AN ABSTRACT OF THE DISSERTATION OF

Catherine L. Searle for the degree of Doctor of Philosophy in Environmental Science presented on February 16, 2011.

Title: Host-Pathogen Dynamics in a Changing Environment: Susceptibility of Amphibians to an Emerging Infectious Disease

Abstract approved:

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Andrew R. Blaustein

Infectious diseases are a growing concern for both humans and wildlife. The negative effects of infectious disease have been exemplified by the recent global amphibian population declines associated with disease outbreaks. Although multiple pathogens and factors play a role in these declines, the aquatic fungal pathogen, *Batrachochytrium dendrobatidis* (Bd), has received considerable attention due to its substantial contribution to amphibian population declines around the world. Bd prevalence and severity appears to be increasing worldwide, either from recent anthropogenic spread of the pathogen or from changes in the environment that have altered host-pathogen dynamics. This dissertation explores the factors that affect host susceptibility to Bd.

I first tested the effects of hormonal stress on susceptibility to Bd (Chapter 2). Using corticosterone, the major chronic stress hormone in amphibians, I was able to mimic the physiological effects of stress without altering other factors that may affect

the host-pathogen relationship. I exposed three species of larval amphibians to corticosterone for two weeks to induce chronic stress before challenging them with exposure to Bd. I found that exposure to corticosterone did not alter infection prevalence or severity in any species, indicating that chronically elevated levels of corticosterone do not affect susceptibility to Bd.

I next examined the interactive effects of the ubiquitous stressor, ultraviolet-B radiation (UVB), and host infection by Bd (Chapter 3). UVB can cause lethal and sublethal effects in amphibians, including increased susceptibility to pathogens. In outdoor mesocosms, I used ambient levels of UVB to stress larval amphibians while simultaneously exposing them to Bd. Although exposure to UVB increased mortality, it did not alter infection.

To investigate the effects of community structure on infection prevalence and severity, I studied how six anuran species (frogs and toads) differed in susceptibility to Bd (Chapter 4). I experimentally exposed post-metamorphic amphibians native to North America to Bd under identical laboratory conditions. All species tested had higher rates of mortality when exposed to Bd compared to unexposed controls. However, the species differed widely in their rates of Bd-associated mortality, even though there was no difference in infection levels among species. I also found that within species, the relationship between body size and infection varied, indicating physiological differences in the way that amphibian species respond to pathogen infection.

Finally, I studied the effects of the amphibian host community on infection. I experimentally exposed larval amphibians to Bd after manipulating host density and species richness in the laboratory (Chapter 5). I recorded five measures of disease risk and found a dilution effect where greater species richness decreased disease risk, even after taking into account changes in density. Together with Chapter 4, this study emphasizes the need to understand the effects of the community on host-pathogen dynamics.

This dissertation provides insight into the effects of stress and community structure on disease dynamics. Although there has been a great effort to understand Bd since it was discovered, the ecology of Bd remains relatively unknown. My research represents an important step in understanding the host-pathogen relationship in a changing environment.

# Host-Pathogen Dynamics in a Changing Environment: Susceptibility of Amphibians to an Emerging Infectious Disease

by
Catherine L. Searle

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APPROVED:

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I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

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Catherine L. Searle, Author

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All co-authors contributed to the writing process. Andrew Blaustein contributed to experimental design and editing of all chapters.

*Chapter 2:* Lisa Belden aided in experimental design of this experiment and performed all corticosterone assays.

*Chapter 3:* Lisa Belden conducted the first experiment in this chapter (mesocosm experiment 1). Betsy Bancroft assisted with all work using artificial ultraviolet radiation in the laboratory. Barbara Han assisted with pathogen culturing and inoculation. Lindsay Biga assisted with implementation of the laboratory experiment.

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## CHAPTER 1: GENERAL INTRODUCTION

