, while walls of the other stages stain reddish-orange (Briggs et al 2004). The most consistent Congo red staining is the inoperculate discharge tube that stains even when zoosporangia are within intact epidermal cells (which do not stain at all or stain pale orange) (Briggs et al 2004). Briggs and Burgin state that the chytrid fungi have walls with a pH of between 5 and 7 (based on the orange to red colour), and the more intense staining may indicate a greater number of β-glucan molecules (typical of other chytrid fungi) (Briggs et al 2004). The advantage to using a Congo Red stain is that it provides a means of easily identifying fungal cells in skin scrapings to quickly reveal the presence of all morphological stages of chytrid fungi, aiding the identification of potential carriers, resistant species and early outbreaks (Briggs et al 2004). Immunohistochemistry is also developed for *Bd* (Van Ells et al 2003). Any of these additional stains may be used to confirm infection where a few indistinct stages are present and/or to rule out other fungal infections such as those with hyphae, but are typically they are not required for diagnosis if the pathologist is familiar with the histologic lesions (Berger et al 1999).

1.2.5 Diagnosis in Live Amphibians

Diagnostic assays for live amphibians have been thoroughly reviewed in the literature. Recommendations are to swab live adult amphibians along the ventral surfaces and digits, live tadpoles around the mouthparts, and perform real-time Taqman PCR (Hyatt et al 2007) using established primers (Boyle et al 2004).

1.2.6 Pathogenesis

There are two proposed theories that are not mutually exclusive, explaining how *Bd* kills frogs. The first is disruption of the cutaneous barrier leading to water/electrolyte imbalances with subsequent cardiac arrest. The second is that *Bd* may produce an unidentified toxin with unknown mechanisms of action, with no visible gross/histopathological lesions.

A group of Australian authors have published the most intense investigation regarding the pathogenesis of chytridiomycosis (Voyles et al 2007). In 2007, various parameters were measured, including blood gases,

electrolytes, enzymes/markers, other blood constituents (albumin, glucose, cholesterol, etc.) as well as hematological parameters in three groups of *Litoria caerulea*: uninfected, clinically diseased with *Bd*, and subclinically infected with *Bd* (Voyles et al 2007). Severely infected frogs had significantly lower blood pH, plasma osmolality and plasma sodium, potassium, magnesium and chloride concentrations (Voyles et al 2007). Since there was no significant change in hematocrit, albumin or urea (indicating unaltered hydration status), they concluded that reduced electrolyte concentrations probably were due to loss from the blood, rather than a consequence of increased water uptake due to disruption of the cutaneous barrier function (Voyles et al 2007).

In 2009, the "epidermal dysfunction hypothesis" was then confirmed, finding that the cardiac electrical activity of the frogs several hours before death resembled patterns associated with cardiac standstill (Voyles et al 2009). Although this pattern can be caused by several factors, including hypothermia, dehydration and hypovolemia, the design of the study allowed these factors to be ruled out, leaving hypoxia and shifts in electrolytes as the remaining possibilities (Voyles et al 2009). Hypoxia could not be completely ruled out, but peripheral blood oxygen saturation only declined in one individual after electrical changes were noted, suggesting that hypoxia did not cause the cardiac electrical abnormalities (Voyles et al 2009). Then, some of the frogs were treated with electrolytes and the righting reflex was restored, whereas no reversal of signs was observed in the untreated frogs (Voyles et al 2009). Renal-associated loss of electrolytes was ruled out based on plasma biochemistry (Voyles et al 2009). Measurements of electrolyte transport across the ventral abdominal skin showed lower sodium and chloride transport in diseased frogs (which also had degenerative epidermal changes on histology) versus the controls (Voyles et al 2009). Therefore, it was concluded that disruption of the osmoregulatory function of skin caused osmotic imbalances leading to cardiac arrest (Voyles et al 2009). How *Bd* actually disrupts electrolyte transport has not been determined. Voyles et al (Voyles et al 2009) propose that there may be alteration of electrolyte channels in the skin, perhaps due to pathogen adhesion to protein-based receptors, or damage to ion channels by a toxin produced by *Bd*, therefore not refuting the second hypothesis of toxin involvement.

As mentioned above, there is often little or no dermal inflammation evident in *Bd*-infected amphibian skin. This may reflect either lack of stimulation, or a lack of a response, of the host immune system. Lack of stimulation might be due to the superficial site of infection, insufficient epidermal damage, or low inherent antigenicity of *Bd* (Berger et al 1999). The lack of host response has been investigated recently, finding that the mature stage of *Bd* produces a heat-stable, non-proteinaceous substance that inhibits activated lymphocyte proliferation *in vitro*, specifically by inducing apoptosis of T-lymphocytes more than B-lymphocytes (unpublished data, Louise Rollins-Smith).

There are a few articles looking at other factors that appear to impact the pathogenicity of *Bd*. An often-quoted study by Morehouse et al (Morehouse et al 2003) found that there is little genetic variation among isolates of *Bd* worldwide. However, Berger et al (Berger et al 2005) found that the pathogenicity varied when *Litoria caerulea* were exposed to three strains of *Bd* as measured by times to death. However, Berger et al considered that differences in *in vitro* management of the isolates, as well as differing times from isolation, could result in the apparent differences in virulence. *Bd* has also been found to be more pathogenic in frogs at lower temperatures, first shown

by Woodhams et al (Woodhams et al 2003) in *Litoria chloris*. Then in 2008, Andre et al (Andre et al 2008) exposed *Litoria muscosa* to *Bd* at 17 and 22°C, with the frogs at 22°C experiencing significantly lower mortality and declines in severity of clinical signs over time despite persistent infection. Since the optimal thermal range for *Bd* growth is 17-25°C (Piotrowski et al 2004), the authors (Andre et al 2008) suggest that the lower mortality at 22°C was due to the effect of temperature on host resistance, rather than an effect on the fungus alone.

1.3: Chytridiomycosis – The Roles of Immunity and Stress

1.3.1 Overview: Resistance and Susceptibility

Despite the extensive information regarding amphibian declines, not all frog species are similarly affected, and the focus of many recent studies is aimed at determining why some species seem to persist while others have massive declines. The extinction risk and population declines of amphibians are taxonomically non-random (Stuart et al 2004, Bielby et al 2006). This is true even at small geographic scales (Bielby et al 2006, Williams et al 1998, Lips et al 2003), suggesting that perhaps there are certain host attributes of specific species that influence susceptibility to fungal infection (Bielby et al 2008).

However, determination of which species are resistant and which are susceptible is difficult. Several authors state in various articles that *Xenopus laevis* is a "resistant" or "carrier" species, based on the facts that populations of this species have not been hit with mass extinctions, and many of them test positive for chytrid infection yet are not affected with clinical signs (Wake et al 2008). In the only experimental infection with *Bd* in this species, *Xenopus* did become infected, although mortality was not observed even with very high doses of viable zoospores (Rollins-Smith et al 2009). Some populations of *Litoria catesbeiana* also appear to prevail with high prevalence/intensity of infection (Hanselmann et al 2004, Sanchez et al 2008). In New Zealand, *Litoria raniformis* was experimentally infected with *Bd*, showing clinical signs of infection; however most of the frogs survived (Carver et al 2010).

Other species suspected to be susceptible typically demonstrate progressive disease and mortality (Nichols et al 2001, Bosch et al 2001, Raverty et al 2001); however, this is not observed in all species of frogs (Davidson et al 2003). Some species experience varying infection intensities and rates of die-off/recovery, at both the individual and population level (Savage et al 2009). Many authors have speculated on an underlying alteration in immune defenses, both acquired (Savage et al 2009, Ramsey et al 2010) and innate (Rollins-Smith et al 2009); however, studies investigating these specific aspects only began to appear in the last decade. Perhaps the key to resolving this issue is to define "resistance" and "susceptibility", to accept that the distinction between the two is not black and white, and realize these concepts are affected by a multitude of factors.

1.3.2 The Role of Immunity

1.3.2.1 Immunity to Fungal Infections in Mammals and Amphibians

Broadly speaking, host susceptibility to a cutaneous fungal infection depends on the composition and structure of its keratin, the nutritional requirements and pathogenicity of the fungal species, the health status of the animal, and the various host defense mechanisms (Chermette et al 2008). These can involve both adaptive and innate immune mechanisms. For fungal infections, it is understood that cell-mediated immunity (CMI) is the main mechanism of defense, with Th1-type CMI required for clearance of a fungal infection and a Th2-type CMI associated with susceptibility to infection (Blanco et al 2008). Many studies in mammals have focused on aspergillosis (caused by the fungus *Aspergillus*), where the fungus is recognized by the immune system, which quickly responds with the innate effector phase followed by a delayed adaptive effector phase for an immunoprotective status and resolution of disease (Blanco et al 2008).

Although CMI has been extensively studied in certain contexts such as aspergillosis, the importance of innate immune mechanisms may be underestimated. Apart from the physical barrier that skin and other mucous membranes provide, there are three important components of the innate response on these surfaces: antimicrobial peptides, antibodies, and commensal microflora. The first two responses are important because they rapidly recognize conserved molecular patterns common to broad groups of microbial species, and activate the adaptive immune system through specific signals (Romani 2004).

Fungal infections in mammals are typically of minor clinical importance and only become a concern when the host is immunosuppressed (such as from exogenous corticosteroid therapy, Cushing's disease, or viral infections) (Chermette et al 2008, Wang et al 2003). The same holds true in amphibians, particularly in captive amphibians, where fungal infections are typically secondary to conditions that diminish host immune-inflammatory defenses, such as inappropriate husbandry or concurrent disease (Williams 1995, Wright 1996). Chytrid fungi are typically non-pathogenic, and *Bd* is the first chytrid fungus known to infect vertebrates (Wake et al 2008). Other fungal infections in amphibians include mucormycosis (*Mucor amphibiorum*), basidiobolomycosis (*Basidiobolus ranarum*), chromomycosis (various saprophytes), saprolegniasis (*Saprolegnia*, water molds), *Ichthyophonus*-like infection (*Ichthyophonus hoferi*-like organism) (Pare 2003). These are considered opportunistic pathogens, that only lead to death of amphibians that are also affected by stressors, disruption of the cutaneous barrier and/or events such as breeding or coming out of hibernation (Pare 2003)

The amphibian immune system includes both adaptive (Du Pasquier et al 1989) and innate (Conlon et al 2004) responses (Carey et al 1999, Apponyi et al 2004). Stice and Briggs suggest that because *Bd* is an intracellular pathogen (Berger et al 1998), and frogs can mount protective adaptive responses to intracellular pathogens (Morales et al 2007), then it is likely that the adaptive immune system is involved in the host response to chytridiomycosis (Stice et al 2010).

1.3.2.2 Adaptive Immunity and Chytridiomycosis

As mentioned above, unpublished work by Louise Rollins-Smith showed that mature *Bd* secretes an unidentified heat-labile, non-protein substance that inhibited particularly T-lymphocyte proliferation *in vitro*. This work was performed in two species, one thought to be resistant to *Bd* (*X. laevis*) and one thought to be more susceptible (*L, pipiens*), with no difference in lymphocyte inhibition between the two (unpublished data, Louise Rollins-Smith). These results suggested that perhaps adaptive immunity is not a key component of infection in susceptible species of amphibians. However, more experimental work is required to determine if the same effects occur *in vivo*. It was suggested that the reason for the lack of difference in lymphocyte inhibition is that the initial infection quickly inhibits the adaptive immune system and prevents development of an appropriate immune response (unpublished data, Louise Rollins-Smith). This would hold true in susceptible species. Yet why do the "resistant" species develop infection without mortality, or not develop infection at all? Perhaps the lymphocytes are only inhibited for a certain period of time? Due to the fact that only the mature zoosporangia produce this lymphocyte-inhibitory substance, infection of the epidermis is required, as mature zoosporangia are thought to survive only in tissue. Therefore, species that have no signs of infection must rely on another component, perhaps substances on the surface of the skin, such as antimicrobial peptides, or antibodies, to prevent infection.

Furthermore, Carver et al (Carver et al 2010) suggested that the lag time in the course of infection between initial clinical signs and full recovery from the disease suggested an adaptive immune response, which is not a new concept (Richmond et al 2009, Robert et al 2009).

Evidence suggests that prior *Bd* infection followed by antifungal therapy does not confer resistance to subsequent challenge (Cashins et al 2013), suggesting that if there is a natural acquired immune response, it is not protective. In terms of the effect of immunization, there is one study that attempted to immunize *L. muscosa* followed by a subsequent exposure to *Bd* (Stice et al 2010). There was no difference in overall mortality, time to infection, infection prevalence or intensity in the frogs that were injected with saline, adjuvant alone, or adjuvant with formalin-killed culture of *Bd* (Stice et al 2010). However, a low concentration of adjuvant was used to avoid irritation to the frogs, perhaps failing to potentiate an immune response, and the authors did not look for increased antibody production post-immunization (work in progress) due to a lack of existing ELISA protocols to determine antibody titers in this species (Stice et al 2010).

1.3.3 Innate Immunity in Frogs

1.3.3.1 General Characteristics of Cutaneous Glands & Regulation of Expression and Secretion

The morphology and physiology of cutaneous glands in frogs and toads are well described (Lacombe et al 2000, Noble et al 1944, Benson et al 1969, Toledo et al 1992, Dockray et al 1975, Holmes et al 1978, Mills et al 1984, Neuwirth et al 1979, Clarke 1997). There are three main types of glands in frog skin, specifically studied in *Phyllomedusa bicolor*: mucous, lipid and serous/granular glands (Lacombe et al 2000). The mucous glands are in all life stages of frogs, stain positively with the PAS reaction, and are most common in the ventral skin. The lipid

glands are also present in all life stages, stain positively with Sudan IV stain, and are most common in the dorsolateral skin (Lacombe et al 2000).

The serous glands, more commonly known as granular glands, venom glands, or poison glands, are more numerous and larger in adults (2 years old) than at 4 months post-metamorphosis, at least in *P. bicolor* (Lacombe et al 2000), and are more common on the dorsal surface of the body, though not confined to this region (Lindley 1969), and on the dorsal part of the extremities (Sjoberg et al 1976). They are surrounded by a single layer of myoepithelial cells and contain spherical 2-5 μm diameter basophilic "storage" granules (Holmes et al 1978, Lindley 1969, Flucher et al 1986). There are two types of granular glands: Type I have a poorly developed endoplasmic reticulum while Type II have a well-developed endoplasmic reticulum (Lacombe et al 2000). Norepinephrine stimulates a dose-dependent discharge of the Type I granular gland, through contraction of the myoepithelial cells (Holmes et al 1978, Nosi et al 2002), which have α- and β- adrenoreceptors and will contract in response to α-adrenergic agonists (adrenaline, noradrenaline, phenylephrine) or high potassium concentrations (Holmes et al 1978). The terminal nerve endings are located between smooth muscle cells and are also in direct contact with the secretory epithelium (Sjoberg et al 1976). Granular glands are also structurally similar in all species of dendrobatid frogs, even though the substances secreted differ in chemical structure and toxicity (Neuwirth et al 1979), as well as in Australian hylid and leptodactylid frogs (Tyler et al 1985). There have been rare reports of some frog species that do not have detectable granular glands, such as *Litoria cancrivora* (Seki et al 1995).

The lipid glands produce oily substances such as sebum, via a holocrine method. In general, serous glands typically produce peptides (as well as other proteins, alkaloids and steroids, bufodienolides and biogenic amines (Clarke 1997)) and release them via a merocrine method. However, although granular glands are serous because they produce proteins (peptides), their method of secretion is more holocrine rather than merocrine. Ultrastructurally, as the gland matures, a syncytial secretory compartment is formed inside the layer of myoepithelium, where the inner epithelial cells break down and many of the nuclei disintegrate, with some of the nuclear material appearing to take part in the formation of early secretion products as well as being expelled (Noble et al 1944, Delfino et al 2006, De Perez et al 1985). The basilar area of the syncytium contains nuclei and major cytoplasmic organelles, while the apical portion contains the large electron-dense granules (De Perez et al 1985). Rather than the typical cytoplasmic involution seen in conventional holocrine glands, it is the mechanical force of the contraction that results in degeneration (Delfino et al 2006).

The peptides are released onto the surface of the skin in high concentrations upon stress or injury, which, via adrenergic stimulation, trigger the myocytes surrounding the glands to contract (Conlon et al 2004, Simmaco et al 1997). Peptide synthesis can be induced in response to bacterial challenge, as seen in *Litoria sylvatica* and *Litoria esculenta* (Matutte et al 2000, Mangoni et al 2001).

Relevant work regarding cutaneous gland secretions has been the discovery that polyadenylated mRNAs encoding dermal granular gland peptides are present within the secretions, stabilized by endogenous nucleic acid-binding amphipathic peptides (Chen et al 2003). This enables proteomic and transcriptome analysis without killing the frogs.

Cutaneous glands can be stimulated experimentally in various ways. Norepinephrine injection (into dorsal lymph sacs), or immersion in a solution of norepinephrine, are non-stressful techniques commonly used to stimulate cutaneous glands in amphibians (Conlon et al 2010). Conlon and Leprince (Conlon et al 2010) suggest that agitated or highly mobile animals can be partially anesthetized by immersion for 5 minutes in crushed ice, with no effect on the concentration and distribution of peptides in the norepinephrine-stimulated secretions. However, not only is immersion in ice not as humane as other techniques for immobilization, a sharp drop in body temperature may not be ideal for experimental situations in which *Bd* is involved, as the low temperature could suppress the immune system.

In *X. laevis* that were experimentally stimulated with either norepinephrine or electrostimulation (discussed later), regeneration of glands had a timeline as follows: 1 week post-stimulation there was still contraction of the myoepithelial layer, no storage granules and a shriveled gland; however cells were proliferating to fill the acinus (Flucher et al 1986). At 2 weeks, cells began to fuse forming small syncytial units, were highly packed with rough endoplasmic reticulum, and the first detectable storage granules were formed (Flucher et al 1986). At three weeks, the secretory cells were in a monolayer and there were many storage granules (Flucher et al 1986). The gland required 4-6 weeks to reach its original size (Flucher et al 1986). In contrast, using mass spectrometry on secretions from the same species stimulated by norepinephrine, replenishment of the whole range of peptides was shown to be complete, in composition, if not in mass, at 2-6 days post-stimulation (Giovanni et al 1987).

Recent work found that two injections of norepinephrine (80 nmol/L) were sufficient to almost completely deplete the granular glands (based on histologic appearance), as well as causing structural damage to mucous glands (unpublished data, Louise Rollins-Smith). It was also discovered that peptides collected from both resting and chased frogs had activity against *Bd* in vitro at fairly low concentrations, with a peak of secreted antimicrobial peptides at 15-30 minutes post-stimulation, followed by a decline suggesting protease activity in the secretions themselves or from other microbes on the skin (unpublished data, Louise Rollins-Smith).

Surface electrical stimulation (SES), or transdermal electrical stimulation, was one of the first non-invasive methods to release cutaneous gland secretions in amphibians (Tyler et al 1992). Electrostimulation has been cited by several authors as having the potential to cause distress in frogs (Conlon 2011). However, the study that is often cited is that of Tyler et al (Tyler et al 1992) in which there is no mention of distress, and electrostimulation is clearly less invasive than norepinephrine injections. Other authors quote the same study as describing a method not harmful to amphibians (Apponyi et al 2004). Electrostimulation, although non-invasive and effective, is difficult to perform in the field and likely produces more variation in results compared to the precise dosing used with norepinephrine injections.

1.3.3.2 Structure and Function of Cutaneous Antimicrobial Peptides in Frog Skin

Antimicrobial peptides in secretions from frog skin were first identified over two decades ago, with the discovery of magainin in the skin of *X. laevis* (Giovanni et al 1987). Magainin is a broad-spectrum antibacterial and antifungal compound (Zasloff 1987). Considering that most frog species are exposed to a moist and warm environment

conducive to the growth of potentially harmful bacterial and fungi in at least one life stage, it is not surprising that the skin of amphibians is an important first-line defense against such agents.

Peptides in secretions from Anura (frogs and toads) have antimicrobial, myotropic, mast cell-degranulating, neuroendocrine, enzyme-inhibiting and pheromonal activities (Lazarus et al 1993). Most of the amphibian antimicrobial peptides, including those of the ranatuerin-1 and -2 families, are hydrophobic and cationic with many lysine residues (Hancock et al 1995), and tend to have an amphipathic α-helical conformation both in solution and when interacting with the phospholipid membrane of microorganisms (Goraya et al 1998). This is thought to aid the peptides in binding to, crossing, disrupting and permeabilizing bacterial membranes to cause bacterial cell death (Goraya et al 1998).

Twenty four antimicrobial peptides, belonging to four known structural families (α-helical, β-stranded, β-hairpin or loop and extended), were isolated from the skin of three species of North American frogs *(L. luteiventris, L. berlandieri* and *L. pipiens*), and were found to have a close phylogenetic relationship (Goraya et al 2000). However, the amino acid sequences of the antimicrobial peptides were highly variable between the species, and species-specific (Goraya et al 2000). All the antimicrobial peptides isolated, apart from the temporins, were inhibitory for a Gram-positive bacterium (*Staphylococcus aureus*), a Gram-negative bacterium (*Eschericia coli*) and a yeast (*Candida albicans*), indicating broad-spectrum antimicrobial activity (Goraya et al 2000).

1.3.3.3 Effect of Cutaneous Antimicrobial Peptides on Chytrid Fungi

The two main factors that contribute to the effectiveness of AMP defenses in the mucus covering frog skin are the relative potencies of each peptide against a pathogen, and the total amount of peptides released by actively secreting frogs (Rollins-Smith et al 2006). Synergistic interactions among host defense molecules are also of importance in other species and on other body surfaces. There have been several studies, first to determine which peptides are most abundant in the secretions of various frog species (via a mild norepinephrine induction), and then to determine the *in vitro* effectiveness of various cutaneous antimicrobial peptides against various life stages of cultured *Bd* using growth inhibition assays (Rollins-Smith et al 2006). The minimum inhibitory concentration (MIC) is determined for both natural peptide mixtures as well as individual peptides (Rollins-Smith et al 2006). Bradykinin, one of the most abundant peptides produced in frog skin, is typically used as a standard against which concentrations of other peptides can be measured (Rollins-Smith et al 2006). It is also helpful to estimate the thickness of the mucous layer and the surface area of the skin, to estimate the amount of peptides within the mucus (Rollins-Smith et al 2006).

Litoria muscosa, highly susceptible to *Bd* in the wild, among other peptides, produces temporin-1M, ranatuerin-2Ma and ranatuerin-2Mb. These inhibit growth of *Bd* zoospores *in vitro*, suggesting that simply having the ability to produce antimicrobial peptides is not sufficient to confer resistance (at least against isolate 119, recovered from *L. muscosa*) (Rollins-Smith et al 2006). *Lithobates pipien*s has eight cutaneous antimicrobial peptides, from the families brevinin (1Pa, 1Pb, 1Pc, 1PD, 1Pe), esculentin (2), ranatuerin (2P) and temporin (1P) (Goraya et al 2000) of which both esculentin and ranatuerin inhibit *Bd* zoospores *in vitro* (Rollins-Smith et al 2006). *Lithobates*

tarahumara produces two peptides, brevinin-1TRa and ranatuerin-2TRa, that inhibit growth of both mature *Bd* cells and immature zoospores (isolate 197, recovered from *D. azureus*), although the peptides were more effective against purified zoospores (Rollins-Smith, 2002).

Although *in vitro* activity of the above-mentioned peptides and peptide mixtures has been firmly established against both immature and mature stages of *Bd*, their *in vivo* role remains undetermined. Therefore, authors have speculated that the link between peptide-based host defenses and amphibian population declines associated with *Bd* infection is unclear and multifactorial (Conlon et al 2009).

1.3.4 The Role of Stress

1.3.4.1 Overview

"Stress" is broadly defined as an environmental or physical pressure that is perceived as straining or exceeding the adaptive capacity of an organism and threatening its well-being (Britannica.com 2013). However, in the context of this work, this definition will be narrowed to focus on the physiologic response to "stressful" stimuli, particularly the various hormonal changes that occur. The most relevant of these is an increase in secretion of corticosteroids from the adrenal cortex.

Corticosteroids are lipophilic steroid hormones that are powerful suppressors of inflammation, and which also cause a decrease in the number of circulating lymphocytes, secondary to lympholysis, or by altering lymphocyte circulation patterns. In some species, particularly the hamster, mouse, rabbit and rat, this can occur at extremely low doses (Goldsby et al 2003). Corticosteroids can also bind indirectly to promoter regions of DNA inducing increased transcription of the inhibitor of nuclear factor-κB (I-κB), a key regulator in the immune response to infection (Goldsby et al 2003). Particularly in lymphocytes, corticosteroids inhibit NF-κB (through I-κB upregulation) and inhibit activation and cytokine production of T-lymphocytes (Goldsby et al 2003). As well, corticosteroids decrease the function of macrophages and neutrophils, reduce their chemotaxis to sites of infection, decrease levels of lysosomal enzymes released by leukocytes, and reduce T-helper-cell activation (Goldsby et al 2003). Congenital or acquired defects in these components of immunity (neutrophils, T-helper-cells), at least in humans, are the main risk factors predisposing to severe candidiasis (Romani 2004). However, there is little published on the effects of steroids on innate immune mechanisms such as antimicrobial peptide activity, particularly in amphibians.

Corticosteroids in amphibians have been studied mostly in the context of metamorphosis, where there are significant increases in the concentration of many endogenous hormones, including glucocorticoids and those produced by the thyroid and the pituitary glands (Rollins-Smith et al 1997). An elevation in plasma glucocorticoid concentration results in lymphocyte apoptosis in the thymus, suggesting that this allows development of immunological tolerance of adult-specific antigens (Rollins-Smith et al 1997).

1.3.4.2 The Role of Stress in Fungal Infections

Various fungal infections in animals are associated with stress, including candidiasis and aspergillosis in birds (Blanco et al 2008). However, fungal agents can cause disease, such as *Candida* infections in dogs and nasal aspergillosis, in animals with no obvious underlying disease or immunosuppression (Blanco et al 2008). As mentioned above, most fungal infections in amphibians, other than chytridiomycosis, are thought to be secondary to a combination of unknown stressors, a cutaneous defect and/or a major life event such as breeding or coming out of hibernation (Pare 2003).

Bd may be associated with increases in concentrations of endogenous glucocorticoids, based on indirect evidence from an outbreak of chytridiomycosis in *Litoria catesbeiana* tadpoles, in which neutrophils were the only circulating leukocytes that were increased in tadpoles with severe depigmentation of keratinized mouthparts. The authors speculate that since monocyte numbers were unchanged, the increase in circulating neutrophils was not simply due to an inflammatory response, which is not characteristic of chytridiomycosis in any case, but rather secondary to increases in endogenous glucocorticoid concentration (Davis et al 2010). Similarly, in a laboratory population of *Litoria caerulea* affected by a *Bd* outbreak, glucocorticoid levels were higher in diseased than healthy frogs (Peterson et al 2013).

1.3.4.3 Causes of Stress in Frogs

Many factors are hypothesized to be stressful to frogs but only a few have been studied experimentally. Relevant to our work, in *Scaphiopus hammondii* the stress of environmental water loss led to an increase in corticosterone concentration (among others), which initiated metamorphosis at a premature stage (Denver 1993, Denver 1997). Rollins-Smith (Rollins-Smith 1998) hypothesized that initiation of metamorphosis at a stage where tadpoles are underweight and have not developed a mature immune system would result in more significant immune system dysfunction, secondary to elevation in corticosterone concentration, leading to a greater risk of infection and death.

Parris and Cornelius (Parris et al 2004) studied the effect of pathogen-mediated stress by infecting *Bufo fowleri* and *Hyla chrysoscelis* with *Bd* during different developmental stages. Both species underwent metamorphosis at smaller body masses when reared together rather than separately, suggesting that there is an indirect increase in interspecific competitive effects in mixed-species environments. This work also suggests that *Bd* may in itself be a stressor, rather than affecting the frog secondary to other stressors.

1.3.4.4 Antimicrobial Peptides and Stress

The effects of glucocorticoids on antimicrobial peptides have been studied in mammals, albeit superficially. In feedlot cattle, the role of the innate pulmonary defense system and how stressors alter its function in the context of respiratory disease has been studied (Mitchell et al 2007). Administration of dexamethasone decreases lipopolysaccharide-induced expression of tracheal antimicrobial peptide (TAP) and lingual antimicrobial peptide mRNA *in vitro* and in bronchial biopsy specimens, representing a possible mechanism by which “stress” impairs innate pulmonary defenses (Mitchell et al 2007).

In mice, there is one report showing that psychological stress increased the endogenous levels of glucocorticoids and decreased the bioavailability of two key antimicrobial peptides in the epidermis through decreased delivery into lamellar bodies, which was correlated with persistence and severity of cutaneous skin infectious caused by Group A *Streptococcus pyogenes* (GAS) (Aberg et al 2007). As well, physiological stress decreases AMP production in the skin of mice, associated with increased susceptibility to infection with *Staphylococcus aureus* and GAS (Radek et al 2010).

In the only published report of the effect of glucocorticoids on the synthesis of antimicrobial peptides in amphibian skin (Simmaco et al 1997), *L. esculenta* were treated with synthetic glucocorticoids, either in a topical cream (clobetasol proprionate at 0.25 g/g body weight) or in an intraperitoneal/subcutaneous injection (water-soluble methylprednisolone at 75 μg/g body weight). Glucocorticoid treatment inhibited the transcription of all genes encoding antibacterial peptides by inducing the synthesis of I-κB,' implying that glucocorticoids play a role in modulating secretion of antimicrobial peptides.

1.4 Rationale and Objectives

This research aimed to examine what may be a fundamental component of the pathogenesis of chytridiomycosis, broadly addressing the question of why frog species that produce antimicrobial peptides known to kill *Bd* in vitro succumb to chytridiomycosis.

The first objective was to determine whether AMP expression within skin gland secretions was altered by injection of glucocorticoids, in a frog species thought to be susceptible to chytridiomycosis, and important in Ontario (*L. pipiens*). The hypothesis was that injection of glucocorticoids would lead to impaired cutaneous gene expression of antimicrobial peptides. Norepinephrine injections were administered to obtain cutaneous gland secretions, and gene expression of antimicrobial peptides known to inhibit *Bd* growth *in vitro* was measured by quantitative RT-PCR on the secretions.

The second objective was to determine whether glucocorticoid administration affects the susceptibility of *L. pipiens* to experimental infection with *Bd*, corresponding with altered levels of AMP expression. This would be the first attempt to correlate the administration of corticosteroids with antimicrobial peptide secretion and the outcome of experimental inoculation of frogs with *Bd*. Further, the study was designed to permit comparison of this work on *L. pipiens* to recently published work on *X. laevis.* The hypothesis was that glucocorticoid administration would result in an increase in fungal load (the number of zoospores) and severity of disease in the susceptible frog species (*L. pipiens*) but not in the resistant frog species (*X. laevis*), and this difference in infection/disease outcome would be associated with corresponding differences in gene expression of those cutaneous antimicrobial peptides known to inhibit *Bd* growth *in vitro* (Giovanni et al 1987).

Chapter 2: The Effect of Glucocorticoids on mRNA Expression of Cutaneous Antimicrobial Peptides in *Lithobates Pipiens*

2.1 Introduction

Global populations of frogs are declining and some species are facing extinction, of which one major cause is the geographic spread of the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*), causing chytridiomycosis (Fisher et al 2009). The relationship between global climate change and increased prevalence of this disease is not direct (Wake et al 2008), as higher environmental temperatures reduce mortality after experimental infection (Gascon et al 2007), suggesting that other factors play a role in the emergence of this disease.

Antimicrobial peptides made in cutaneous glands are secreted to the skin surface (the usual location of *Bd* infection). Antimicrobial peptides from some species of frogs that are susceptible to chytridiomycosis are nonetheless effective in killing *Bd* fungi *in vitro* (McCallum 2007), so it seems that these frog species ought to be resistant to chytridiomycosis.

However, it has been shown that corticosteroids suppress antimicrobial peptide expression in skin of *Litoria esculenta* (Stuart et al 2004), as occurs with tracheal antimicrobial peptide in cattle (Mitchell et al 2007). Therefore, corticosteroid-induced reductions in the synthesis of these antimicrobial peptides may predispose frogs to chytridiomycosis.

In support of this hypothesis, levels of antimicrobial peptides are low in infected, compared to uninfected, frogs in a single population (Woodhams et al 2012). Further, experimental reduction of skin peptides and skin commensals exacerbated the response to *Bd* challenge (Woodhams et al 2012). Finally, frogs in geographic areas with endemic *Bd* infection have been shown to experience seasonal mortalities (Longo et al 2013), suggesting that environmental factors may influence host susceptibility. This study was intended to address the broad hypothesis that spread and increased prevalence of chytridiomycosis is a manifestation of climate change-related alteration in environmental temperatures, with stress-induced impairment of antimicrobial peptide expression predisposing to fungal infection. This hypothesis is not inconsistent with the observed geographic spread of *Bd*, if it is assumed that increased susceptibility of otherwise resistant frog species could promote spread of infection.

Initially, I evaluated the effect of glucocorticoid treatment on gene expression of cutaneous antimicrobial peptides that are known to inhibit the in vitro growth of *Bd*, using a frog species considered susceptible to chytridiomycosis (*L. pipiens*) (Voordouw et al 2010). Ranatuerin-2P and esculentin-2 were studied because they both had previously been sequenced in *L. pipiens* (Goraya et al 2000), and, individually, both are known to inhibit *Bd* growth *in vitro* (Rollins-Smith et al 2002). I hypothesized that exogenous glucocorticoid administration would inhibit the gene

expression of these antimicrobial peptides, and if true, that this inhibition of the innate immune response may predispose to chytridiomycosis.

Showing that glucocorticoids impair expression and synthesis of cutaneous antimicrobial peptides would be a stepping stone to understanding how “stress” on a higher level may be involved in predisposing to chytridiomycosis. In the future, it might help to clarify the relationship between climate change (an environmental “stressor”) and this disease; affect intervention strategies that currently focus on limiting spread of the fungus; indicate potential mechanistic reasons to explain the differences in susceptibility between amphibian species; and provide an experimental platform to test the effect of other environmental factors (e.g. environmental temperatures, toxins, UV light, viral infection) on innate immune defences against this pathogen.

2.2 Materials and Methods

2.2.1 Experimental Subjects

Wild-caught frogs were obtained from Wards of Canada Limited (www.wardsci.com) and housed at the Central Animal Facility, University of Guelph. This study was approved by the Animal Care Committee of the University of Guelph, operating under the auspices of the Canadian Council for Animal Care (Animal Use Protocol number 10R085). Thirteen frogs (*L. pipiens*) were separated arbitrarily into two groups (6 in the treatment group, 7 in the control group). The sex of the frogs was determined later at necropsy, with three males and three females in the treatment group, and two males and five females in the control group. Upon arrival, frogs were half-submerged in 100 mg/L oxytetracycline (Oxy-Vet 100LP, Vetoquinol, Quebec, Canada) for 1 hr at room temperature (see below) to prevent bacterial infection of the skin (Denardo et al 1995). Individual frogs were identified using digital photographs to record unique cutaneous spot patterns. The acclimatization period was 23 days.

Frogs were held in two plastic tanks of 144 cm^2 surface area, each with 2/3 terrestrial habitat made of potting soil, artificial plants and plastic structures for enrichment/hiding, and 1/3 aquatic habitat consisting of about 10 L of dechlorinated water 8 cm deep that was changed every three days. Frogs were free-fed live crickets, earthworms and mealworms in surplus every two days. Fluorescent lighting was on a 12:12 hour abrupt-change photoperiod. Humidity was approximately 40% and ambient temperature was 22-23ºC.

2.2.2 Collection of Samples

Frogs were manually restrained using sterile nitrile gloves and each was weighed every 48 hours. Starting on Day 0, an injection of either 0.075 mg/g body weight of the corticosteroid methylprednisolone (M0639, Sigma-Aldrich, Canada; treated group) or the same volume of 0.9% saline solution (control group) was made into the dorsal lymph sac of the conscious frog at the time of weighing. This dosing schedule was based on a prior study in *L. esculenta* (Simmaco et al 1997) as well as unpublished work of Dr. Rollins-Smith.

All frogs were sampled on Day 0 (after the initial steroid/saline injection), and then every 8 days until the end of the experiment at Day 40, in order to detect *Bd* infection, and to collect skin gland secretions. For detecting *Bd* infection, the ventral abdomen, ventral thighs and interdigital skin of the hind limbs of individual frogs were rubbed using a single sterile cotton swab, for a total of 10 strokes in each location. Swab tips were placed in sterile 1.5 mL microcentrifuge tubes and held at -20°C. To collect skin gland secretions, after being swabbed, frogs were placed individually in sterile clear plastic bags (Zip-Loc, sandwich size), and 40 nmol/g norepinephrine bitartrate (A0937-1G, Sigma-Aldrich, Canada) was injected through the bag into the dorsal lymph sac of the conscious frog. After 10 minutes, frogs were removed from the bags and acclimated in a dark plastic bin for an additional 10 minutes before being placed back in the main tank. When frogs were removed from the bags, cutaneous secretions in the bag were then gently squeezed into a 1.5 ml microcentrifuge tube containing 300 µl of RNALater solution (Ambion, Austin, Texas), which was stored initially at 4°C for 24-48 hrs, then placed at -80°C for long-term storage.

2.2.3 Fungal Culture

Cultures of *Bd* were required for positive controls when analyzing the cotton swabs, as well as for the second experiment (inoculation trial). Cultures of *Bd* strain JEL 423 on 1% tryptone agar plates, provided by Dr. Joyce Longcore (University of Maine, Maine, USA), shipped at ambient temperature, were imported under Canadian Food Inspection Agency import permit number A2011-00601-4. Agar cubes from these plates, approximately 8 mm^3, were placed in 250 mL flasks of tryptone broth and incubated for eight months at 17°C as stock cultures. Other agar cubes were transferred to fresh 1% tryptone agar plates, sealed with paraffin film and stored at 17°C. Plates and flasks were observed several times weekly for growth, which was detected as white fuzzy colonies on agar, or flecks suspended in clear broth. Subcultures, done only every 3-4 weeks to minimize the likelihood of contamination, were prepared by diluting broth cultures 1:10 into fresh tryptone broth, or transferring 1 mL of broth culture onto fresh tryptone agar plates, which were then dried and sealed with paraffin film.

2.2.4 Pathologic Examination

Frogs were euthanized at the end of Day 40, by full submergence in water containing benzocaine (E1501, Sigma-Aldrich, Canada; first dissolved in ethanol [100 g/L] prior to being mixed with water) 100 mg/L for at least 20-30 minutes. In the post-mortem room of the University of Guelph Animal Health Laboratory, complete whole-body post-mortem examinations, including examination for cutaneous lesions and evidence of systemic disease, were performed on each frog within an hour after euthanasia. Samples for histopathology (skin from the dorsal lumbar region and the ventral abdominal/thigh region, lung, liver, spleen, kidney, heart, and testis or ovary and oviduct, as well as additional abnormal tissues from individual frogs) were fixed in formalin, processed routinely by the University of Guelph Animal Health Laboratory, stained with hematoxylin and eosin (HE), and examined by a single blinded observer (LT). In the sections of skin, the following observations were recorded: granular glands (full/ partially full/ empty), dermal inflammation (presence/absence and cell type), hyperkeratosis (presence/absence), epidermal hyperplasia (presence/absence), and intra-epidermal fungal zoosporangia (presence/absence).

2.2.5 Measurement of Antimicrobial Peptide Gene Expression

Total RNA was isolated using an RNeasy Mini Kit (QIAGEN, Mississauga, Ontario) from cutaneous secretions collected and stored as described above. Prior to cDNA synthesis, genomic DNA contamination was eliminated using DNase I Incubation Mix (QIAGEN, Mississauga, Ontario), and RNA yield and quality were analyzed by spectrophotometry at 260 and 280 nm (NanoDrop, Thermo Scientific, Wilmington, Delaware) (Appendix 2).

The first-strand cDNA was synthesized using the Superscript III® Reverse Transcriptase Kit (QIAGEN, Mississauga, Ontario). Samples were processed in batches of ten to fifteen. The cDNA synthesis was performed using a target of 100 ng total RNA, 1 μL Oligo (dT), 1 μL dNTP mix, and sterile distilled water to a total of 13 μL. Samples were incubated at 65°C for 5 min. Then, 4 μL 5X First Strand Buffer, 1 μL 0.1M DTT, 1 μL RNase OUT and 1 μL Superscript III RT were added, and samples were incubated at 50°C for 1 hr, followed by 70°C for 15 min to inactivate the reverse transcriptase. Then, 1 μL RNase H was added to remove any residual RNA, samples were incubated at 37°C for 20 min, and stored at -20°C (Appendix 2).

The primers for amplification of the coding regions of brevinin and ranatuerin were based on available partial mRNA sequences for *L. pipiens* brevinin 1Pf (GenBank: DQ276961.1) and *L. pipiens* ranatuerin 2P (GenBank: AJ427747.1). Primers were designed to optimize the product size for real-time PCR using Primer-BLAST (Primer 3 and BLAST, NCBI) (Table 2-1). The Sigma Tm calculator (http://www.sigma-genosys.com/calc/DNACalc.asp) was used to predict primer-dimers and secondary structures. M-fold (http://mfold.rit.albany.edu/) was used to predict secondary structures.

Elongation factor-1α (EF1-α) and 60S ribosomal protein L8 (RPL8) were used as reference genes, using previously published primers (for *X. laevis*) for amplification of the coding regions (Table 2-1) (Crespi et al 2004, Oka et al 2008). Gene transcripts for β-actin or 18S ribosomal RNA could not be amplified during preliminary testing of other reference gene candidates, despite many attempts using various primers from several frog species. The RT-qPCR procedure was optimized using samples of skin from a healthy *L. pipiens* frog, initially using PCR, gel electrophoresis and sequencing of the amplified product (University of Guelph Laboratory Services, Guelph, Ontario). The amplified product was used to make the qPCR standard curves, using 12 serial dilutions from 10^{-1} to 10^{-12}. A standard curve was generated for each primer set, performed in triplicate. Efficiencies for each standard curve are listed in Table 2-1.

RT-qPCR analysis of the test samples was performed using 1 μL of amplified cDNA (or PCR-grade water, for the negative control wells) added to a premix consisting of 5 μL SYBR Green 1 (Roche Applied Science, Eugene, Oregon), 0.5 μL of 20 μM of each forward and reverse primer, and 3 μL of PCR-grade water for a total volume of 10 μL. Real-time quantifications were performed using the Lightcycler® 480 system (Roche Applied Science, Salt Lake City, Utah). Each assay was performed in duplicate with the following program parameters: pre-incubation at 95°C for 5 minutes; amplification consisting of 45 cycles of denaturation at 95°C for 20 seconds, annealing at 57°C for 15 seconds, and elongation at 72°C for 20 seconds; a melting curve from 45°C to 95°C; and cooling at 40°C for

10 seconds. The fluorescence threshold value was calculated using Lightcycler® 480 system software.

Test samples were run in duplicate. cDNA (1:3 dilution) from one sample (frog ID: June 22 #10) was used as a calibrator for each 96-well plate. Basal expression of the brevinin and ranatuerin genes was normalized with expression of reference genes EF1-α and RPL8. Concentrations of brevinin and ranatuerin relative to the reference gene for each sample were calibrator-normalized. Representative melting curves are shown in Appendix 4.