Infectious diseases are a growing global concern. In addition to the social and economic impacts of infectious diseases in humans (e.g. Gubler 2002, Fendrick et al. 2003), pathogens also severely affect populations, communities and ecosystems. Within communities, pathogens may act as keystone species, regulating dominant species to increase overall biodiversity (e.g. Wills et al. 1997, Kiesecker et al.1999). However, pathogens can also cause epidemics that result in population declines and reductions in biodiversity (Daszak et al. 2000, Anderson et al. 2004). Infectious disease may increase in prevalence or severity when pathogens are introduced to a naive host species or environmental conditions shift (Daszak et al. 2000). Globally, disease epidemics appear to be increasing in both humans and wildlife (Harvell et al. 2002). Many factors are likely contributing to this increase. For example, environmental stressors such as climate change, pollution, invasive species and habitat destruction can make hosts more susceptible to pathogens (Harvell et al. 2002, Lafferty and Holt 2003, Bradley and Altizer 2007). When hosts are exposed to stressful conditions, they may become physiologically stressed and lack sufficient energy to launch an effective immune response (Rigby and Moret 2000, Kiesecker 2002, Christin et al. 2003) making them more susceptible to pathogens. Changes in community structure can also lead to an increase in epidemics. Pathogens are functioning members of ecosystems, and the

loss or gain of a species in a community can affect all organisms in a system. To mitigate the negative effects of infectious diseases, it is essential to understand the complex and often context-dependent interactions between hosts and their pathogens.

Infectious diseases in amphibians are a particular concern because amphibian populations are declining worldwide (Houlahan et al. 2000, Stuart et al. 2004). Many factors are contributing to amphibian population declines, but disease-related die-offs and declines have received considerable attention and have been documented in many locations (reviewed by Daszak et al. 2003). Amphibians are vulnerable to a wide variety of pathogens (Kiesecker et al. 2004) and the degree to which a pathogen affects an amphibian population or species can be highly context-dependent (e.g. Taylor et al. 1999, Berger et al. 2004, Rojas et al. 2005, Johnson et al. 2007). Thus, amphibians are an ideal group of organisms to study the interactions between disease and changes to the environment.

The aquatic fungal pathogen, *Batrachochytrium dendrobatidis* (hereafter referred to as "Bd"), is of particular concern due to its association with population declines of amphibians around the world (e.g. Lips et al. 2006, Rachowicz et al. 2006, Vredenburg et al. 2010). Bd can infect many, and possibly all, amphibian species by living within their keritanized structures, which includes the mouthparts of larvae and the keratinized epidermal cells of post-metamorphic amphibians (Longcore 1999, Piotrowski et al. 2004). Amphibians appear to be the only taxonomic group affected by Bd; no hosts have been found outside the class Amphibia. Infection by Bd at the larval stage can lengthen the larval period, reduce mass at metamorphosis and cause mortality

in some species (e.g. Garner et al. 2009, Parris and Cornelius 2004, Blaustein et al. 2005b). Larval infection is generally (but not always) non-lethal, but can cause mortality during or after metamorphosis (e.g. Rachowicz and Vredenburg 2004). During post-metamorphic stages, keratin is distributed throughout the epidermis, allowing infection to become more widespread on the host. Bd infection at the post-metamorphic stage is generally more severe than in larvae and can lead to lethargy, loss of appetite, seizures and high rates of mortality (Berger et al. 1998, Pessier et al. 1999, Carey et al. 2006, Voyles et al. 2009). Mass die-offs attributed to Bd have generally involved mortality at the post-metamorphic stage (Lips et al. 2006, Rachowicz et al. 2006, Vredenburg et al. 2010, but see Mendelson et al. 2004), but larvae may serve as reservoirs, facilitating the persistence of Bd through time (Briggs et al. 2010). Thus, the Bd-host relationship is multifaceted and varies with developmental stage.

Globally, Bd appears to be increasing in range and severity (Fisher and Garner 2007, Skerratt et al. 2007). There are two major hypotheses to explain this increase: (1) Bd has recently been introduced around the world, exposing naïve amphibians to a new pathogen or (2) changes to the environment have shifted the host-pathogen relationship such that amphibians are more susceptible to the negative effects of Bd. These hypotheses are not mutually exclusive and there is evidence for both causes (Fisher et al. 2009, James et al. 2009, Rachowicz et al. 2005). Additionally, although many population declines of amphibians have been associated with Bd, species in some locations appear to be persisting despite the presence of Bd (Daszak et al. 2005, Kriger and Hero 2006). Why some populations decline from Bd while others persist is not

well-understood. Many factors may be involved including species differences in immunological resistance to Bd, abiotic environmental stress, community biodiversity, and the amount of time that Bd has been present in a host population.