2.2.6 Quantification of *Batrachochytrium* Infection

Cutaneous *Bd* infection was quantitated by qPCR, because the results would be confounded if any frogs were indeed infected during the study. Swab tips remained in the original 1.5 mL microcentrifuge tube for the digestion process. For each sample, 500 µL strong digestion buffer (see Appendix 1) and 50 µL proteinase K (Qiagen) were added and the samples were placed in a thermal shaker for 24 hours at 65°C then 10 minutes at 99°C. After cooling at room temperature, DNA was extracted using the DNeasy Plant Handbook protocol (Qiagen). Eluted DNA was stored at minus 20°C until further processing.

For the positive control, *Bd* zoospores and zoosporangia were harvested from the culture plates by pipetting 1 mL distilled water onto the surface of the agar and incubating for 1 minute prior to filtering the fluid through a mesh (Fisher Scientific >20 µm coarse-porosity filter paper) to separate zoospores from zoosporangia. The suspension of zoospores was washed once, and the pellet was re-suspended in 100 µL water. An aliquot was stained by diluting 1:10 with iodine, and zoospores were enumerated using a Neubauer haemocytometer. The zoospores were diluted to make a 10-fold dilution series from 10^0 to 10^6 zoospores/mL as a standard curve for the subsequent qPCR reactions (Figure 2-1). DNA was extracted and quantified as described above. PCR was carried out using the previously described primers and protocol (Boyle et al 2004) (see Appendix 3), with gel electrophoresis and subsequent sequencing of the product to confirm that the primers resulted in specific amplification.

Quantitative PCR as described above was used to determine the amount of *Bd* DNA in the skin swabs, as well as in samples of water from each tank at the beginning and end of the experiment. Each qPCR assay was performed in duplicate with the following program parameters: pre-incubation at 50°C for 2 minutes and 95°C for 10 minutes, followed by 50 cycles of denaturation at 95°C for 15 seconds, annealing at 60°C for 1 minute, elongation at 72°C for 15 seconds, followed by a melting curve from 45°C to 95°C, then cooling for 40°C for 10 minutes. All qPCR assays were performed in a mixture containing 1 µL of cDNA preparation, 6 µ L premix including SYBR Green 1 Master (2X concentration) (Roche Applied Science, Eugene, Oregon), 0.5 µ L of 10 µM of each forward and reverse primer, and 3 µ L of PCR grade water. A sample of water run through the DNA extraction process was used as an additional negative control. Quantitative PCR was performed using the Lightcycler® 480 system (Roche Applied Science, Salt Lake City, Utah). The fluorescence threshold value was calculated using Lightcycler® 480 system software.

2.2.7 Statistical Analysis

A generalized linear mixed model was used to fit the data using Proc MIXED (SAS 9.2) with fixed effects of time, treatment and the covariates EF1-α and RPL8; plate was considered a random effect. Outcome variables include brevinin and ranatuerin gene expression levels in relation to each of the reference genes EF1-α and RPL8, expressed as a ratio. There were repeated measures over time on individual frogs, so to handle this, various error structures were entertained and the error structures with the smallest AIC were chosen (among those that converged, as offered by SAS: variance component, ar(1), arh(1), toeplitz, banded toeplitz, unstructured and banded unstructured error structures). All two-way interactions among time, treatment and the covariates were entertained as were the three-way interactions between treatment, time and the covariates; in addition to the quadratics of EF1-α and RPL8 were also included in the model. Effects that were not significant with $p>0.05$ were removed from the model except the main-effects primary factors of interest. Residual analyses were performed to examine the ANOVA assumptions. This included formally testing the residuals for normality using the four tests offered by SAS (Shapiro, etc). In addition, the residuals were plotted against the predicted values and the explanatory variables used in the model. Such analyses may reveal outliers, unequal variances or the need for data transformation. ANOVA assumptions were adequately met. There was no need for data transformations. Data are shown as mean ± standard error of the mean (SEM) unless otherwise indicated.

2.3 Results

2.3.1 Clinical and Pathologic Observations

Frogs in the two treatment groups were of similar body weight on day 0 (P=0.74, Student's t-test), and gained weight over the 40-day course of the experiment (Figure 2-2). Maximal increases in body weight, 11.8 ± 1.4 g for the steroid treatment group and 14.9 ± 0.9 for the control treatment group, were not different between groups (P=0.10, Student's t-test). One frog (#1) had a right-sided head tilt for the course of the experiment but did not have any weight loss or anorexia, and therefore remained in the study.

At post-mortem examination, no gross lesions were found. Histologically, frog 1 (which had a head tilt during the experiment) had a unilateral (right-sided) neutrophilic otitis interna with no involvement of the brain. No other histologic lesions were found in any of the other organs.

2.3.2 Measurement of Antimicrobial Peptide Gene Expression

The two reference genes were significantly correlated (Pearson r=0.965, P<0.0001, Figure 2-2) with no overall significant effect of treatment. There was a treatment by time interaction for two of the time points (32 and 40 days).

Each of the two target genes (brevinin, ranatuerin) was compared individually to each of the two reference genes

(EF1-α, RPL8) using the normalized values. Within each paired comparison, the means of the normalized values for the treatment group were compared to the means of the normalized values for the control group at individual time points. The normalized values were defined as the ratio of the crossing point of the target gene to the reference gene, in relation to the calibrator value. A higher crossing point reflects a lower level of mRNA expression. Therefore, a higher normalized value reflects an increase in mRNA expression of the target gene, assuming a constant reference gene expression. The evaluation of these normalized values for each target: reference gene pair is subsequently referred to as the "ratio model".

Results of the various ratio model comparisons are presented in Figure 2-4, and individual data points for each frog are found in Appendix 5. Over the six time points, the level of target gene expression appeared to increase within the steroid treatment group. In the brevinin/EF1-α ratio model, there was an overall significant (P<0.0272) increase in gene expression in the corticosteroid treatment group (0.6266 ± 0.0246, mean ± SEM) compared to the control treatment group (0.3855 ± 0.0459). Although not statistically significant, an overall increase in gene expression in the corticosteroid treatment group was also found in the brevinin/RPL8 ratio model (P =0.1235), ranatuerin/RPL8 ratio model (P =0.1678), and ranatuerin/EF1-α ratio model (P =0.1120).

2.3.3 Quantification of Batrachochytrium Fungus in Cutaneous Swabs

The amount of fungal DNA on the swabs collected from all frogs at Day 0 all had crossing points above 30.00, and therefore were below the assay limit of 10^3 zoospores/mL. However, the RT-qPCR product of one sample (P10, or frog #10) had a melting point identical to that of the positive control (80°C), indicating the presence of *Bd* DNA. Therefore, this sample was considered to be positive but for a very small quantity of *Bd* DNA (less than 10^2 zoospores/mL).

2.4 Discussion

This experiment investigated the effect of glucocorticoids on gene expression of cutaneous antimicrobial peptides that are known to inhibit the *in vitro* growth of *Bd*, using a frog species (*L. pipiens*) that is considered susceptible to this pathogen (Voordouw et al 2010). I hypothesized that exogenous glucocorticoid administration would inhibit the gene expression of these important peptides, and in turn that glucocorticoid-induced suppression of this innate immune response may predispose to infection with *Bd*. Unexpectedly, the data showed the opposite effect, that corticosteroid administration was associated with increased antimicrobial peptide gene expression in the norepinephrine-induced cutaneous secretions of *L. pipiens*.

Lithobates pipiens was studied because it was reported to be susceptible to chytridiomycosis. For example, this species suffered massive die-offs in the Colorado Rockies that were originally thought to be due to bacterial infection, but later analysis of museum specimens confirmed chytrid fungal infection (Rollins-Smith et al 2002)

(citing unpublished data from Carey and Pessier). Second, *L. pipiens* is common in Ontario, so the health of this species is considered important. Further, this species is easily obtained through commercial suppliers, and is known to produce two antimicrobial peptides that have anti-chytrid activity in vitro (Rollins-Smith et al 2002, Pask et al 2013). In contrast, "susceptible" frog species that have experienced massive die-offs are either extinct or in low numbers, and not readily available for laboratory work.

Samples of norepinephrine-induced cutaneous secretions were used for analysis of gene expression in this study. Norepinephrine, an α-adrenergic agonist, stimulates contraction of myoepithelial cells that leads to dose-dependent discharge of the Type I granular gland (Holmes et al 1978, Nosi et al 2002). The granular glands have a holocrine method of secretion, resulting in discharge of cellular mRNA from the glandular epithelium, thus providing a method for sequential analysis of gene expression.

In this study, injection of norepinephrine resulted in visible accumulation of secretions within 3-5 minutes, confirming the effect of the norepinephrine. Quantitative RT-PCR proved reliable in measuring gene expression of the secreted AMPs of interest. Amplification efficiencies for RT-qPCR were about 2.0, which is considered excellent. Crossing points for the reference genes—EF1-α and RPL8—were not affected by corticosteroid administration, and they were highly correlated, indicating that these genes were valid for reference use in this study.

The amount of isolated RNA was not normalized prior to cDNA synthesis, nor was the concentration of cDNA in each PCR reaction normalized. As a result, individual crossing points could not be directly compared to each other, but comparison of the ratios of target: reference gene levels (e.g. brevinin: EF1-α) was considered valid.

Counterintuitively, antimicrobial peptide gene expression was greater in norepinephrine-stimulated secretions from frogs that were administered corticosteroid, compared to frogs that were administered saline. This effect was statistically significant for the antimicrobial peptide brevinin, using EF1-α as the reference gene. Although not statistically significant, a similar effect was seen for brevinin using RPL8 as the reference gene, and for the antimicrobial peptide ranatuerin relative to either of the reference genes. Furthermore, this effect appeared to be time-dependent: steroid- and saline-treated groups were similar at 0, 8 and 16 days, but differences between the groups were apparent at 24, 32 and 40 days of treatment. The reason for this apparent time-dependence was not investigated. The timing of this effect was unexpected, because previously reported inhibitory effects of corticosteroids on antimicrobial peptide gene expression in cultured bovine tracheal epithelial cells were demonstrated at 40 hours (Mitchell et al 2007), and the inhibitory effects of corticosteroid on antimicrobial peptide levels in the skin of frogs were evident at seven days of treatment (Simmaco et al 1997). The fact that one frog appeared to be infected with low levels of *Bd* did not influence the statistical analysis.

The current findings differed from those previously reported (Simmaco et al 1997), where topical or systemic administration of glucocorticoids resulted in reduced gene expression of antimicrobial peptides in the skin of frogs. There are several methodological difference that may explain this discrepancy: the use of *L. pipiens* rather than *L.*

esculenta, the analysis of brevinin and ranatuerin rather than esculentin, collection of secretions using norepinephrine rather than electrical stimulation, the use of saline-treated controls rather than untreated control frogs, and quantitation of gene expression using RT-qPCR rather than Northern blot. Other variables also may also have contributed to the differences, including the nutritional and infection status of the frogs, the acclimatization period, the laboratory environment and housing, and the degree of handling stress. Nonetheless, the precise reasons for the discrepancy between the two studies were not determined.

Chronic exposure to corticosteroids is considered to be immunosuppressive, but shorter-term administration of corticosteroids augments some aspects of the innate immune response in mammals. For example, dexamethasone has several anti-inflammatory effects on neutrophils but has been shown to increase neutrophil migration into the uterus (Konig et al 2006) and lung (Mitchell et al 2007). Although psychological stress reduces cathelicidin and β-defensin expression in mice, it has been shown to increase expression of the antimicrobial neuropeptide catestatin (Martin-Ezquerra et al 2011). Finally, glucocorticoids increase production of the surfactant proteins A and D by lung epithelial cells *in vitro*, and may increase the response to signalling through Toll-like receptors (Schleimer 2004). Thus, there is precedent for glucocorticoid-mediated enhancement of antimicrobial peptide defences.

The findings of this *in vivo* study are subject to several limitations. First, it should be considered that stress or infections with agents other than *Bd* might have had an effect on antimicrobial peptide gene expression or modified the response to glucocorticoid administration. This possibility was mitigated by the long acclimatization period of 23 days, during which no overt evidence of infection with any agent was observed, and attention was paid to environmental enrichment conditions to minimize environmental stress. Further, frogs were treated with an antibiotic prior to the study, and evidence of bacterial infection was not found at post-mortem examination. Nonetheless, the frogs were subjected to restraint and injection stress every other day, and one frog had evidence of a low level of *Bd* infection based on PCR testing. A second caveat is that the RT-qPCR method used in this study would not account for post-translational modifications that may affect secretion, function or half-life of the antimicrobial peptides.

This study identified increased antimicrobial peptide gene expression in skin of *L. pipiens* following 24-40 days of treatment with the corticosteroid methylprednisolone. These findings do not support the hypothesis that environmental stress predisposes frogs to chytridiomycosis by causing glucocorticoid-induced suppression of antimicrobial peptide defences.

Table 2-1. Primers for RT-qPCR measurement of antimicrobial peptide gene expression.

Gene	Forward Primer (5´-3´)	Reverse Primer (5´-3´)	Amplicon size (bp)	Efficiency
EF1-α (Reference gene)	CACACTGCTCACAT TGCTTGC	ACAATGGCAGCAT CTCCAGAC	209	2.069
RPL8 (Reference gene)	CACAGAAAGGGGC TGCTAAG	CAGGATGGGTTTGT CAATACG	254	2.092
Brevinin (Target gene)	TGAAACGGATGTT GAAGTGG	GTGATTGCCATCTG GTGTGC	178	2.086
Ranatuerin (Target gene)	CCAAAGATGTTCA CCATGA	CATATGTCCGGCCA AATTCT	187	1.987

Table 2-2. Mean crossing points (Cp) for qPCR quantification of *Bd* zoospore load on Day 0 of the experiment.

Steroid Treatment Group		Control Group	
Frog ID	Mean Cp	Frog ID	Mean Cp
1	34.00	3	37.93
2	34.23	5	35.29
4	36.54	6	34.54
7	35.80	11	33.35
9	38.68	12	34.65
10	31.21	13	35.14
		14	33.14

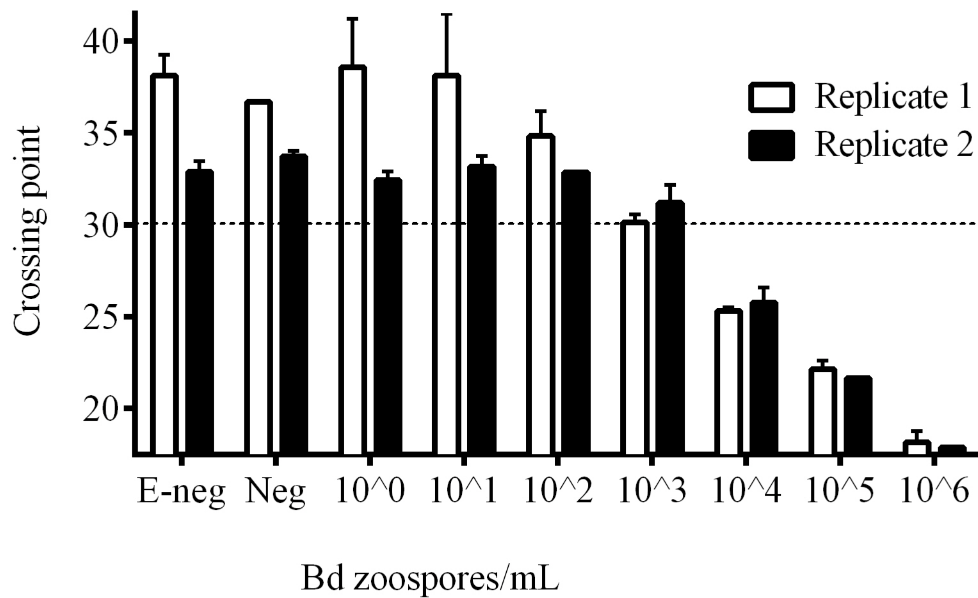


Figure 2-1. Standard curve dilutions for quantifying *Bd* DNA in extracts of cutaneous swabs using qPCR. DNA equivalent to 10^0 to 10^6 *Bd* zoospores/mL were analyzed. Negative controls included omission of DNA template (Neg) and a sample of water run through the DNA extraction process (E-neg). Samples falling above the dashed horizontal line were considered negative, based on difference from the negative controls and melting temperatures of the amplification product that differed from 80.2 ± 0.1^{o}C.

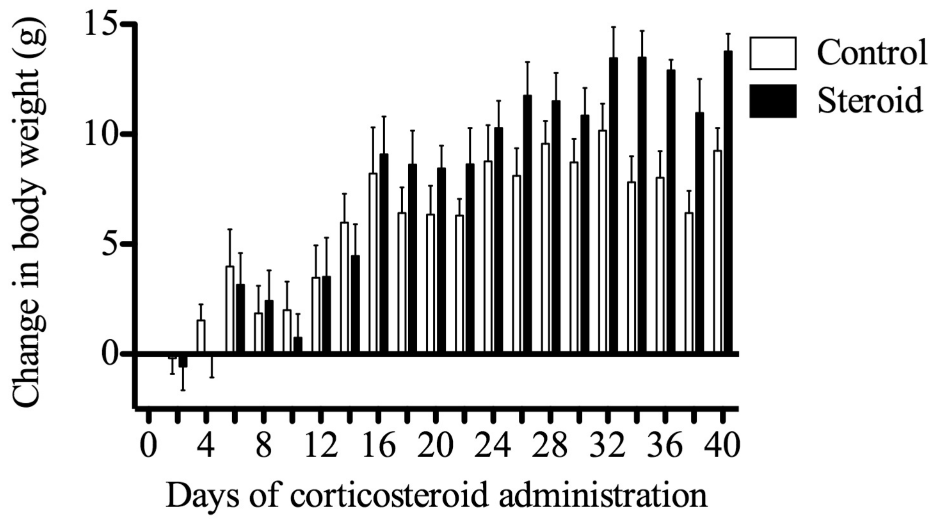


Figure 2-2. Mean change in body weight from Day 0 ± SEM for steroid-treated and control groups at each time point over the course of the experiment.

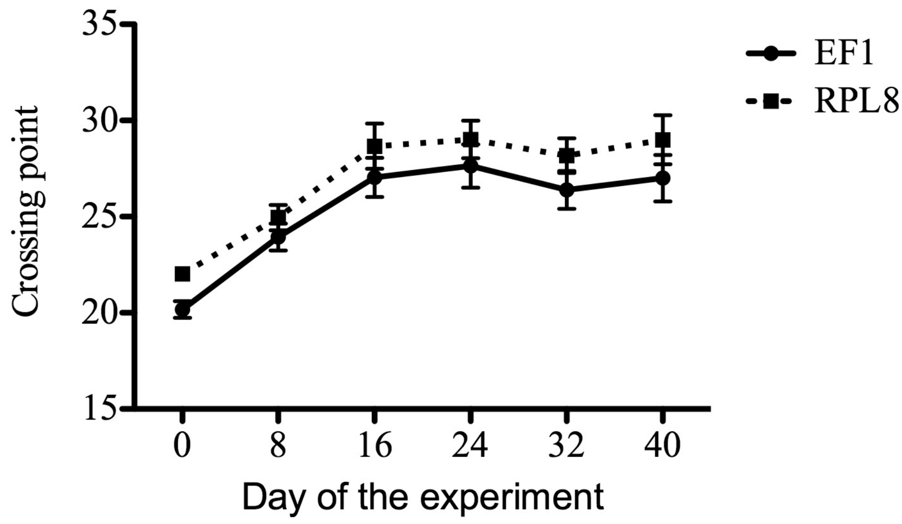


Figure 2-3. RT-qPCR crossing points of the two reference genes (EF1-α and RPL8) at the six sampling times during the experiment.

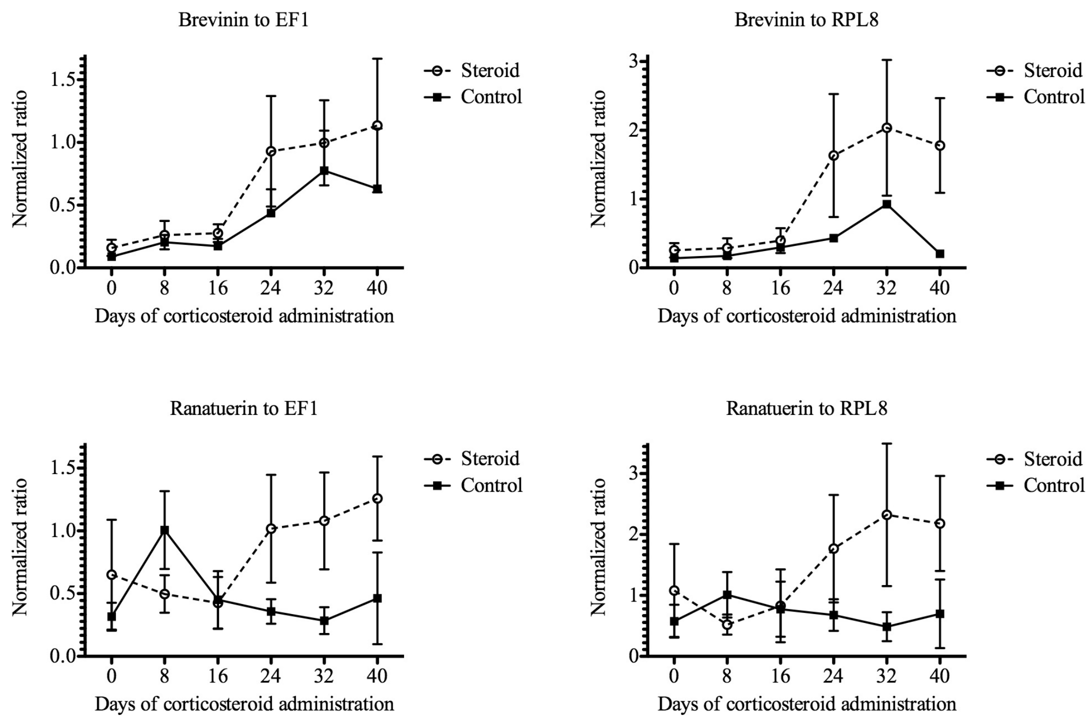


Figure 2-4. Brevinin and ranatuerin gene expression in norepinephrine-induced cutaneous secretions of frogs administered corticosteroid or saline. Comparison of steroid to control treatment groups, based on normalized ratios of the target gene (brevinin or ranatuerin) to the reference gene (EF1-α or RPL8) at the six sampling times (mean ± SEM of two technical replicates).

Chapter 3: Experimental Infection with *Batrachochytrium dendrobatidis*

3.1 Introduction

The overall objective of the investigation described in this chapter was to determine whether glucocorticoid administration affects the susceptibility of *L. pipiens* to experimental infection with *Bd*, corresponding with glucocorticoid-stimulated alterations in AMP expression. Although there are scattered studies of corticosteroids and of antimicrobial peptides in frogs experimentally infected with *Bd,* this is the first experiment examining these three elements simultaneously. Further, the study was designed to permit comparison of findings in *L. pipiens* to recently published work on *X. laevis* (Ramsey et al 2010). The general hypothesis was that glucocorticoid administration would result in an increase in fungal load (the number of zoospores) and severity of disease in the susceptible frog species (*L. pipiens*) but not in the resistant frog species (*X. laevis*), and that this difference in infection/disease outcome would be associated with corresponding differences in gene expression of those cutaneous antimicrobial peptides known to inhibit *Bd* growth *in vitro*. The study reported in this chapter was a pilot experiment to establish the *Bd* challenge model in *L. pipiens*, and to provide baseline data to permit rational design of a proposed larger subsequent experiment.

3.2 Materials and Methods

3.2.1 Experimental Subjects

The study was approved by the Animal Care Committee of the University of Guelph, operating under the auspices of the Canadian Council for Animal Care (Animal Use Protocol number 10R085). Eighteen frogs (*L. pipiens*) were obtained from Wards of Canada Limited (www.wardsci.com) and housed in the Biosafety Level 2 containment unit of the University of Guelph Central Animal Facility for a 78-day acclimatization period as well as the 24-day experimental period. Upon arrival, frogs were half-submerged in an aqueous solution of 100 mg/L of oxytetracycline (Oxy-Vet 100LP, Vetoquinol, Quebec, Canada) for 1 hour to prevent bacterial infection (a widely used method). Individual frogs were identified using digital photographs to record unique spot patterns of the skin on the dorsum.

During acclimatization, two frogs had unilateral diffuse eyelid edema, and swabs of the affected eyelids were obtained. Cytologic examination of smears revealed necrotic debris and sloughed epithelial cells with no bacteria or neutrophils, and both frogs were treated empirically with topical 0.3% gentamycin ophthalmic solution (Garamycin, Merck) (1 drop once daily for 8 days). One of the ill frogs (#18) was found dead in the tank prior to the start of the

experiment, and was not included in the study. Another frog (#16) was found dead the morning after the initial inoculation with *Bd*, and was also excluded from the experiment.

Frogs were haphazardly separated into four groups of 3-5 animals (Table 3-1), housed in separate tanks placed approximately two feet apart within the same room. The tank design, water quality and exchange, amenities, and frog handling, feeding and weighing were as described in Chapter 2. Any interaction with the frogs, either related to husbandry or related to the experiment itself, commenced with Tank A (control), then Tank B, C and D to avoid cross-contamination between tanks. The sex of the frogs was determined at the time of necropsy, revealing two or three males and two or three females in each group (Table 3-1).

3.2.2 Experimental Infection with *Batrachochytrium*

A second shipment of cultures of *Bd* strain JEL 423 was obtained from Dr. Joyce Longcore (University of Maine, Maine, USA), under Canadian Food Inspection Agency import permit number A-2012000169-4, as described for the previous importation in Chapter 2, and zoospores were propagated and collected as described there.

The suspension of zoospores was microcentrifuged at 7500 rpm for 30 minutes, the supernatant was decanted, and the pellet resuspended in 25 mL of sterilized water. Zoospores were enumerated using a haemocytometer as described in Chapter 2. Zoospores were determined to be alive by microscopically observing swimming movement in an aliquot of the zoospore suspension that was stained 1:1 with iodine solution.

The zoospore suspension was diluted in sterile distilled water to form the inoculum of 0 (tank A), 10^4 (tank B), 10^5 (tank C) or 10^6 (tank D) zoospores per tank. All frogs within each treatment group were placed together in a plastic container containing 2 L of fresh dechlorinated water to a level in which the frogs were half immersed. The inoculum was added and the frogs were left in this bath for 24 hours. The actual zoospore concentration was calculated following this first challenge, revealing that the zoospore concentration was less than originally intended (Appendix 6). Therefore, the frogs were exposed a second time, 48 hours after the start of the first inoculation, as needed to achieve the total inoculation dose intended (0, 10^4, 10^5 or 10^6 zoospores),

Cutaneous swabs were collected from each of the 16 frogs as described in Chapter 2. Swabs were collected on day minus 78 (soon after the frogs arrived), day 0 (just prior to fungal exposure), and on days 8, 16 and 24 after the first *Bd* exposure. After the swabs were obtained, norepinephrine was injected into the dorsal lymph sac and the resulting cutaneous secretions were collected as described in Chapter 2. Before swabbing, each animal was weighed in grams to two decimals.

3.2.3. Pathologic Examination

Frogs were euthanized at the end of Day 24 and post-mortem procedures were identical to those described in Chapter 2, with the only exception being that post-mortem examinations were conducted within the post-mortem room of the Biosafety Level 2 containment unit of the University of Guelph Central Animal Facility, rather than the

post-mortem room of the University of Guelph Animal Health Laboratory.

3.2.4. Quantification of Fungal Infection

The tip of the cutaneous swabs remained in the original 1.5 mL microcentrifuge tube for the digestion process. For each sample, 500 μL strong digestion buffer (Appendix 1) and 50 μL proteinase K (Qiagen) were added, and the samples were placed in a thermal shaker for 24 hours at 65°C, then 10 minutes at 99°C. After cooling to room temperature, DNA was extracted using the DNeasy Plant Handbook protocol (Qiagen). Eluted DNA was stored at -20°C. The qPCR procedure for quantification of *Bd* was as described in Chapter 2. Samples were analysed in duplicate. The positive control was a preparation of DNA extracted from *Bd* cultures, as described in Chapter 2. Negative controls were an "extraction negative" (extraction buffer without sample), and "PCR negative" (primers and water but no DNA). The assay was considered negative if the crossing point was higher than the highest crossing point in the standard curve (Crossing point=33.06, corresponding to 100 *Bd* zoospores/mL). For the standard curve, mean crossing points for positive samples ranged from 16.3 to 32.8, with a product melting temperature of 79.92 to 80.31°C (Figure 3-2; Appendix 7). The standard curve was linear in the range of 10^2 to 10^6 zoospores/mL. In contrast, a crossing point was only detected for one of the two samples at 10^1 zoospores/mL (Crossing point=36.6), and the products had a higher crossing point (89.48°C and 82.96°C). Crossing points were not detected for any of the negative control samples, and melting temperatures of these products were higher (88.53-89.83°C) or lower (74.87-77.21°C) than those listed above.

3.3. Results

3.3.1 Clinical and Pathologic Observations

No abnormal behaviour was observed throughout the course of the experiment. Body weights were recorded on arrival (day minus 78, shortly after arrival), and on days 0, 8, 16 and 24 relative to the first *Bd* exposure (Figure 3-1). Three measurements were excluded as outliers (frog 2, day minus 78 and day 8; frog 3, day 8). Mean body weights ± SEM in grams over the course of the experiment were: Group A, 56.4 ± 3.9; Group B, 59.7 ± 2.3; Group C, 70.6 ± 4.6; and Group D, 61.2 ± 3.1 (mean ± SEM), and the differences between groups were significant (P=0.009, repeated measures ANOVA).

No gross lesions were observed on post-mortem examination. Microscopic findings in individual frogs, including some that supported or confirmed *Bd* infection, are summarized in Table 3-1. Within the sections of skin, there was up to threefold thickening (hyperplasia) of the epidermis (Figures 3-12 and 3-13), with variable amounts of parakeratotic hyperkeratosis (Figure 3-11), jumbling of the basal layer and variable numbers of individual necrotic or degenerating keratinocytes. Dermal inflammation, when present, was superficial and mostly comprised of mixed mononuclear cells (Figure 3-10). Intraepidermal fungal zoosporangia were identified in three frogs (Table 3-1; Figures 3-5 to 3-7). These ranged from 6-15 μm in diameter with refractile walls that stained positively with PAS

and GMS. In only one section was a discharge papilla captured. The granular glands varied from empty to full of proteinaceous secretions (Figures 3-8 to 3-9). Lesions were not clearly associated with degree of Bd exposure, and were found in frogs within the unexposed control group as well (Table 3-1).

Findings in visceral organs were presumed to be clinically insignificant, and included trematodes in the liver associated with dilation and fibrosis of bile ducts (7/16 frogs, Table 3-1; Figures 3-14 and 3-15), and/or in the lung (5/16 frogs, Table 3-1; Figures 3-16 and 3-17), involving some animals in all experimental groups.

3.3.2. Quantification of Fungal Infection

The presence of *Bd* DNA was measured by quantitative PCR. In contrast to the standard curve samples, PCR amplicons from DNA extracts of skin swabs had melting point peaks at 76, 80 and 84°C. Consistent with the above standard curve, standards (positive control) used in this assay had melting points of 78.49-80.03°C. Amplified products from samples with each of the above melting points were sequenced, and a Blast search was performed for each sequence to identify most closely related nucleotide sequences (Table 3-2). Three samples matched to the expected *Bd* gene (internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2) and these products had melting points of 84.1-84.59°C. A positive match to *Bd* was made in frog 9 from time 0 (before *Bd* challenge), as well as one frog each at 8 and 24 days after challenge. However, other amplified sequences—from all three melting temperature peaks—matched to the same gene of other fungi but not to *Bd*.

Using the quantitative PCR assay on DNA extracts of cutaneous swabs, positive tests were obtained from some animals in all experimental groups. The frequency of positive test results did not consistently depend on experimental group (0, 10^4, 10^5, 10^6 zoospore challenge) or time (minus 78, 0, 8, 16, 24 days after challenge) (Table 3-1, Figure 3-3).

The PCR crossing points of skin swab samples obtained from males and females differed significantly (P=0.049, 2-way ANOVA), with lower qPCR crossing points (higher fungal DNA concentrations) in males (Table 3-1, Figure 3-4). Despite mixing of male and female frogs within each experimental group, there was little evidence of increasing fungal loads in female frogs over time, with the exception of frog 9 at day 24 (Table 3-1).

Bd zoosporangia were detected histologically in only 3 of 16 frogs (Table 3-1): numbers 2 and 9 from the control group (which also had sequencing data matching to *Bd*), and number 11 from the treatment group (Figure 3-5). The two of these frogs with day 24 qPCR data had crossing point values (and product melting points) of 26.35 (Tm=83.05°C) and 29.91 (Tm=84.85°C) at that time. By inspection, the presence or absence of histologic lesions in the skin (dermal inflammation; basal cell degeneration, dysplasia or necrosis; epidermal thickening; and hyperkeratosis) was not correlated with *Bd* qPCR results (no formal analysis was done).

The presence of *Bd* in tank water was analyzed by qPCR (Table 3-4). Crossing points were all above 33.06 and were therefore considered negative for the presence of *Bd*, except for tank B (10^4 *Bd* zoospores/mL, after infection) with

an equivocal crossing point of 32.1 and a melting temperature of the amplicon of 84.38 ^{o}C. Furthermore, crossing points for water collected at the end of the experiment from tank A (uninfected) were lower than those from the 10^5 and 10^6 *Bd* challenge groups.

3.4 Discussion

The study reported in this chapter was a pilot experiment to establish the *Bd* challenge model in *L. pipiens*, and to provide baseline data to permit rational design of a more definitive subsequent experiment. Unexpectedly, some of the frogs used in this study were found, after the fact, to have been infected with *Bd* prior to the experimental challenge. Although this provided a serendipitous opportunity to study the naturally occurring disease in the environment of a research facility, analysis was complicated for reasons discussed below.

Experimental challenge of this species with *Bd* has, to our knowledge, only been reported in *L. pipiens* that had recently undergone metamorphosis (Paetow et al 2012, Gahl et al 2012), but not in adults of this species. This study objective was not achieved, because retrospective analysis of the *Bd* infection status indicated that some of the frogs were infected with *Bd* prior to the experimental challenge, making it impossible to evaluate an effect of the experimental challenge. As well, the methods used to quantify *Bd* load in the skin showed some cross-reactivity with other fungi. Nonetheless, the study reports useful data on histologic lesions, inconsistency in detection of *Bd* with repeated sampling of infected individuals, evidence that uninfected in-contact frogs may not develop *Bd* infection, and questions of specificity about a previously published method to detect *Bd* DNA.

The reasons for this lack of specificity were not thoroughly investigated. It is notable that crossing points in this assay were high compared to other qPCR and RT-qPCR assays. That is, the PCR reaction required many cycles before generation of a detectable product, compared to other comparable assays. It is speculated that this could confer a lack of specificity to the assay, as it would favor amplification of gene sequences that hybridized poorly to the primers. It is likely that this problem could be overcome by designing new primers, by using a fluorescent probe for more specific detection of the amplified *Bd* gene sequence, or by high resolution melt curve analysis. However, this was abandoned because *Bd* infection was detected prior to experimental analysis.

Lack of specificity of the *Bd* qPCR assay is problematic for interpreting the results of this study. Evaluation of the standard curve, prepared using *Bd* but free of contamination by other fungi, showed a linear relationship between numbers of *Bd* zoospores and crossing point in the qPCR assay. Thus, the qPCR assay was considered valid for detection and quantification of pure cultures of *Bd*. However, after completion of the in vivo study and of the laboratory analysis, it was discovered that the amplification products of the *Bd* qPCR assay had variable melting temperatures, which corresponded to amplification of various fungal genes. This finding indicates a lack of specificity of the assay, and is a major limitation to interpreting the data.

Irrespective of the above lack of specificity, the *Bd* qPCR assay did identify *Bd* DNA in frogs prior to experimental

challenge with *Bd*, and this was confirmed as *Bd* by sequencing in one of these frogs at time 0. The frogs were housed in the isolation unit and biosecurity level 2 protocols were followed. It is therefore likely that the frogs were infected with *Bd* prior to purchase. A previous study in Prince Edward Island found evidence of *Bd* infection in 5 of 20 *L. pipiens* frogs (4 of 17 adults), based on qPCR testing. Infection load was considered to be lower in *Lithobates pipiens* compared to *L. clamitans* (Forzan et al 2010). Another study, of an isolated population of *L. pipiens* in British Columbia, detected *Bd* infection in 13.1% (42/320) of *L. pipiens* tested using PCR (Voordouw et al 2010). Thus, in the present study, it is plausible that these wild-caught frogs were infected with *Bd* prior to entry into the experiment.

Male frogs in this study had a greater prevalence and degree of positive qPCR test results compared to the females, both at day 0 and at all later time points. If this represents a greater load of *Bd* in the male frogs (a conclusion that is limited by the lack of specificity of the *Bd* qPCR assay), this finding suggests that the male frogs were infected at a higher level or prevalence in the facility of the supplier.

Frogs in the present study that had higher fungal loads (lower crossing points) at day 0 tended to have higher fungal loads at later time points, consistent with a chronic infection of a subset of frogs in this study. However, this was not perfectly consistent. Some frogs initially tested positive and later had a negative test, while others were the opposite. This could occur if frogs eliminated infection but were subsequently re-infected. While I cannot rule out this explanation, it more likely reflects some combination of a low level of infection, differences in fungal shedding at different times, and variability in the swabbing technique, such that infected individuals may sometimes test negative. This finding has implications with respect to declaring individual frogs free of infection, for example prior to release from quarantine when entering new collections. As above, this conclusion is limited by the lack of specificity of the *Bd* qPCR assay, and the presence of other fungi may have complicated these results.

Despite being in the same tank as chronically infected males, most females remained negative for *Bd*, with qPCR crossing points greater than 33.06. This observation is considered valid, because a lack of assay specificity should not interfere with interpretation of a negative test result. This observation of persistently high crossing points could be explained by a lack of transmission, because of low fungal loads, and more importantly, low level shedding of *Bd* into the water by the infected males. An alternative explanation is that these females were resistant to infection. We are not aware of any evidence that female sex hormones affect resistance to *Bd*, although this possibility remains.

In the present study, frogs that were infected with *Bd* did not experience mortality or clinical signs of illness that could be attributed to this infection. In previous studies, *Bd* infection of frogs that had recently undergone metamorphosis was not considered to contribute to mortality of frogs during the experiment, nor did it have an effect on growth or organ weights. Similarly, infection of recently post-metamorphic *L. pipiens* frogs to *Bd* did not result in mortality (Gahl et al 2012). Increased frequency of ecdysis was the only reported effect of *Bd* infection (Paetow et al 2012). Thus, despite reports of natural mortality associated with *Bd* infection (Johnson et al 2011), experimental *Bd* infection of otherwise healthy *L. pipiens* frogs may not induce any substantial mortality or clinically detectable effect.

It has been suggested that *L. pipiens* may be a carrier of *Bd*, with implications for the health of other amphibians exposed to the same environment. However, despite detecting *Bd* in skin swabs at all time points of the present experiment, qPCR testing of the tank water did not detect evidence of infection (with one equivocal test result). Thus, although *L. pipiens* survives with chronic *Bd* infection of the skin, fungal loads appear to be low and this may limit the level of *Bd* contamination of water. The implications of this finding for in-contact amphibian species of greater susceptibility to *Bd* are uncertain.

The microscopic appearance of *Bd* within the epidermis of frogs in this study was similar to that previously reported (Nichols et al 2001, Bradley et al 2002, Pessier et al 1999, Voyles et al 2009). *Bd* was detected by microscopic examination in only 3 of the 16 exposed frogs in this study. There was no apparent relationship between infection status by qPCR and the presence of histologic lesions in the skin of frogs in this study. This lack of cutaneous inflammation or epidermal changes is consistent with the low mortality and lack of observed clinical signs, suggesting that *L. pipiens* can be chronically infected with *Bd* without eliciting a substantial host reaction that would lead to development of clinically apparent disease.

In conclusion, this study did not succeed in establishing an infection model of *Bd* in *L. pipiens* that could be used experimentally, because frogs were infected on arrival prior to the infection challenge. Despite infection of some frogs with *Bd*, other in-contact animals remained free of infection throughout the experiment, as assessed by qPCR and histopathology for detection of *Bd*. Further, infected frogs did not show any clinical signs, and histologic changes in the skin were not correlated to the histologic presence of *Bd* in the skin or to the *Bd* qPCR test results. Other findings of the study were limited by a lack of specificity of the assay, with unexpected amplification of gene sequences from fungi other than *Bd*.

Table 3-1. The effect of *Bd* zoospore challenge and time on histopathology findings and *Bd* qPCR crossing points. Cells are formatted to show dark grey for values less than or equal to 29.0, light grey for values 29.1 to 32.9 and white for values greater than or equal to 33. Time is shown as days after the first challenge with *Bd*; day 0 is pre-challenge. 1 or P = Presence of lesions; 0 or A = absence of lesions. n/a: data not available.

Experimental parameters				Histopathology findings*							Bd-qPCR Crossing points			
Tank ID	Zoospore challenge	Sex	Frog ID	Dermal inflammation	Basal cell degeneration	Epidermal thickening	Hyperkeratosis	PAS, GMS stains	Liver trematodes	Lung trematodes	Day 0	Day 8	Day 16	Day 24
A	0	F	2	1	1	2	2	+	0	0	32.0	29.0	45.0	45.0
A	0	F	7	1	1	0	1	-	0	1	32.8	34.4	45.0	33.0
A	0	F	9	0	1	2	0	+	1	0	30.4	30.6	38.8	26.4
A	0	M	13	0	1	0	1	-	1	1	24.3	26.0	28.0	21.7
A	0	M	17	1	1	1	1	-	1	0	25.1	32.3	27.9	28.0
B	10,000	F	6	2	1	0	1	-	0	1	33.0	29.9	45.0	41.8
B	10,000	F	8	2	2	0	2	-	1	0	45.0	33.5	36.7	29.5
B	10,000	M	10	1	2	3	1	-	0	0	25.7	35.4	42.6	21.6
B	10,000	M	14	3	2	3	3	-	1	0	45.0	37.8	45.0	44.0
C	100,000	F	3	0	2	2	0	-	0	0	45.0	30.6	32.4	45.0
C	100,000	F	5	0	1	1	0	-	0	1	34.9	n/a	31.6	31.7
C	100,000	M	11	0	1	3	1	+	1	0	45.0	31.5	26.5	29.9
C	100,000	M	15	0	2	3	1	-	0	1	26.4	30.5	24.1	45.0
D	1,000,000	F	1	1	0	0	0	-	1	0	45.0	45.0	30.7	45.0
D	1,000,000	F	4	0	1	0	0	-	0	0	45.0	44.0	45.0	45.0
D	1,000,000	M	12	3	2	3	3	-	0	1	26.4	26.3	28.8	45.0

Table 3-2. Sequencing results from selected PCR amplicons, for evaluating the specificity of the quantitative PCR assay for *Bd*. The findings represent data from 8 samples and are shown as date of sampling and frog identification number, crossing point of the quantitative PCR reaction, melting point (ºC) of the PCR amplicon, as well as the forward and reverse seqencing results of the amplicon. The sequencing results are shown as the top one or two matches in a Blast search of the NCBI database, the NCBI reference number of this match, and the E-vlaue from NCBI as an indicator of the strength of the match.