Amphibians are exposed to many stressors in nature. Factors such as changing temperature, habitat destruction, competition for food and environmental contamination can create physiological stress (e.g. Cushman 2006, Relyea 2006, Alford et al. 2007, Jones et al. 2011, Blaustein et al. *in press*). This stress can decrease the overall health of an organism and make it more susceptible to infectious disease (reviewed in Daszak et al. 2001, Lafferty and Holt 2003). One hypothesis for the recent increase in Bd prevalence around the world is that changes to the environment are making amphibians more susceptible to Bd (Skerratt et al. 2007). For example, increased nighttime temperatures due to climate change may create conditions that favor Bd growth and lead to more amphibians becoming infected and dying from Bd (Pounds et al. 2006). In other amphibian pathogens such as the water mold, *Saprolegnia ferax*, exposure to stressors such as ultraviolet-B radiation (UVB) increases the prevalence of this pathogen in amphibians eggs (Kiesecker et al. 2001). Thus, many stressors including climate change, pollution, UVB and habitat loss have the potential to affect infection with Bd. It is essential to investigate the interactions between Bd infection and the environmental stressors.

Community changes can also influence infectious diseases in amphibians. For example, Parris and Cornelius (2004) tested competition between two species of larval amphibians and found that one species (*Bufo fowleri*) negatively affected development

of another species (*Hyla chrysoscelis*), but only when Bd was present. In multi-host pathogens like Bd, species may differ in their susceptibility to infection and the diversity of hosts in a community may be very important. Ecological theory predicts that in most cases, increased biodiversity will decrease the severity of infectious disease (Keesing et al. 2006, Keesing et al. 2010). When more species are present, a "dilution effect" may occur where the abundance of potential hosts diffuses the negative impacts on any one species (e.g., Schmidt and Ostfeld 2001). Thus, a diverse ecosystem should allow hosts to experience lower disease prevalence and severity. However, some systems have demonstrated the opposite "amplification effect" where greater diversity actually increases the prevalence of an infectious disease (e.g., Power and Mitchell 2004). In amphibians, Han (2008) found that infection severity by Bd in a toad species (*Anaxyrus boreas*) decreased in the presence of other species. However, this trend was not found in two other anuran species, indicating that more studies are necessary to understand the relationship between biodiversity and infection severity in amphibians.

*Organization of dissertation*

My dissertation work examined the factors that affect amphibian susceptibility to Bd. In Chapter 2, I experimentally examined the effects of hormonal stress on susceptibility to Bd in larval amphibians. By manipulating the stress hormone, corticosterone, I was able to induce physiological effects of stress on the host without changing any environmental factors which could alter other aspects of the amphibian-Bd relationship.

In Chapter 3, I investigated the effects of the stressor, UVB, on Bd infection in larval amphibians in outdoor mesocosms. Exposure to ambient levels of UVB has many negative effects on amphibians (reviewed in Croteau et al. 2008) and negatively affects the immune system in other vertebrates (e.g. Noonan and DeFabo 1992, Noonan and Hoffman 1994). Thus, exposure to UVB may increase amphibian susceptibility to Bd.

In Chapter 4, I examined species differences in susceptibility to Bd. In nature, some populations and species appear to be declining from Bd while others persist, which may be due to differences in habitat among species or from physiological differences in the ability of species to cope with Bd infection. I experimentally tested post-metamorphic amphibians from six species for susceptibility to Bd under identical laboratory conditions. I then documented the relationships between infection, mortality, body size and feeding rates.

Finally, in Chapter 5, I studied the effects of host density and species richness (a taxonomic measure of biodiversity) on infection in larval toads. Global losses of biodiversity are changing ecological communities in ways that can affect disease dynamics in natural populations. I tested for evidence of a relationship between species richness and disease risk to demonstrate either a dilution effect or an amplification effect.

My dissertation provides new information on the ecology of Bd with implications for infectious diseases in other natural systems. Understanding the factors that affect susceptibility to Bd can lead to informed management decisions to control

disease outbreaks and prevent future amphibian population declines. Additionally, understanding the relationships between stressors, community structure and disease on different species will aid in the understanding of emerging infectious diseases in other natural systems (including humans). This knowledge will help mitigate further losses of biodiversity from infectious diseases.