Date & frog	**Crossing point**	**Melting point**	**Dire-ction**	**Sequence quality**	**Top matches in Blast search**	**Reference sequences**	**E values**
0-2	32.22	80	F		No results		
			R	0.2	Uncultured fungus, *Alternaria*	GU721751, JQ346908	7.55E-155, 3.50e-155
0-3	45	76.71	F	45.3	*Trichurus, Doratomyces*	JF429664, FJ914695	0, 0
			R	12.6	*Doratomyces, Trichurus*	GU566278, FJ914659	0, 9.44e-159
0-9	30.56	84.48	F	0.2	No results		
			R	0	*Batrachochytrium dendrobatidis*	JQ582924	1.04E-43
8-4	31.75	80.12	F	12.7	*Rhodotorula, Sporidiobolales*	AB025988, EF060587	0, 0
			R	10.3	*Rhodotorula, Rhodosporidium*	AB025975, JN662395	0,0
8-14	21.53	84.1	F	3.4	*Batrachochytrium dendrobatidis*	JQ582916	1.16E-147
			R	6.2	*Batrachochytrium dendrobatidis*	JQ582926	1.66E-66
16-1	45	76.34	F	0.7	Various incl *Nothobranchius*	GAIB01157510	8.36E-10
			R	17.6	Various non-fungal sequences	NM_001256143	3.43E-148
24-5	30.45	84.59	F	0.2	No results		
			R	4	Uncultured fungus, *Alternaria*	GU721935, JQ346904	0, 0
24-10	30.65	85.06	F	3	*Batrachochytrium dendrobatidis*	JQ582920	0
			R	46.5	*Batrachochytrium dendrobatidis*	JQ582915	2.14E-45

Table 3-3. The effect of sex of the frog on *Bd* qPCR crossing points. Cells are formatted to show higher *Bd* concentrations in dark grey, lower concentrations in light grey and crossing points above the detection limit in white (negative test). Blank cells are missing data points. Time is shown as days after the first challenge with *Bd*; day 0 is pre-challenge. n/a: data not available.

Experimental parameters				Bd-qPCR Crossing points			
Sex	Tank ID	Zoospore challenge	Frog ID	Day 0	Day 8	Day 16	Day 24
F	A	0	2	32.0	29.0	45.0	45.0
F	A	0	7	32.8	34.4	45.0	33.0
F	A	0	9	30.4	30.6	38.8	26.4
F	B	10,000	6	33.0	29.9	45.0	41.8
F	B	10,000	8	45.0	33.5	36.7	29.5
F	C	100,000	3	45.0	30.6	32.4	45.0
F	C	100,000	5	34.9	n/a	31.6	31.7
F	D	1,000,000	1	45.0	45.0	30.7	45.0
F	D	1,000,000	4	45.0	44.0	45.0	45.0
M	A	0	13	24.3	26.0	28.0	21.7
M	A	0	17	25.1	32.3	27.9	28.0
M	B	10,000	10	25.7	35.4	42.6	21.6
M	B	10,000	14	45.0	37.8	45.0	44.0
M	C	100,000	11	45.0	31.5	26.5	29.9
M	C	100,000	15	26.4	30.5	24.1	45.0
M	D	1,000,000	12	26.4	26.3	28.8	45.0

Table 3-4. Analysis of tank water for *Bd* using a qPCR assay. The values shown are crossing points (Cp) for 2 technical replicates, as well as the calculated estimate of the concentration (zoospores/mL) in the sample. Values below the lower limit of detection (85.1 zoospores) were considered negative (Neg).

	Zoospore challenge	Pre-challenge		Post-challenge	
		Mean Cp	*Bd* zoospores	Mean Cp	*Bd* zoospores
Tank A	0	38.24	Neg	32.83	97
Tank B	10^4	32.10	145.66	32.51	116
Tank C	10^5	33.58	Neg	31.45	210
Tank D	10^6	40.76	Neg	35.31	Neg

Figure 3-1. Body weight of the frogs over the course of the experiment.

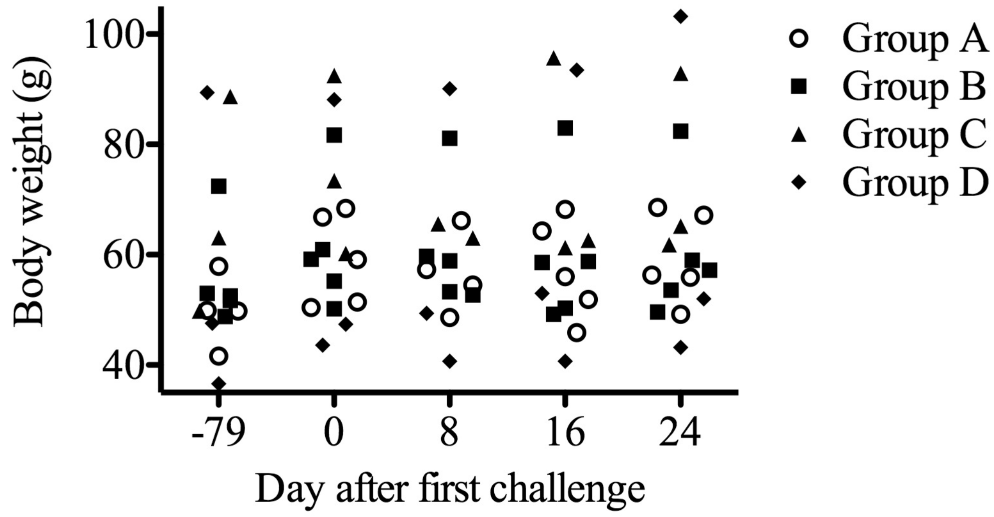


Figure 3-2 Quantitative PCR assay to detect known concentrations of *Bd* zoospores.

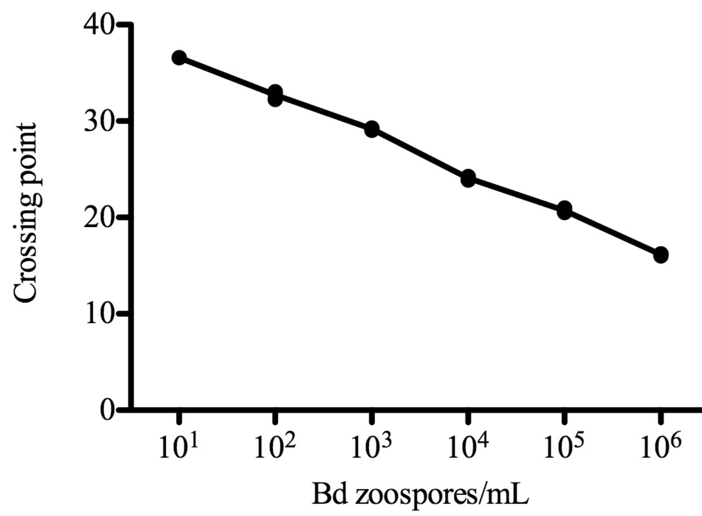


Figure 3-3. Detection of *Bd* infection by qPCR in the four experimental groups (challenge with 0, 10^4, 10^5, or 10^6 zoospores/mL of *Bd*) across the time course of the study. The data show the qPCR crossing points (mean ± SEM, for 3-5 frogs per group).

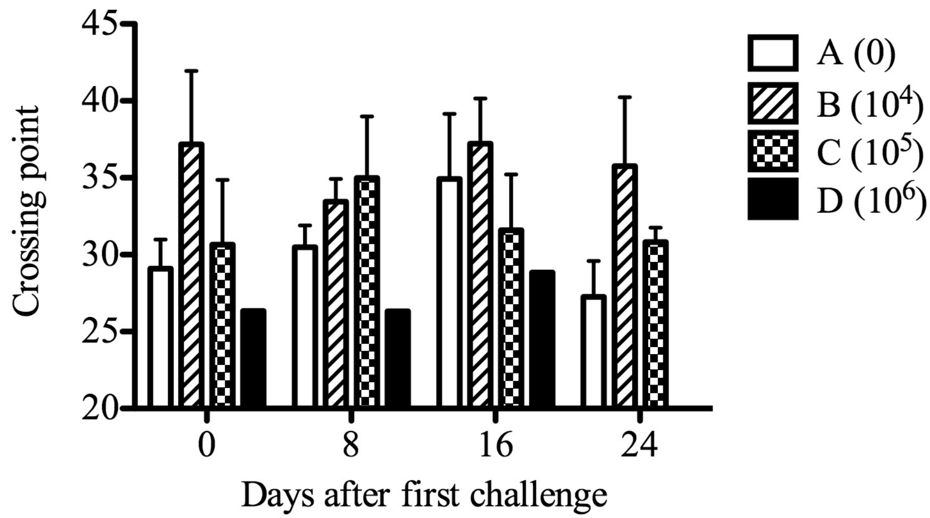
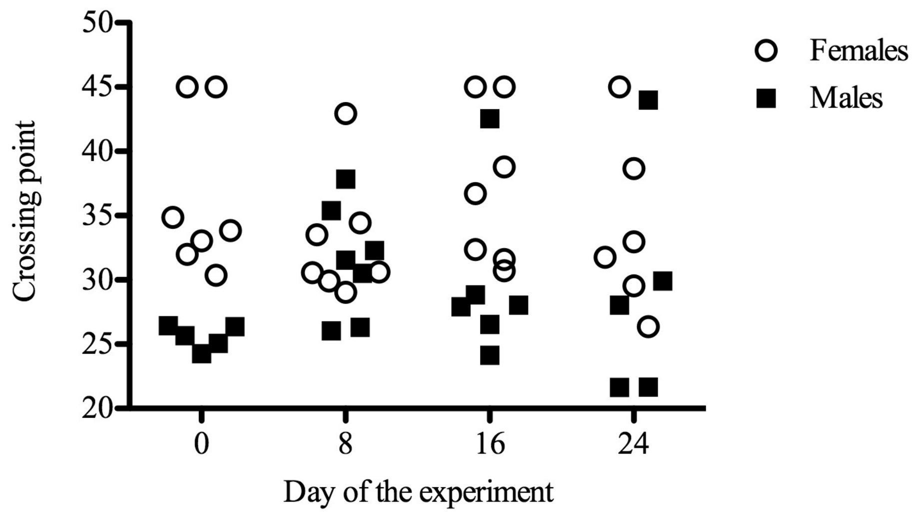
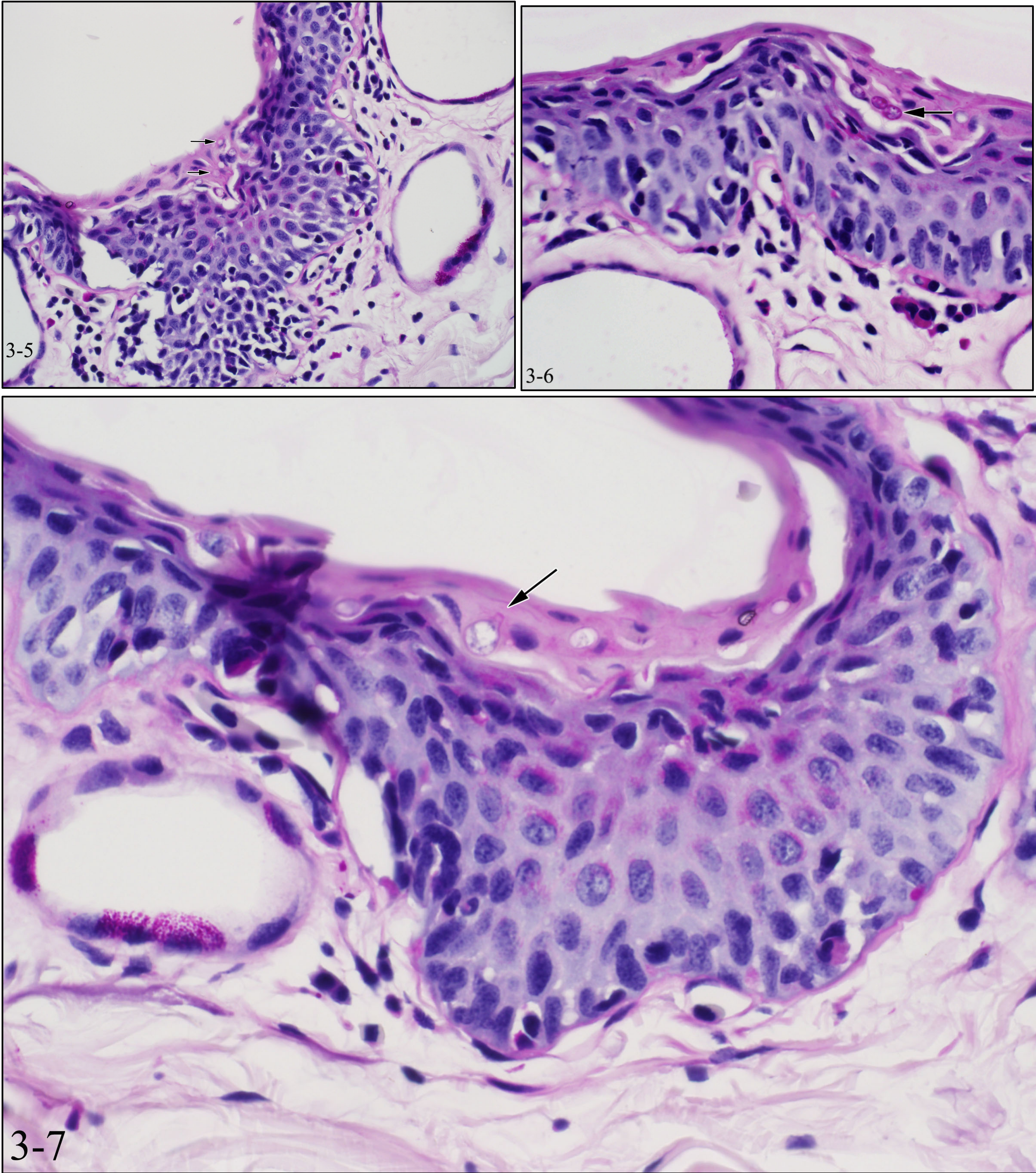


Figure 3-4. *Bd* qPCR crossing points and their relation to sex of the frog and time during the experiment.



Figures 3-5 to 3-7. *Bd* zoospores (arrows) within the epidermis of ventral abdominal skin. PAS reaction. Figure 3-7 (bottom) shows a zoospore with a discharge papilla (arrow). Frog 17 (Tank A, control group).



Figures 3-8 to 3-9. Ventral abdominal skin. The granular glands (arrows) in figure 3-8 (top, frog 1, Tank D, 10^6 zoospores) are discharged and collapsed, whereas the two shown in figure 3-9 (bottom, frog 11, Tank C, 10^5 zoospores) are filled with granular content. The mucous glands (M) are located in the more superficial dermis. HE.

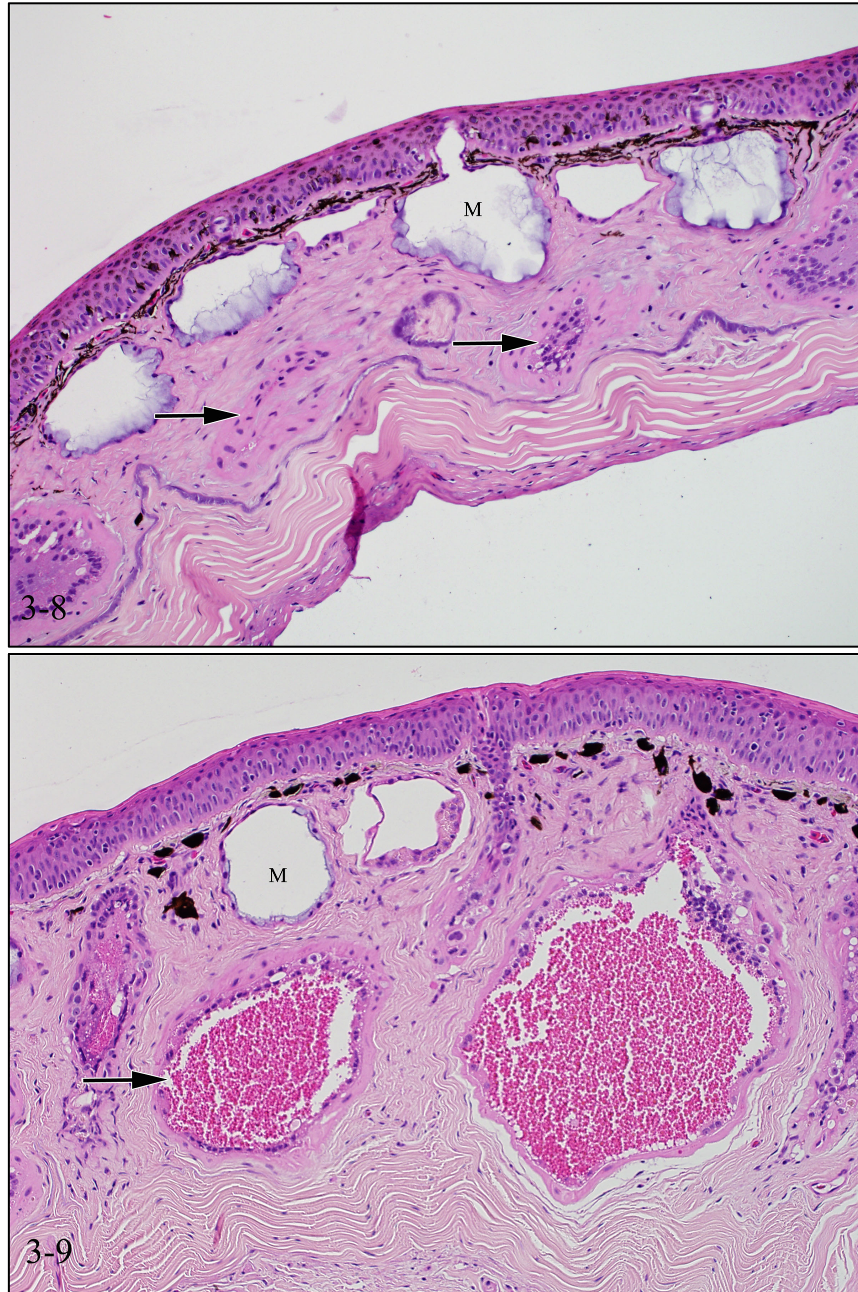


Figure 3-10. Ventral abdominal skin. There is dermal inflammation characterized by infiltration of lymphocytes, with epidermal dysplasia and parakeratosis. HE. Frog 17 (Tank A, control group).

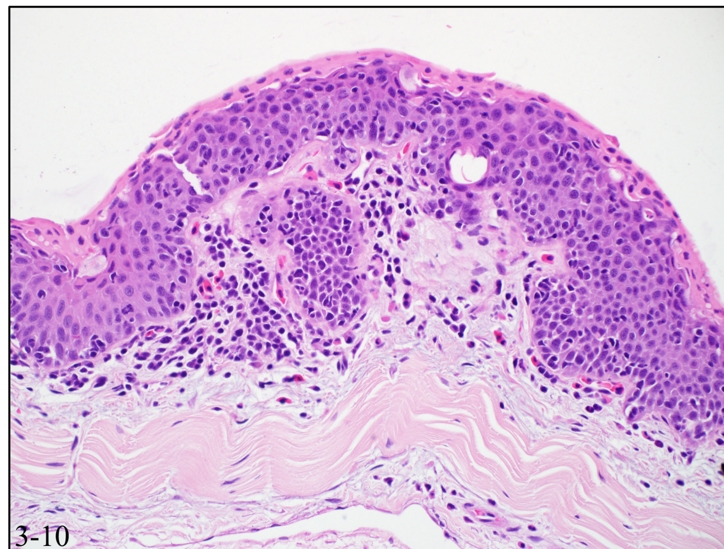


Figure 3-11. Ventral abdominal skin. Parakeratotic hyperkeratosis, with retention of nuclei of the stratum corneum, with vacuoles suspicious of *Bd* zoospores (arrow). HE. Frog 13 (Tank A, control group).

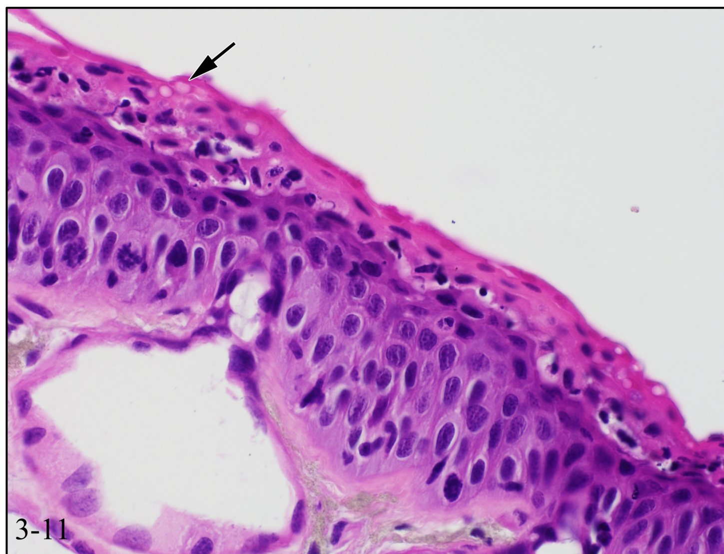


Figure 3-12. Ventral abdominal skin. The epidermis has increased cellularity leading to thickening of the tissue. HE. Frog 12 (Tank D, 10^6 zoospores).

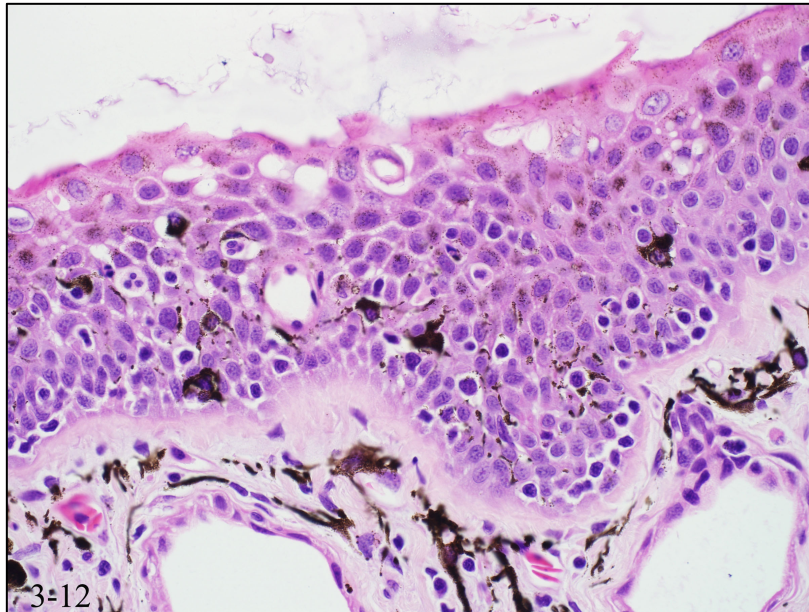
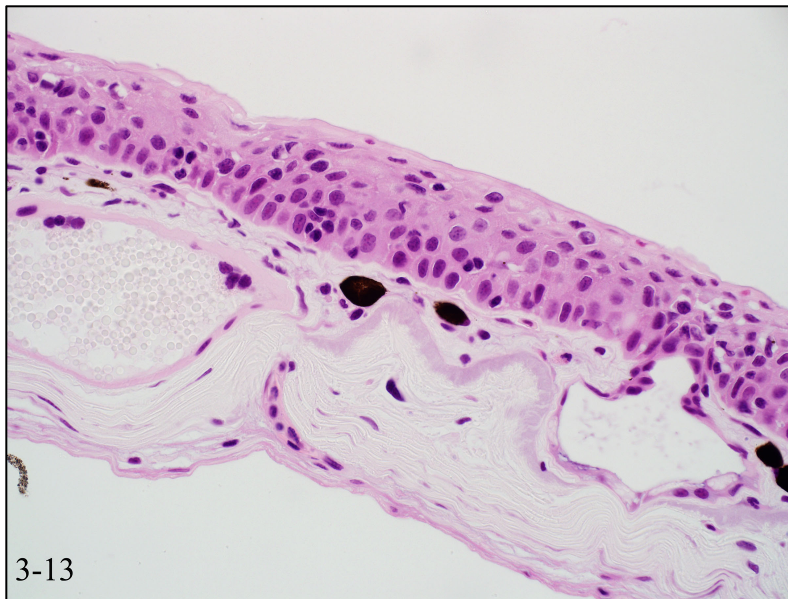
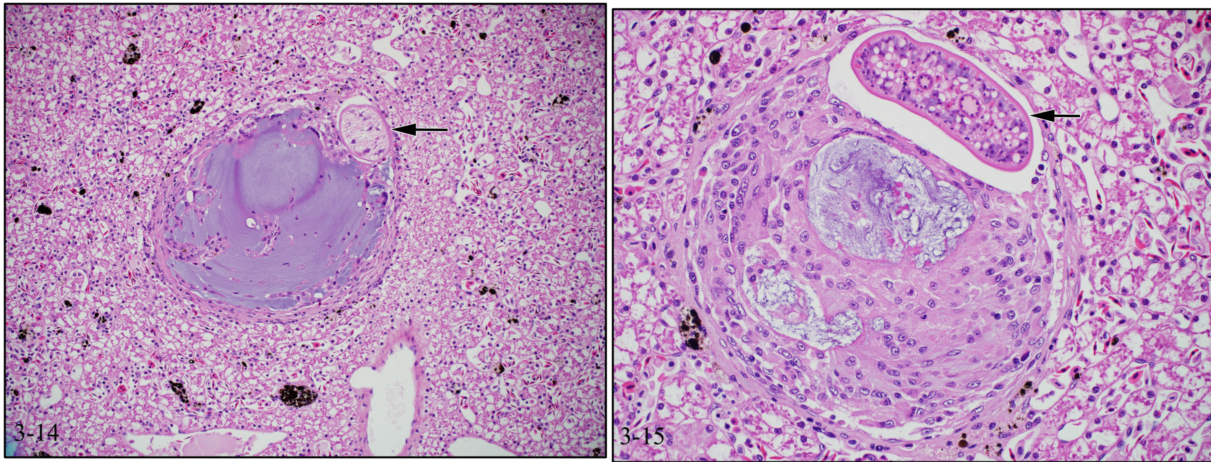


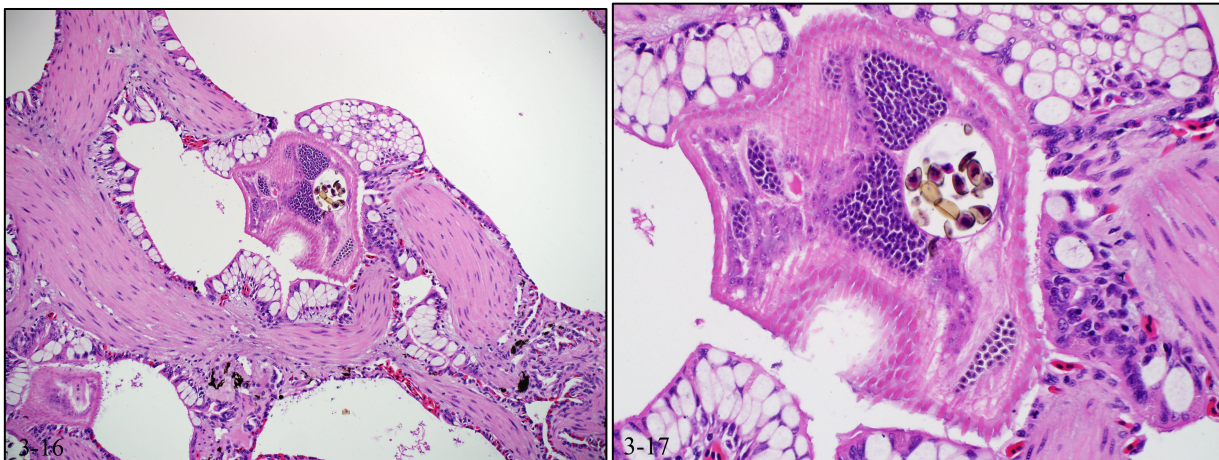
Figure 3-13. Ventral abdominal skin. Normal epidermis. HE. Frog 12 (Tank D, 10^6 zoospores).



Figures 3-14 and 3-15. Trematodes (arrows) adjacent to granulomas in the liver. HE. Frog 8 (Tank B, 10^4 zoospores).



Figures 3-16 and 3-17. Trematode parasite (arrows) containing pigmented ova, in the lung. HE. Frog 5 (Tanks C, 10^5 zoospores).



Chapter 4. General Discussion

It seems paradoxical that certain frog species secrete antimicrobial peptides that are known to kill *Bd* in vitro onto their skin surface, yet are susceptible to disease when infected by this fungus. The research described herein addresses the hypothesis that exogenous or endogenous influences result in dysregulation of antimicrobial peptide gene expression, and that the suppression of these innate defenses permits colonization of the skin with *Bd*, leading to disease. Specifically, it was hypothesized that injection of glucocorticoids would reduce antimicrobial peptide gene expression in cutaneous secretions from *L. pipiens*, permitting an increased fungal load and more severe disease following challenge with *Bd*. If this hypothesis were shown to be valid, it might suggest more appropriate methods to manage the disease, partially explain the differences in susceptibility to *Bd* between amphibian species, and establish an experimental approach to testing the effects of other environmental factors on innate immune defenses against this pathogen (Brown et al 2013).

The research comprising Chapter 2 examined the effect of systemic administration of glucocorticoids on antimicrobial peptide gene expression in the skin of *L. pipiens*. The dose of glucocorticoids used was somewhat arbitrary. There is a paucity of published information not only on doses and administration of glucocorticoids to frogs in a laboratory setting, but also on the rationale behind the doses chosen, as natural plasma glucocorticoid levels in wild populations are unknown. The true serum parameters may never be known, at least with current technology, as frogs would have to be handled for sampling, and these are inherently stressful events. It remains uncertain whether experimental administration of glucocorticoids is adequately representative of the low-grade, chronic stressors that may be contributing to disease susceptibility. However, the objective of this experiment was not to mimic natural environmental stress, but rather to determine if there were a relationship between increased systemic glucocorticoid levels and changes in mRNA expression of particular antimicrobial peptides. The dosing schedule of glucocorticoid used in this study was based on a published study in frogs,(Simmaco et al 1997) as well as on unpublished work from Dr. Rollins-Smith.

The environmental conditions used to house the frogs were chosen to minimize the effects on frog health, stress, and corticosteroid levels, yet permit growth of *Bd* in the challenge experiment. The temperature of the room (16°C) was a compromise between the optimum for *Bd* growth (17-22°C) and the ideal maintenance temperature for *Lithobates pipiens* (10-15°C, Mary Fowler and Marcus Litman, personal communication). The photoperiod of the facility was set at 12 hours of light with abrupt change to 12 hours of dark, and this could not be changed for the experiment.

This investigation reported in Chapter 2 revealed that gene expression of the antimicrobial peptide brevinin was greater in corticosteroid-treated frogs than in those administered saline, and the findings were similar, albeit not statistically significant, for the antimicrobial peptide ranatuerin. This was contrary to the hypothesis, and conflicted with prior reports of esculentin expression in *L. esculenta* (Simmaco et al 1997). The reasons for this discrepancy are discussed in Chapter 2. The findings imply that, at least in *L. pipiens*, glucocorticoids and presumably stress do

not suppress this aspect of innate immunity against cutaneous infection with *Bd*. On the other hand, they reveal the potential for this innate immune defence to be enhanced. This finding has implications with respect to developing methods to stimulate innate resistance to *Bd* in at-risk populations of frogs, for example, by introducing cutaneous bacterial flora that enhance secretion of antimicrobial peptides.

The research comprising Chapter 3 was a pilot study to establish a *Bd* infection challenge model in *L. pipiens*. There have not been many experimental infections of frogs with *Bd* reported in the literature, and those that are published vary widely in all parameters. How a *Bd* isolate is chosen seems to be based largely on availability (Rollins-Smith, personal communication). Currently, few *Bd* isolates are available and there is a move to form a central database of isolates (Collins 2010). In the few published experimental infections of frogs, isolates used include isolate 197 (Nichols et al 2001) (isolated from the *Dendrobates azureus*, by Joyce Longcore (Longcore et al 1999)), isolate 119 (isolated from *R. muscosa*, by the Briggs laboratory), an isolate from juvenile *Litoria lesueuri* in Australia (Carver et al 2010) or isolate JEL275 (isolated from the boreal toad by Longcore) (Carey et al 2006). However, it is generally assumed that the particular isolate does not influence the outcome of experimental infections due to low genetic variability among identified strains of *Bd* (Morehouse et al 2003).

The concentration of zoosporangia used to inoculate frogs in published literature varies from 100 zoospores in 10 mL of water (unpublished data Marantelli and Berger) to 3000 zoospores in 800 mL aqueous suspension (Berger et al 1998) to 5000 zoospores in 150 mL culture broth (Woodhams et al 2007) to 100,000 zoospores in 1 mL DS solution (artificial pond water: 1 mM KH2PO4, 0.1 mM MgCl2, 0.02 mM CaCl2)(Carver et al 2010). As well, the frequency of exposure of the frogs to zoosporangia ranges from every 15 minutes for 15 hours (Woodhams et al 2007) to once for 24 hours (unpublished data, Marantelli and Berger) to every other day for 30 days or until dead (Nichols et al 2001) to once for 120 minutes (Carver et al 2010). There is a fine balance between attempting to mimic natural environmental exposure and ensuring frogs become infected for purposes of the particular experiment. In the present study, frogs were exposed to 10^4, 10^5 or 10^6 zoospores per tank for 24 hours, and the exposure was repeated in 48 hours with the same number of zoospores. The timeline for the experiment in Chapter 3 mirrored that in Chapter 2, for reasons explained above. Swabs for quantifying fungal load were taken every 8 days, based on the life cycle of *Bd*.

The objective to establish the parameters of an experimental infection protocol in a pilot study was not met. After the study was completed, analysis of the cutaneous swabs revealed that some animals were infected with *Bd* prior to the experimental challenge. Thus, it was not possible to conclude which outcomes were the effect of prior infection and which were the result of the experimental challenge. Further, the effect of prior infection on the response to subsequent exposure is neither described in the literature nor obvious from analysis of the present data.

The initial intent was to conduct a third experiment that measured the effect of corticosteroid administration on response to experimental challenge with *Bd*. However, because the findings of the first experiment were opposite to those expected, and to some extent because of the difficulties in obtaining *Bd*-free frogs and establishing the infection model, this third experiment was abandoned.

Despite this outcome, this study reported in Chapter 3 did demonstrate the histologic lesions consistent with cutaneous *Bd* infection, in frogs that tested positive by qPCR, as well as in-contact frogs that tested negative by qPCR, implying that histology may be more sensitive than the qPCR protocol used here for detecting the presence of *Bd* infection. Finally, this study provided evidence that uninfected frogs in contact with infected animals may not develop detectable *Bd* infection, and that infected frogs may not develop disease.

References

Aberg KM, Radek KA, Choi EH, Kim DK, Demerjian M, Hupe M, Kerbleski J, Gallo RL, Ganz RL, Mauro T, Feingold KR, Elias PM. 2007. Psychological stress downregulates epidermal antimicrobial peptide expression and increases severity of cutaneous infections in mice. The Journal of Clinical Investigation. 117(11):3339-3349.

Alford RA. 2001. Testing the novel pathogen hypothesis. Speare R. editor. In: Developing management strategies to control amphibian diseases: decreasing the risks due to communicable diseases. School of Public Health and Tropical Medicine, James Cook University, Townsville, Australia. p 20.

Andre SE, Parker J, Briggs CJ. 2008. Effect of temperature on host response to Batrachochytrium dendrobatidis infection in the mountain yellow-legged frog (Rana muscosa). Journal of Wildlife Diseases. 44(3):716-720.

Apponyi MA, Pukala TL, Brinkworth CS, Maselli VM, Bowie JH, Tyler MJ, Booker GW, Wallace JC, Carver JA, Separovic F, Doyle J, Llewellyn LE. 2004. Host-defence peptides of Australian anurans: structure, mechanism of action and evolutionary significance. Peptides. 25:1035-1054.

Beard KH, O'Neill EM. 2005. Infection of an invasive frog Eleutherodactylus coqui by the chytrid fungus Batrachochytrium dendrobatidis in Hawaii. Biological Conservation. 126:591-595.

Benson BJ, Hadley ME. 1969. In vitro characterization of adrenergic receptors controlling skin gland secretion in two anurans Rana pipiens and Xenopus laevis. Comparative Biochemistry and Physiology. 30:857–864.

Berger L, Hyatt AD, Speare R, Longcore JE. 2005. Life cycle stages of the amphibian chytrid Batrachochytrium dendrobatidis. Diseases of Aquatic Organisms. 68:51-63.

Berger L, Marantelli G, Skerratt LF, Speare R. 2005. Virulence of the amphibian chytrid fungus Batrachochytrium dendrobatidis varies with the strain. Diseases of Aquatic Organisms. 68(1):47-50.

Berger L, Speare R, Daszak P, Green DE, Cunningham AA, Goggin CL, Slocombe R, Ragan MA, Hyatt AD, McDonald KR, Hines HB, Lips KR, Marantelli G, Parkes H. 1998. Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. Proceedings of the National Academy of Sciences. 95:9031-9036.

Berger L, Speare R, Hines HB, Marantelli G, Hyatt AD, McDonald KR, Skerratt LF, Olsen V, Clarke JM, Gillespie G, Mahony M, Sheppard N, Williams C, Tyler MJ. 2004. Effect of season and temperature on mortality in amphibians due to chytridiomycosis. Australian Veterinary Journal. 82(7):434-439.

Berger L, Speare R, Hyatt AD. 1999. Chytrid fungi and amphibian declines: overview, implications and future

directions. In: Campbell A. (ed) Declines and disappearances of Australian frogs. Environment Australia: Canberra. p. 23-33.

Berger L, Speare R, Kent A. 1999. Diagnosis of chytridiomycosis of amphibians by histological examination. Zoos Print Journal. 15:184-190.

Berger L. 2001. Diseases in Australian Frogs. Doctoral Dissertation, James Cook University.

Bielby J, Cooper N, Cunningham AA, Garner TWJ, Purvis A. 2008. Predicting susceptibility to future declines in the world's frogs. Conservation Letters. 1:82-90.

Bielby J, Cooper N, Cunningham AA, Garner TWJ, Purvis A. 2008. Predicting susceptibility to future declines in the world's frogs. Conservation Letters. 1:82-90.

Bielby J, Cunningham AA, Purvis A. 2006. Taxonomic selectivity in amphibians: ignorance, geography or biology? Animal Conservation. 9:135–143.

Bishop PJ, Speare R, Poulter R, Butler M, Speare BJ, Hyatt A, Olsen V, Haigh A. 2009. Elimination of the amphibian chytrid fungus Batrachochytrium dendrobatidis by Archey's frog Leiopelma archeyi. Diseases of Aquatic Organisms. 84:9-15.

Blanco JL, Garcia ME. 2008. Immune response to fungal infections. Veterinary Immunology and Immunopathology. 125:47-70.

Bosch J, Carrascal LM, Duran L, Walker S, Fisher MC. 2007. Climate change and outbreaks of amphibian chytridiomycosis in a montane area of Central Spain; is there a link? Proceedings of the Royal Society of Biological Sciences. 274:253–260.

Bosch J, Martinez-Solano I, Garcia-Paris M. 2001. Evidence of a chytrid fungus infection involved in the decline of the common midwife toad (Alytes obstetricans) in protected areas of central Spain. Biological Conservation. 97:331–337.

Boyle DG, Boyle DB, Olsen V, Morgan JAT, Hyatt D. 2004. Rapid quantitative detection of chytridiomycosis (Batrachochytrium dendrobatidis) in amphibian samples using real-time Taqman PCR assay. Diseases of Aquatic Organisms. 60:141-148.

Bradford DF. 1991. Mass mortality and extinction in a high-elevation population of Rana muscosa. Journal of Herpetology. 25(2):174-177.

Bradley GA, Rosen PC, Sredl MJ, Jones TR, Longcore JE. 2002. Chytridiomycosis in native Arizona frogs. Journal of Wildlife Diseases. 38(1):206-212.

Briggs C, Burgin S. 2004. Congo Red, an effective stain for revealing the chytrid fungus, Batrachochytrium dendrobatidis, in epidermal skin scrapings from frogs. Mycologist. 18(3):98-103.

Britannica.com. 2013. "stress." (www.britannica.com/EBchecked/topic/568921/stress)

Brown JR, Miller T, Kerby JL. 2013. The interactive effect of an emerging infectious disease and an emerging contaminant on Woodhouse's toad (Anaxyrus woodhousii) tadpoles. Environ Toxicol Chem. 32(9): 2003-2008.

Carey C, Bruzgul JE, Livo LJ, Walling ML, Kuehl KA, Dixon BF, Pessier AP, Alford RA, Rogers KB. 2006. Experimental exposures of boreal toads (Bufo boreas) to a pathogenic chytrid fungus (Batrachochytrium dendrobatidis). EcoHealth. 3(1):5-21.

Carey C, Cohen N, Rollins-Smith L. 1999. Amphibian declines: an immunological perspective. Developmental and Comparative Immunology. 23:459-472.

Carver S, Bell BD, Waldman B. 2010. Does chytridiomycosis disrupt amphibian skin function? Copeia. 3:487-495.

Cashins SD, Grogan LF, McFadden M, Hunter D, Harlow PS, Berger L, Skerratt LF. 2013. Prior infection does not improve survival against the amphibian disease chytridiomycosis. PLOS ONE. 8(2):e56747.

Chen T, Farragher S, Bjourson AJ, Orr DF, Rao P, Shaw C. 2003. Granular gland transcriptomes in stimulated amphibian skin secretions. Biochemical Journal. 371(1):125-130.

Chermette R, Ferreiro L, Guillot J. 2008. DERMATOPHYTOSES IN ANIMALS. Mycopathologia. 166:385-405.

Clarke BT. 1997. The natural history of amphibian skin secretions, their normal functioning and potential medical applications. Biological Reviews. 72:365-379.

Collins JP. Conference presentation. 2010. Amphibian Pathogens Annual Meeting. Tempe, Arizona,

Conlon JM, Iwamuro S, King JD. 2009. Dermal cytolytic peptides and the system of innate immunity in anurans. Trends in Comparative Endocrinology and Neurobiology: Annals of the New York Academy of Sciences. 1163:75–82.

Conlon JM, Kolodziejek J, Nowotny N. 2004. Antimicrobial peptides from ranid frogs: taxonomic and phylogenetic markers and a potential source of new therapeutic agents. Biochimica et Biophysica Acta. 1696:1–14.

Conlon JM, Leprince J. 2010. Identification and analysis of bioactive peptides in amphibian skin secretions. Methods in Molecular Biology. 615:145-57.

Conlon JM. 2011. The contribution of skin antimicrobial peptides to the system of innate immunity in anurans. Cell and Tissue Research. 343(1):201-212.

Crespi EJ, Vaudry H, Denver RJ. 2004. Roles of corticotropin-releasing factor, neuropeptide Y and corticosterone in the regulation of food intake in Xenopus laevis. Journal of neuroendocrinology. 16(3):279–288.

Daszak P, Berger L, Cunningham AA, Hyatt AD, Green DE, Speare R. 1999. Emerging infectious diseases and amphibian population declines. Emerging Infectious Diseases. 5(6):735-48.