# CHAPTER 2

## Exogenous treatment with the stress hormone, corticosterone, does not affect *Batrachochytrium dendrobatidis* infection in amphibian hosts

Catherine L. Searle, Lisa K. Belden, Andrew R. Blaustein

**Abstract**

Infectious diseases are increasing in prevalence and severity worldwide. In amphibians, the fungal pathogen, *Batrachochytrium dendrobatidis* (Bd), has been associated with amphibian population declines in many locations. Recent emergence and spread of Bd may be due to a number of factors, including environmental stressors that increase host susceptibility to Bd. Physiological stress can increase circulating levels of the hormone, corticosterone, which at chronic levels can result in immunosuppression and increased susceptibility to pathogens. Using the larvae of three amphibian species (*Anaxyrus boreas* [*Bufo boreas*]*, Rana cascadae,* and *Lithobates catesbeianus* [*Rana catesbeiana*]), we chronically elevated corticosterone levels through exogenous exposure, followed by a challenge with Bd. After pathogen exposure we measured whole-body corticosterone, mortality, growth, development, and infection. In all species, infection prevalence (percent infection) and infection severity were not affected by exposure to corticosterone, even though we successfully elevated stress hormone levels. Upon termination of the experiment, species differed in their overall levels of corticosterone with *A. boreas* experiencing the highest whole-body corticosterone and *L. catesbeianus* experiencing the lowest. Exposure to corticosterone reduced mass and development in *A. boreas* and reduced mass and length in *R. cascadae*. In contrast, Bd-exposure increased length in all species and mass in *R. cascadae,* indicating that Bd may have some positive effects on larval growth. This study indicates that elevated levels of corticosterone do not make larval amphibians more susceptible to Bd infection.

## Introduction

Infectious diseases are a growing global concern, particularly when epidemics result in population declines and reduced biodiversity (e.g. Daszak 2000, Anderson et al. 2004). Globally, epidemics are increasing in both humans and wildlife (Harvell et al. 2002, Jones et al. 2008) and may be facilitated by anthropogenic changes to the environment. Environmental changes including pollution, invasive species, climate change, and habitat destruction can alter disease dynamics and shift host-pathogen relationships in complex and unpredictable ways (Patz et al. 2000, Daszak et al. 2001, Jones et al. 2008, Martin et al. 2010).

Changes in the environment affect both hosts and pathogens. For hosts, environmental changes may act as stressors, modifying host behavior and physiology. For example, changes in the environment may physiologically stress hosts such that their overall health decreases and they lack sufficient energy to launch an effective immune response (reviewed in Daszak et al. 2001, Lafferty and Holt 2003). In this manner, environmental changes can make hosts more susceptible to pathogens. In vertebrates, this stress response is primarily regulated by the adrenal hormones, catecholamines and glucocorticoids. The immediate stress response is regulated by catecholamines (e.g. epinephrine and norepinephrine) which are released within seconds of exposure to a stressful situation. However, after longer periods of stress (minutes to days), glucocorticoids (e.g. corticosterone and cortisol) are released from the adrenal cortex. The effect of glucocorticoids on vertebrates is complex, but generally glucocorticoids suppress non-vital functions and mobilize energy stores

necessary for immediate survival (Romero 2002). At chronic levels glucocorticoids can inhibit functions like reproduction and immunity (Cohn 1997, Dhabhar 2000, Tilbrook et al. 2000, French et al. 2007), but can also be involved in redistribution of immune function (Martin et al. 2006).

In amphibians, the main stress hormone is corticosterone. In addition to its role in amphibian metamorphosis (Denver 1997, Hayes 1997), amphibians will elevate corticosterone in response to capture (Coddington and Cree 1995), crowding and food deprivation (Glennemeier and Denver 2002c, Cooperman et al. 2004, Crespi and Denver 2005), reproductive pheromones (Schubert et al. 2009), confinement (Glennemeier and Denver 2002a, Belden et al. 2003, Belden et al. 2010) and pollution (Hopkins et al. 1999, Hayes et al. 2006, Peterson et al. 2009). Corticosterone can be artificially elevated in larval amphibians by adding exogenous hormone to water, where it is absorbed through their skin and gills. Chronic elevation of corticosterone in amphibians can decrease reproductive activity (Moore and Miller 1984), slow growth and development (Glennemeier and Denver 2002b) and alter immune function (Rollins-Smith et al. 1997, Belden and Kiesecker 2005). For example, chronically elevated corticosterone causes a decrease in lymphocytes in the spleen, thymus, and circulation (Rollins-Smith and Blair 1993, Belden and Kiesecker 2005). Therefore, it is predicted that chronic elevation of corticosterone will increase susceptibility to pathogens. A study by Belden and Kiesecker (2005) demonstrated that elevated levels of corticosterone in larval amphibians increased infection by a trematode parasite.

However, few studies have directly investigated the relationship between corticosterone and infection in amphibian-pathogen systems.