Daszak P, Cunningham AA, Hyatt AD. 2003. Infectious disease and amphibian population declines. Diversity and Distributions. 9:141-150.

Daszak P, Strieby A, Cunningham AA, Longcore JE, Brown CC, Porter D. 2004. Experimental evidence that the bullfrog (Rana catesbeiana) is a potential carrier of chytridiomycosis, an emerging fungal disease of amphibians. Herpetological Journal. 14:21-27.

Davidson EW, Parris M, Collins JP, Longcore JE, Pessier AP, Brunner J. 2003. Pathogenicity and transmission of chytridiomycosis in tiger salamanders (Ambystoma tigrinum). Copeia. 601-607.

Davidson EW, Parris M, Collins JP, Longcore JE, Pessier AP, Brunner J. 2003. Pathogenicity and transmission of chytridiomycosis in tiger salamanders (Ambystoma tigrinum). Copeia. 601–607

Davis AK, Keel MK, Ferreira A, Maerz JC. 2010. Effects of chytridiomycosis on circulating white blood cell distributions of bullfrog larvae (Rana catesbeiana). Comparative Clinical Pathology. 19(1):49-55.

De Perez G, Hindelang C. 1985. Ultrastructure of venom glands in frog (Rana esculenta). Archives d'anatomic microscopique et de morphologique experimentale. 74:216-227.

Delfino G, Drewes RC, Magherini S, Malentacchi C, Nosi D, Terreni A. 2006. Serous cutaneous glands of the Pacific tree-frog Hyla regilla (Anura, Hylidae): patterns of secretory release induced by norepinephrine. Tissue and Cell. 38:65-77.

Denardo D. 1995. Amphibians as laboratory animals. Institute for Laboratory Animal Research Journal. 37:173-181.

Denver RJ. 1993. Acceleration of anuran amphibian metamorphosis by corticotropin releasing hormone-like peptides. General and Comparative Endocrinology. 91:38-51.

Denver, RJ. 1997. Environmental stress as a developmental cue: corticotropin-releasing hormone is a proximate mediator of adaptive phenotypic plasticity in amphibian metamorphosis. Hormones and Behaviour. 31:169-179.

Dockray GJ, Hopkins CR. 1975. Caerulein secretion by dermal glands in Xenopus laevis. Journal of Cell Biology. 64:724–733.

Du Pasquier L, Schwager J, Flajnik M. 1989. The immune system of Xenopus. Annual Review of Immunology.

7:251-275.

Fellers GM, Green DE, Longcore JE. 2001. Oral chytridiomycosis in the mountain yellow-legged frog (Rana muscosa). Copeia. 4:945-953.

Fisher MC, Garner TW, Walker SF. 2009. Global emergence of Batrachochytrium dendrobatidis and amphibian chytridiomycosis in space, time, and host. Annu Rev Microbiol. 63:291-310.

Flucher BE, Lenglachner-Bachinger C, Pohlhammer K, Adam H, Mollay C. 1986. Skin peptides in Xenopus laevis: morphological requirements for precursors processing in developing and regenerating granular skin glands. Journal of Cell Biology. 103:2299-2309.

Forzan MJ, Vanderstichel R, Hogan NS, Teather K, Wood J. 2010. Prevalence of Batrachochytrium dendrobatidis in three species of wild frogs on Prince Edward Island, Canada. Diseases of Aquatic Organisms. 91:91-96.

Gahl MK, Longcore JE, Houlahan JE. 2012. Varying responses of northeastern North American amphibians to the chytrid pathogen Batrachochytrium dendrobatidis. Conservation Biology. 26(1):135-141.

Garcia TS, Romansic JM, Blaustein AR. 2006. Survival of three species of anuran metamorphs exposed to UV-B radiation and the pathogenic fungus Batrachochytrium dendrobatidis. Diseases of Aquatic Organisms. 72:163-169.

Garner TWJ, Walker S, Bosch J, Hyatt AD, Cunningham AA, Fisher MC. 2005. Chytrid fungus in Europe. Letter to the Editor. Emerging Infectious Diseases. 11(10):1639-1641.

Gascon C, Collins JP, Moore RD, Church DR, McKay JE, Mendelson III JR (eds). 2007. Appendix I of the Amphibian Conservation Action Plan (ACAP). In: Amphibian Conservation Summit Proceedings. IUCN/SSC Amphibian Specialist Group. Gland, Switzerland and Cambridge, UK. p 59.

Giovannini M, Poulter L, Gibson B. 1987. Biosynthesis and degradation of peptides derived from Xenopus laevis prohormones. Biochemical Journal. 243:113-120.

Gleason FH, Lilje O. 2009. Structure and function of fungal zoospores: ecological implications. Fungal Ecology. 2:53-59.

Goldsby RA, Kindt TJ, Osborne BA, Kuby J. 2003. Anti-Inflammatory Agents. In: Immunology. 5th ed. New York (NY): W.H. Freeman and Company. p 357-358.

Goraya J, Knoop FC, Conlon JM. 1998. Ranatuerins: antimicrobial peptides isolated from the skin of the American bullfrog, Rana catesbeiana. Biochemical and biophysical research communications. 250(3):589-592.

Goraya J, Wang Y, Li Z, O'Flaherty M, Knoop FC, Platz JE, Conlon JM. 2000. Peptides with antimicrobial activity

from four different families isolated from the skins of the North American frogs Rana luteiventris, Rana berlandieri and Rana pipiens. European Journal of Biochemistry. 267(3):894-900.

Hancock REW, Falla T, Brown MH. 1995. Cationic bactericidal peptides. Advances in Microbial Physiology. 37:135-175.

Hanselmann R, Rodríguez A, Lampo M, Fajardo-Ramos L, Aguirre AA, Kilpatrick AM. 2004. Presence of an emerging pathogen of amphibians in introduced bullfrogs Rana catesbeiana in Venezuela. Biological Conservation. 120:115–119.

Harris RN, Brucker RM, Walke JB, Becker MH, Schwantes CR, Flaherty DC, Lam BA, Woodhams DC, Briggs CJ, Vredenburg VT, Minbiole KPC. 2009. Skin microbes on frogs prevent morbidity and mortality caused by a lethal skin fungus. The International Society for Microbial Ecology Journal. 3:818-824.

Harris RN, James TY, Lauer A, Simon MA, Patel A. 2006. Amphibian pathogen Batrachochytrium dendrobatidis is inhibited by the cutaneous bacteria of amphibian species. EcoHealth 3:53–56.

Hill WA, Newman SJ, Craig L, Carter C, Czarra J, Brown JP. 2010. Diagnosis of Aeromonas hydrophila, Mycobacterium species, and Batrachochytrium dendrobatidis in an African Clawed Frog (Xenopus laevis). Journal of the American Association for Laboratory Animal Science. 49(2):215-220.

Holmes C, Balls M. 1978. In vitro studies on the control of myoepithelial cell contraction in the granular glands of Xenopus laevis skin. General and Comparative Endocrinology. 36:255–263.

Houlahan JE, Findlay CS, Schmidt BR, Meyer AH, Kuzmin SL. 2000. Quantitative evidence for global amphibian population declines. Nature. 404:752-758.

Hyatt D, Boyle DG, Olsen V, Boyle DB, Berger L, Obendorf D, Dalton A, Kriger K, Hero M, Hines H, Phillot R, Campbell R, Marantelli G, Gleason F, Colling A. 2007. Diagnostic assays and sampling protocols for the detection of Batrachochytrium dendrobatidis. Diseases of Aquatic Organisms. 73(3):175-192.

Johnson PTJ, McKenzie VJ, Peterson AC, Kerby JL, Brown J, Blaustein AR, Jackson T. 2011. Regional decline of an iconic amphibian associated with elevation, land-use change, and invasive species. Conservation Biology. 25(3):556-66.

König T, Schuberth HJ, Leibold W, Zerbe H. 2006. Dexamethasone depresses the expression of L-selectin but not the in vivo migration of bovine neutrophils into the uterus. Theriogenology. 65:1227-1241.

Kriger KM, Hero JM. 2006. Survivorship in wild frogs infected with chytridiomycosis. EcoHealth. 3:171-177.

Kriger KM, Hero JM. 2008. Altitudinal distribution of chytrid (Batrachochytrium dendrobatidis) infection in

subtropical Australian frogs. Austral Ecology. 33:1022–1032.

Lacombe C, Cifuentes-Diaz C, Dunia I, Auber-Thomay M, Nicolas P, Amiche M. 2000. Peptide secretion in the cutaneous glands of South American tree frog Phyllomedusa bicolor: an ultrastructural study. European Journal of Cell Biology. 79:631-641

LaMarca E, Lips K, Lotters S, Puschendorf R, Ibanez R, Rueda-Almonacid J, Schulte R, Marty C, Castro F, Manzanillo-Puppo J, Garcia-Perez J, Bolanos F, Chavez G, Pounds A, Toral E, Young B. 2005. Catastrophic population declines and extinction in neotropical harlequin frog (Bufonidae: Atelopus). Biotropica. 37:190–201.

Lauer A, Simon MA, Banning JL, Lam B, Harris RN. 2008. Diversity of cutaneous bacteria with antifungal activity isolated from female four-toed salamanders. The International Society for Microbial Ecology Journal. 2:145–157.

Lazarus LH, Attila M. 1993. The toad, ugly and venomous, wears yet a precious jewel in his skin. Progress in Neurobiology. 41:473-507.

Lindley BD. 1969. Nerve stimulation and electrical properties of frog skin. Journal of General Physiology. 53(4):427-449.

Lips KR, Brem F, Brenes R, Reeve JD, Alford RA, Voyles J, Carey C, Livo L, Pessier AP, Collins JP. 2006. Emerging infectious disease and the loss of biodiversity in a Neotropical amphibian community. Proceedings of the National Academy of Sciences. 103(9):3165-3170.

Lips KR, Reeve JD, Witters LR. 2003. Ecological traits predicting amphibian population declines in central America. Conservation Biology. 17(4):1078-1088.

Lips KR. 1998. Decline of a tropical montane amphibian fauna. Conservation Biology. 12:106-117.

Lips KR. 1999. Mass mortality and population declines of anurans at an upland site in western Panama. Conservation Biology. 13:117-125.

Longcore JE, Pessier AP, Nichols DK. 1999. Batrachochytrium dendrobatidis gen. et sp. nov., a chytrid pathogenic to amphibians. Mycologia. 91(2):219-227.

Longo AV, Ossibof RJ, Zumidio KR, Burrowes PA. 2013. Lability in host defenses: terrestrial frogs die from chytridiomycosis under enzootic conditions. Journal of Wildlife Diseases. 491(1):197-199.

Lotters S, Kielgast J, Bielby J, Schmidtlein S, Bosch J, Veith M, Walker SF, Fisher MC, Rodder D. 2010. The link between rapid enigmatic amphibian decline and the globally emerging chytrid fungus. EcoHealth. 6(3):358-372.

Mangoni NL, Miele R, Renda TG, Barra D, Simmaco M. 2001. The synthesis of antimicrobial peptides in the skin

of Rana esculenta is stimulated by microorganisms. The FASEB Journal. 15(8):1431-1432.

Martin-Ezquerra G, Man MQ, Hupe M, Rodriguez-Martin M, Youm JK, Trullas C, Mackenzie DS, Radek KA, Holleran WM, Elias PM. 2011. Psychological stress regulates antimicrobial peptide expression by both glucocorticoid and b-adrenergic mechanisms. European Journal of Dermatology. 21(Suppl 2):48-51.

Matutte B, Storey KB, Knoop FC, Conlon JM. 2000. Induction of synthesis of an antimicrobial peptide in the skin of the freeze-tolerant frog, Rana sylvatica, in response to environmental stimuli. FEBS Letters. 483(2-3):135-138.

McCallum ML. 2007. Amphibian decline or extinction? Current declines dwarf background extinction rate. Journal of Herpetology. 41(3):483-491.

Mills JW, Prum BE. 1984. Morphology of the exocrine glands of the frog skin. The American Journal of Anatomy. 171:91-106.

Mitchell GB, Al-Haddawi MH, Clark ME, Beveridge JD, Caswell JL. 2007. Effect of corticosteroids and neuropeptides on the expression of defensins in bovine tracheal epithelial cells. Infection and Immunity. 75(3):1325-1334.

Mitchell GB, Al-Haddawi MH, Clark ME, Beveridge JD, Caswell JL. 2007. Effect of corticosteroids and neuropeptides on the expression of defensins in bovine tracheal epithelial cells. Infection and Immunity. 75(3):1325-34.

Morales HD, Robert J. 2007. Characterization of primary and memory CD8 T-cell responses against ranavirus (FV3) in Xenopus laevis. Journal of Virology. 81:2240–2248.

Morehouse EA, James TY, Ganley ARD, Vilgalys R, Berger L, Murphy J, Longcore JE. 2003. Multilocus sequence typing suggests the chytrid pathogen of amphibians is a recently emerged clone. Molecular Ecology. 12:395-403.

Moss AS, Reddy NS, Dortaj IM, San Francisco MJ. 2008. Chemotaxis of the amphibian pathogen Batrachochytrium dendrobatidis and its response to a variety of attractants. Mycologia. 100(1):1-5.

Muths E, Corn PS, Pessier AP, Green DE. 2003. Evidence for disease-related amphibian decline in Colorado. Biological Conservation. 110:357–365.

Neuwirth M, Daly JW, Myers CW, Tice LW. 1979. Morphology of the granular secretory glands in the skin of poison-dart frogs (Dendrobatidae). Tissue Cell. 11:755-771.

Nichols DK, Lamirande EW, Pessier AP, Longcore JE. 2001. Experimental transmission of cutaneous chytridiomycosis in Dendrobatid frogs. Journal of Wildlife Diseases. 37(1):1-11.

Noble GA, Noble ER. 1944. On the histology of frog skin glands. Transactions of the American Microscopical Society. 63(3):254-263.

Nosi D, Terreni A, Alvarez BB, Delfino G. 2002. Serous gland polymorphism in the skin of Phyllomedusa hypochondrialis azurea (Anura, Hylidae): response by different gland types to norepinephrine stimulation. Zoomorphology. 121:139-148.

Oka T, Tooi O, Mitsui N, Miyahara M, Ohnishi Y, Takase M. 2008. Effect of atrazine on metamorphosis and sexual differentiation in Xenopus laevis. Aquatic toxicology. 87(4):215–226.

Ouellet M, Mikaelian I, Pauli BD, Rodrigue J, Green DM. 2005. Historical evidence of widespread chytrid infection in North American amphibian populations. Conservation Biology. 19(5):1431-1440.

Paetow LJ, McLaughlin JD, Cue RI, Pauli BD, Marcogliese DJ. 2012. Effects of herbicides and the chytrid fungus Batrachochytrium dendrobatidis on the health of post-metamorphic Northern leopard frogs (Lithobates pipiens). Ecotoxicology and Environmental Safety. 80(1):372-380.

Pare JA. 2003. Fungal diseases of amphibians: an overview. Veterinary Clinics of North America: Exotic Animal Practice. 6(2):315-326.

Parker JM, Mikaelian I, Hahn N, Diggs HE. 2002. Clinical diagnosis and treatment of epidermal chytridiomycosis in African clawed frogs (Xenopus tropicalis). Comparative Medicine. 52(3):265-268.

Parris MJ, Cornelius TO. 2004. Fungal pathogen causes competitive and developmental stress in larval amphibian communities. Ecology. 85:3385–3395.

Pask JD, Cary TL, Rollins-Smith LA. 2013. Skin peptides protect juvenile leopard frogs (Rana pipiens) against chytridiomycosis. The Journal of Experimental Biology. 216:2908-2916.

Pessier AP, Nichols DK, Longcore JE, Fuller MS. 1999. Cutaneous chytridiomycosis in poison dart frogs (Dendrobates spp.) and White's tree frogs (Litoria caerulea). Journal of Veterinary Diagnostic Investigation. 11:194–199.

Peterson JD, Steffen JE, Reinert LK, Cobine PA, Appel A, Rollins-Smith L, Mendonca MT. 2013. Host stress response is important for the pathogenesis of the deadly amphibian disease, chytridiomycosis, in Litoria caerulea. PLOS ONE. 8(4):e62146.

Piotrowski JS, Annis SL, Longcore JE. 2004. Physiology of Batrachochytrium dendrobatidis, a chytrid pathogen of amphibians. Mycologia. 96(1):9-15.

Puschendorf R, Bolanos F, Chaves G. 2006. The amphibian chytrid fungus along an altitudinal transect before the

first reported declines in Costa Rica. Biological Conservation. 132(1):136-142.

Rachowicz LJ, Knapp RA, Morgan JAT, Stice MJ, Vredenburg VT, Parker JM, Briggs CJ. 2006. Emerging infectious disease as a proximate cause of amphibian mass mortality. Ecology. 87(7):1671-1683.

Rachowicz LJ, Vredenburg VT. 2004. Transmission of Batrachochytrium dendrobatidis within and between amphibian life stages. Diseases of Aquatic Organisms. 61:75-83

Rachowicz LJ. Hero JM, Alford RA, Taylor JW, Morgan JAT, Vredenburg VT, Collins JP, Briggs CJ. 2005. The novel and endemic pathogen hypotheses: competing explanations for the origin of emerging infectious diseases of wildlife. Conservation Biology. 19(5):1441-1448.

Radek KA, Elias PM, Taupenot L, Mahata SK, O'Connor DT, Gallo RL. 2010. Neuroendocrine nicotinic receptor activation increases susceptibility to bacterial Infections by suppressing antimicrobial peptide production. Cell Host and Microbe. 7:277-289.

Ramsey JP, Reinert LK, Harper LK, 2010. Immune Defenses against Batrachochytrium dendrobatidis, a fungus linked to global amphibian declines, in the South African Clawed Frog, Xenopus laevis. Infection and Immunity. 78(9):3981-3992.

Raverty S, Reynolds T. 2001. Cutaneous chytridiomycosis in dwarf aquatic frogs (Hymenochirus boettgeri) originating from southeast Asia and in a western toad (Bufo boreas) from northeastern British Columbia. Canadian Veterinary Journal. 42:385-386.

Retallick RWR, McCallum H, Speare R. 2004. Endemic infection of the amphibian chytrid fungus in a frog community post-decline. PLoS Biology. 2(11):1965-1971.

Richmond JQ, Savage AE, Zamudio KR, Rosenblum EB. 2009. Toward immunogenetic studies of amphibian chytridiomycosis: linking innate and acquired immunity. BioScience. 59:311-320.

Robert J, Ohta Y. 2009. Comparative and developmental study of the immune system in Xenopus. Developmental Dynamics. 238:1249–1270.

Rollins-Smith LA, Barker KS, Davis AT. 1997. Involvement of glucocorticoids in the reorganization of the amphibian immune system at metamorphosis. Developmental Immunology. 5(2):145-52.

Rollins-Smith LA, Carey C, Longcore J, Doersam JK, Boutte A, Bruzgal JE, Conlon JM. 2002. Activity of antimicrobial skin peptides from ranid frogs against Batrachochytrium dendrobatidis, the chytrid fungus associated with global amphibian declines. Developmental & Comparative Immunology. 26(5):471-479.

Rollins-Smith LA, Ramsey JP, Reinert LK, Woodhams DC, Livo LJ, Carey C. 2009. Immune defenses of Xenopus

laevis against Batrachochytrium dendrobatidis. Frontiers in Bioscience. S1:68-91.

Rollins-Smith LA, Woodhams DC, Reinert LK, Vredenburg VT, Briggs CJ, Nielsen PF, Conlon JM. 2006. Antimicrobial peptide defenses of the mountain yellow-legged frog (Rana muscosa). Developmental and Comparative Immunology. 30:831-842.

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

Romani L. 2004. Immunity to fungal infections. Nature Reviews Immunology. 4(1): 11-24.

Rosenblum EB, Stajich JE, Maddox N, Eisen MB. 2008. Global gene expression profiles for life stages of the deadly amphibian pathogen Batrachochytrium dendrobatidis. Proceedings of the National Academy of Sciences. 105 (44):17034-17039.

Sanchez D, Chacon-Ortiz A, Leon F, Han BA, Lampo M. 2008 Widespread occurrence of an emerging pathogen in amphibian communities of the Venezuelan Andes. Biological Conservation. 141:2898–2905.

Sanchez D, Chacon-Ortiz A, Leon F, Han BA, Lampo M. 2008. Widespread occurrence of an emerging pathogen in amphibian communities of the Venezuelan Andes. Biological Conservation. Short Communication. 141(11):2898-2905.

Savage A, Jaeger JR. 2009. Isolation and characterization of microsatellite markers in the lowland leopard frog (Rana yavapaiensis) and the relict leopard frog (R. onca), two declining frogs of the North American desert southwest. Molecular Ecology Resources. 9:199-202.

Savage AE, Zamudio KR. 2011. MHC genotypes associate with resistance to a frog-killing fungus. Proceedings of the National Academy of Sciences of the United States of America, 108(40): 16705–16710.

Schleimer RP. 2004. Glucocorticoids suppress inflammation but spare innate immune responses in airway epithelium. Proc Am Thorac Soc. 1:222-230.

Seki T, Kikuyama S, Yanaihara N. 1995. Morphology of the skin glands of the crab-eating frog (Rana cancrivora). Zoological Science, 12(5): 623–626.

Simmaco M, Boman A, Mangoni ML, Mignogna G, Miele R, Barra D, Boman HG. 1997. Effect of glucocorticoids on the synthesis of antimicrobial peptides in amphibian skin. FEBS Letters. 416(3):273-275.

Sjoberg E, Flock A. 1976. Innervation of skin glands in the frog. Cell Tissue Research. 172:81-91.

Skerratt LF, Berger L, Speare R, Cashins S, McDonald KR, Phillott AD. 2007. Spread of chytridiomycosis has caused the rapid global decline and extinction of frogs. EcoHealth. 4:125-134.

Stice MJ, Briggs CJ. 2010. Immunization is ineffective at preventing infection and mortality due to the amphibian chytrid fungus Batrachochytrium dendrobatidis. Journal of Wildlife Diseases. 46(1):70–77.

Stuart SN, Chanson JS, Cox NA, Young BE, Rodrigues ASL, Fischman DL, Waller RW. 2004. Status and trends of amphibian declines and extinctions worldwide. Science. 306:1783-1786.

Toledo RC, Jared C, Brunner Junior A. 1992. Morphology of the large granular alveoli of the parotid glands in toad (Bufo ictericus) before and after compression. Toxicon. 30:745–753.

Tyler MJ, Miller CA. 1985. Surface architecture of the dorsal epidermis in Australian frogs. Transactions of the Royal Society of Southern Australia. 109(2):45-48.

Tyler MJ, Stone DJ, Bowie JH. 1992. A novel method for the release and collection of dermal, glandular secretions from the skin of frogs. Journal of Pharmacological and Toxicological Methods. 28(4):199-200.

Une Y, Kadekaru S, Tamukai K, Goka K, Kuroki T. 2008. First report of spontaneous chytridiomycosis in frogs in Asia. Diseases of Aquatic Organisms. 82:157-160.

Van Ells T, Stanton J, Strieby A, Daszak P, Hyatt AD, Brown C. 2003. Use of immunohistochemistry to diagnose chytridiomycosis in dyeing Poison Dart frogs (Dendrobates tinctorius). Journal of Wildlife Diseases. 39(3):742-745.

Voordouw MJ, Adam D, Houston B, Govindarajulu P, Robinson J. 2010. Prevalence of the pathogenic chytrid fungus, Batrachochytrium dendrobatidis, in an endangered population of northern leopard frogs, Rana pipiens BMC Ecology. 10(6):1-10.

Voyles J, Berger L, Young S, Speare R, Webb R, Warner J, Rudd D, Campbell R, Skerratt LF. 2007. Electrolyte depletion and osmotic imbalance in amphibians with chytridiomycosis. Diseases of Aquatic Organisms. 77:113-118.

Voyles J, Young S, Berger L, Campbell C, Voyles WF, Dinudom A, Cook D, Webb R, Alford RA, Skerratt LF, Speare R. 2009. Pathogenesis of chytridiomycosis, a cause of catastrophic amphibian declines. Science. 326:582-585.

Wake DB, Vredenburg VT. 2008. Are we in the midst of a sixth mass extinction? A view from the world of amphibians. Proceedings of the National Academy of Sciences. 105 (Supplement 1):11466-11473.

Wang H, Ding Y, Li X, Yang L, Zhang W, Kang W. 2003. Fatal aspergillosis in a patient with SARS who was treated with corticosteroids. New England Journal of Medicine. 349:507-508.

Williams DL. 1995. Amphibian dermatology. In: Kirk's current veterinary therapy XII. Bonagura JD (ed). WB Saunders, Philadelphia, PA. p.1375–1379.

Williams SE, Hero JM. 1998. Rainforest frogs of the Australian wet tropics: guild classification and the ecological similarity of declining species. Proceedings of the Royal Society of London Biological Sciences. 265:597-602.

Woodhams DC, Alford RA, Marantelli G. 2003. Emerging disease of amphibians cured by elevated body temperature. Diseases of Aquatic Organisms. 55(1):65-67.

Woodhams DC, Alford RA. 2005. Ecology of chytridiomycosis in rainforest stream frog assemblages of tropical Queensland. Conservation Biology. 19(5):1449-1459.

Woodhams DC, Ardipradja K, Alford RA, Marantelli G, Reinert LK, Rollins-Smith LA. 2007. Resistance to chytridiomycosis varies among amphibian species and is correlated with skin peptide defenses. Animal Conservation. 10(4):409-417.

Woodhams DC, Bell SC, Kenyon N, Alford RA, Rollins-Smith LA. 2012. Immune evasion or avoidance: fungal skin infection linked to reduced defence peptides in Australian green-eyed treefrogs, Litoria serrata. Fungal Biol. 116(12): 1203-11.

Woodhams DC, Bigler L, Marschang R. 2012. Tolerance of fungal infection in European water frogs exposed to Batrachochytrium dendrobatidis after experimental reduction of innate immune defenses. BMC Veterinary Research. 8:197.

Woodhams DC, Vredenburg VT, Simon MA, Billheimer D, Shakhtour B, Shyr Y et al. 2007. Symbiotic bacteria contribute to innate immune defenses of the threatened mountain yellow-legged frog, Rana muscosa. Biological Conservation. 138:390–398.

Wright KM. 1996. Amphibian husbandry and medicine. In: Reptile medicine and surgery. Mader DR (ed). WB Saunders, Philadelphia, PA. p. 436–459.

Yang HJ, Baek HJ, Speare R, Webb R, Park SK, Kim TH, Lasater KC, Shin SP, Son SH, Park JH, Min MS, Kim YJ, Na K, Lee H, Park SC. 2009 First detection of the amphibian chytrid fungus Batrachochytrium dendrobatidis in free-ranging populations of amphibians on mainland Asia: survey in South Korea. Diseases of Aquatic Organisms. 86:9-13.

Zasloff M. 1987. Magainins, a class of antimicrobial peptides from Xenopus skin: isolation, characterization of two active forms, and partial cDNA sequence of a precursor. Proceedings of the National Academy of Sciences. 84(15):5449-5453.

Appendices

Appendix 1. Preparation of Laboratory Materials.

Buffers to extract DNA from *Bd*. These buffers are used to digest extracts of cutaneous swabs, to prepare fungal DNA for qPCR.

Digestion Buffer: 10mM Tris HCl + 25m MEDTA + 0.1M NaCl + 0.5% SDS

Strong Digestion Buffer: 100mM Tris HCl + 20m EDTA + 0.5M NaCl + 1% SDS

Norepinephrine 1g powder.

Target dose is 40nmol/g BW. Reconstituted powder in 20ml saline for a 50mg/ml stock (157mmol/L). Diluted this by 1/40 with saline for the working solution. Therefore, a 30g frog will get 280uL of working solution. Remaining doses calculated based on a 30g frog.

Methylprednisolone 100mg powder.

Target dose is 0.075mg/g BW. Reconstituted powder in 300uL ethanol and added 9700uL saline for a total volume of 10mL for the working solution. Therefore a 30g frog gets 225uL of working solution. Remaining doses calculated based on a 30g frog.

Calculating the inoculum dose for the second study.

25mL H20 + zoospores, 1:1 iodine dilution to count.

Average of 12 zoospores per square, in 5 squares = 60 zoospores per 500nL = 0.12/1nL x 1,000,000 = 120,000 zoospores in 1mL diluted = 240,000 zoospores/1 mL. For each tank calculated # of zoospores needed for target zoospores/mL, to give a total of x μL to put in infection bath.

Appendix 2. Details of RNA Extraction and cDNA Preparation (Chapter 2).

Date RNA isolated	Sample Name	RNA concentration (ng/µl)	260/ 280	260/230	Amount of RNA added	Date cDNA made	cDNA concentration (ng/µl)
August 25 AM	June 6 – 1	6.8	1.98	0.47	11 µL RNA	Nov 4	1370.1
	June 6 – 2	56.2	2.08	2.24	2 µL RNA	Nov 4	2258.0
	June 6 – 3	9.6	2.08	1.92	11 µL RNA	Nov 4	1634.3
	June 6 – 9	65.8	2.11	1.60	2 µL RNA	Nov 4	837.3
	June 6 – 10	4.9	2.33	0.22	11 µL RNA	Nov 4	694.2
	June 6 - 11	19.8	2.12	0.49	5 µL RNA	Nov 4	661.6
August 25 PM	June 6 – 4	24	2.20	1.31	11 µL RNA	Nov 4	1601.7
	June 6 – 5	11.6	2.39	0.80	10 µL RNA	Nov 4	1230.4
	June 6 – 6	22.0	2.21	0.93	5 µL RNA	Nov 4	1242.6
	June 6 – 7	19.0	2.23	0.90	5 µL RNA	Nov 4	1079.3
	June 6 – 12	2.3	1.66	0.02	11 µL RNA	May 24a	463.5
	June 6 – 13	3.1	1.47	0.39	11 µL RNA	May 24a	n/a
August 26 PM	June 14 – 1	8.7	2.13	1.35	11 µL RNA	May 23	1204.6
	June 14 – 2	24.1	2.12	2.01	5 µL RNA	May 23	1153.5
	June 14 – 3	3.3	2.45	0.64	11 µL RNA	May 23	1403.6
	June 14 – 4	26.0	2.14	0.60	5 µL RNA	May 23	1147.6
	June 14 – 5	2.9	2.40	0.10	11 µL RNA	May 23	1606.5
	June 6 – 14	4.1	1.75	0.84	11 µL RNA	May 23	1085.2
August 29 PM	June 14 – 6	17.2	2.22	0.27	5 µL RNA	May 23	1162.5
	June 14 – 7	71.6	2.08	0.73	2 µL RNA	May 23	1002.5
	June 14 – 9	89.5	2.06	2.02	2 µL RNA	May 23	996.3
	June 14 – 10	28.3	2.01	0.85	5 µL RNA	May 23	971.2
	June 14 – 11	15.4	2.02	0.09	11 µL RNA	May 23	1494.0
	June 14 – 12	1.5	2.09	0.02	11 µL RNA	May 23	1440.9
	June 14 – 13	4.1	2.08	0.10	11 µL RNA	May 23	1092.1
	June 14 – 14	3.0	3.23	0.01	11 µL RNA	May 23	1123.8
August 30	June 22 – 1	0.2	-0.30	0.05	11 µL RNA	Aug 31	1281.7

	June 22 – 3	12.7	2.01	1.34	11 μL RNA	May 23	1251.6
	June 22 – 4	4.2	1.62	0.26	11 μL RNA	Aug 31	1160.5
	June 22 – 5	8.2	2.18	0.67	11 μL RNA	May 23	1196.9
	June 22 – 6	21.4	2.00	0.28	5 μL RNA	Aug 31	1193.5
	June 22 – 7	12.5	2.02	1.60	11 μL RNA	May 23	1213.0
	June 22 – 9	68.1	2.11	0.82	2 μL RNA	May 23	692.9
August 30 PM	June 22 – 10	176.5	2.12	1.46	1 μL RNA	Aug 31	977.7
	June 22 – 11	14.6	2.18	0.14	11 μL RNA	May 23	1488.7
	June 22 – 12	55.7	2.12	1.96	2 μL RNA	Aug 31	826.7
	June 22 – 13	1.9	1.34	0.06	11 μL RNA	May 23	1272.9
	June 22 – 14	1.3	8.91	0.01	11 μL RNA	May 23	1172.1
	May 13 – 7	87.4	2.22	2.43	2 μL RNA	Sept 5	1372.6
	May 13 – 14	12.7	2.30	2.00	Sample not found		
	May 13 – 6	92.9	2.15	1.66	1 μL RNA	Sept 5	1094.0
August 31 AM	May 13 – 2	76.3	2.09	2.16	2 μL RNA	Sept 5	1741.8
	May 13 – 3	16.5	2.40	0.27	1 μL RNA	Sept 5	1239.6
	May 13 – 4	67.3	2.09	0.88	2 μL RNA	Sept 5	1267.0
	May 13 – 5	11.9	2.08	1.40	11 μL RNA	Sept 5	1445.0
	May 13 – 9	378.3	2.10	2.34	1 μL RNA	Sept 5	1449.8
	May 13 – 11	19.4	2.22	0.51	5 μL RNA	Sept 5	1211.0
	May 13 – 12	4.8	2.24	0.30	11 μL RNA	Sept 5	1241.8
	May 13 – 13	214.7	2.12	2.28	1 μL RNA	Sept 5	1029.2
September 5 PM	May 13 – 10	4.0	2.30	0.25	11 μL RNA	May 23	1012.9
	May 21 – 4	3.3	2.65	0.08	11 μL RNA	May 23	933.5
	May 21 – 5	6.9	2.02	0.61	11 μL RNA	May 23	980.6
	May 21 – 6	47.0	2.13	1.78	6 μL RNA	May 23	731.4
	May 21 – 7	5.4	2.08	0.50	11 μL RNA	May 23	870.0
	May 21 – 12	4.1	2.02	0.51	11 μL RNA	May 24a	536.5
	May 21 – 13	2.0	1.48	0.12	11 μL RNA	May 24a	450.9
	May 21 – 14	1.4	1.16	0.26	11 μL RNA	May 24a	543.2
September 7 AM	May 21 – 3	1.2	25.92	-0.53	11 μL RNA	May 24a	533.5
	May 21 – 11	3.5	3.44	0.01	11 μL RNA	May 24a	709.4
	May 21 – 2	15.1	1.98	3.60	11 μL RNA	May 24a	689.5
	May 21 – 10	3.7	3.08	0.08	11 μL RNA	May 24a	633.4
	May 21 – 1	20.3	2.08	1.20	5 μL RNA	May 24a	787.1
	May 21 – 9	2.4	2.18	0.53	11 μL RNA	May 24a	639.8

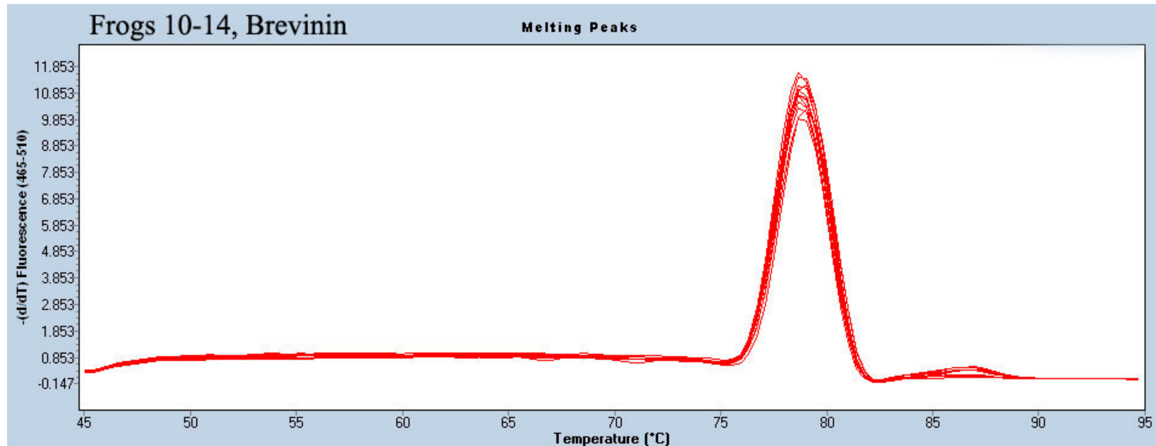
	May 29 – 7	9.5	2.12	1.72	11 μL RNA	May 24a	641.2
	May 29 - 14	4.9	2.04	0.69	11 μL RNA	May 24a	620.1
September 8 AM	May 13 – 1	27.4	2.12	1.81	4 μL RNA	May 24b	717.1
	May 29 – 3	30.8	1.83	0.62	4 μL RNA	May 24b	480.4
	May 29 – 4	25.5	2.04	2.57	4 μL RNA	May 24b	591.8
	May 29 – 5	11.4	2.20	0.64	10 μL RNA	May 24b	327.4
	May 29 – 6	25.8	2.16	1.83	4 μL RNA	May 24b	500.1
	May 29 – 10	3.8	2.11	0.91	11 μL RNA	May 24b	944.2
	May 29 – 11	57.0	2.13	1.56	2 μL RNA	May 24b	491.3
	May 29 – 13	68.4	2.07	1.54	2 μL RNA	May 24b	498.9
September 9 PM	May 29 – 1	2.6	2.24	0.07	11 μL RNA	May 24b	1117.5
	May 29 – 2	771.7	2.16	2.37	1 μL RNA	May 24b	416.7
	May 29 – 9	28.1	2.10	2.33	4 μL RNA	May 24b	784.5
	May 29 – 12	11.2	1.84	1.77	10 μL RNA	May 24b	951.0

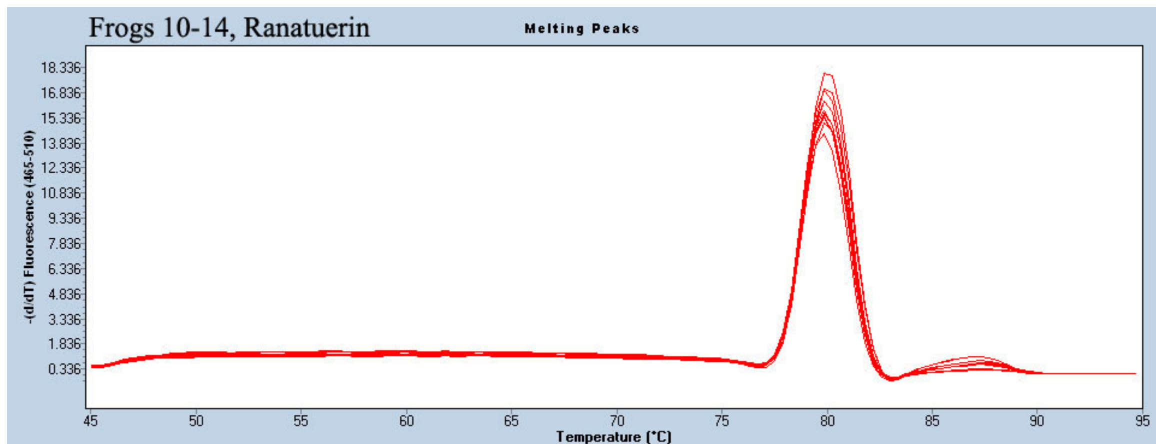
Appendix 3. Primers for qPCR Quantification of *Bd*.

Primers for qPCR quantification of *Bd* (Boyle et al 2004).

Target Gene	Forward Primer (5´-3´) "BOB5"	Reverse Primer (5´-3´) "BOB6"	Amplicon size (bp)	Standard Curve Efficiency
18S, 28S DNA	ATGCTTAAGT TCAGCGGG	CCGATTGAATG GCTTAGTGAGA CC	740	1.768

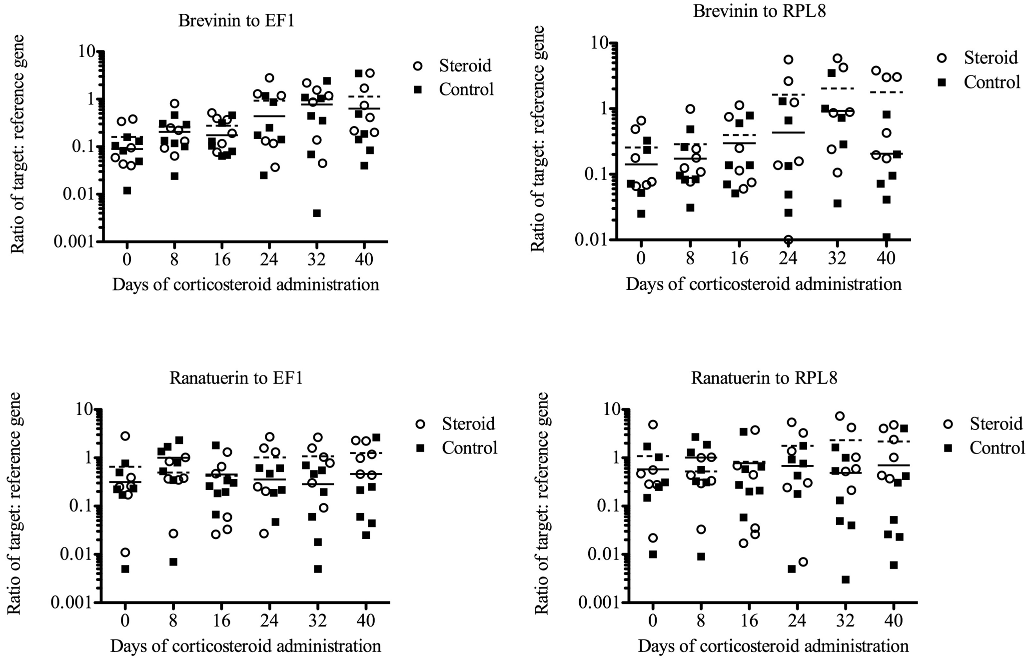
Appendix 4. Representative Example of Melting Curves of the RT-qPCR products, Showing Data for Brevinin and Ranatuerin (Chapter 2).





Appendix 5. Quantitative RT-qPCR Data for Individual Frogs (Chapter 2).

Data for steroid-treated frogs are shown as open circles, and the mean for each time point is indicated by the horizontal dashed lines. Data for sham-treated (control) frogs are shown as closed squares, and the mean for each time point is indicated by the horizontal solid lines.



Appendix 6. *Bd* Zoospore Counts for Preparation of Fungal Inoculum (Chapter 3).

Flask #	# zoospores in 5 extra large squares (in 500nL)	# zoospores per 1 nL of iodine dilution	# zoospores per 1 mL of iodine dilution	# zoospores per 1 mL of broth
1	18	0.036	36000	360000
2	15	0.030	30000	300000
3	13	0.026	26000	260000
4	9	0.018	18000	180000
5	2	0.004	4000	40000
6	10	0.020	20000	200000
7	9	0.018	18000	180000
8	3	0.006	6000	60000
9	5	0.010	10000	100000
10	5	0.010	10000	100000
11	20	0.040	40000	400000
12	1	0.002	2000	20000

Appendix 7. Crossing Points for the Standard Curve, for *Bd* qPCR (Chapter 3, see Figure 3-2).

Zoospores/mL	Crossing points of three technical replicates		
10^6	16.03	16.20	16.20
10^5	20.53	20.65	20.98
10^4	n/a	23.89	24.26
10^3	n/a	29.05	29.29
10^2	32.25	32.83	33.06
10^1	36.60	n/a	n/a
Negative (no template)	n/d	n/d	n/d
Negative (extract control)	n/d	n/d	n/d

n/a: data not available; n/d: crossing point not detected