One amphibian pathogen which has received considerable attention in the last decade is *Batrachochytrium dendrobatidis* (Bd). Bd was first discovered by Berger et al. (1998), described by Longcore et al. (1999), and infects keratinized structures of amphibians. While amphibian eggs do not contain keratin, larval mouthparts and post-metamorphic amphibian skin are keratinized and can be infected. Both lethal and sublethal effects of Bd infection have been documented in larval and post-metamorphic stages (e.g. Blaustein et al. 2005b, Carey et al. 2006). Little evidence has been found to indicate that amphibians can express adaptive immune responses to Bd (Rivas et al. 2009, Rosenblum et al. 2009, Stice and Briggs 2010, but see Ramsey et al. 2010). However, innate responses, including anti-microbial peptides and natural microbial skin assemblages, may provide some resistance (Rollins-Smith et al. 2002, Harris et al. 2006, Woodhams et al. 2007, Lam et al. 2010).

Abotic factors such as temperature, pH and humidity can influence Bd growth and infection (Berger et al. 2004, Piotrowski et al. 2004, Andre et al. 2008). For example, at the post-metamorphic stage, treatment with X-irradiation decreased leukocyte counts and increased Bd infection (Ramsey et al. 2010), indicating that stressors can alter the immune system and subsequently affect susceptibility to Bd. Increased susceptibility to Bd from environmental stressors may be one reason that Bd has globally increased in its range and prevalence (Skerratt et al. 2007).

This study investigates the effects of elevated levels for corticosterone on infection by Bd using a comparative framework with the larvae of three species of amphibians (*Anaxyrus boreas* [formerly *Bufo boreas*]*, Rana cascadae* and *Lithobates catebeianus* [formerly *Rana catesbeiana*]). These species were chosen because they demonstrate a range of susceptibility to Bd, with *A. boreas* experiencing high rates of mortality at the larval and post-metamorphic stages (Blaustein et al. 2005b, Carey et al. 2006) and *L. catesbeianus* identified as a species tolerant of Bd infection (Daszak et al. 2004, Blaustein et al. 2005b). We exposed each species to exogenous corticosterone at chronic levels before subsequently challenging them with Bd. By using exogenous corticosterone, we can identify how physiological stress may affect susceptibility without altering other aspects of the Bd-amphibian relationship (e.g. transmission, host behavior, pathogen virulence). This study has implications for how environmental stress may influence Bd susceptibility.

## Materials and Methods

Amphibian eggs were collected from natural oviposition sites in the Willamette Valley and Cascades Mountain Range of Oregon, USA. Western toad eggs (*Anaxyrus boreas*) were collected in June, 2008 from Lost Lake in Linn County (elevation 1220m), Cascades frog eggs (*Rana cascadae*) were collected in May, 2009 from Parrish Pond in Linn County (elevation 1130m) and American bullfrog eggs (*Lithobates catesbeianus*) were collected in June 2009 from Finley National Wildlife Refuge in Benton County (elevation 90m). After collection, all eggs were immediately brought to

the laboratory at Oregon State University for rearing in 38L aquaria filled with dechlorinated water. The temperature in the laboratory is maintained at 13.5-14.5ºC with a light regime that mimics outdoor conditions. After hatching, animals were fed a 3:1 ratio (by volume) of ground rabbit chow to fish food. Density of larvae was approximately 200 per aquarium and water in the aquaria was changed weekly.

Species were not tested concurrently, but each species was tested using the same protocol and at the same developmental stage (stages 25-26; Gosner 1960). Larvae were held individually in 1L plastic containers filled with 500mL dechlorinated water for the duration of the experiment. Individuals were randomly assigned to treatments, with each treatment replicated 20 times for a total of 160 individuals for each species. The experiment was a 4 x 2 factorial design with 4 levels of corticosterone and 2 pathogen treatments (control [unexposed], exposed). Corticosterone treatments were (1) water control (water added), (2) ethanol control (30 µL of 95% ethanol in each 500 mL container), (3) low corticosterone (0.01 µM solution [0.00000173 g dissolved in 30 µL 95% ethanol in each 500 mL container]), and (4) high corticosterone (0.1 µM solution [0.0000173g dissolved in 30 µL 95% ethanol in each 500 mL container]). Treatment with exogenous corticosterone at these levels elevates whole-body corticosterone within or slightly above those naturally occurring in amphibians under chronic stress (Belden et al. 2005, 2010). Larvae were first exposed to corticosterone treatments for 15 days prior to Bd exposure. Water was changed every three days with concentrations of corticosterone (purchased from Sigma-Aldrich) re-established at each water change (Appendix A).

After 15 days of corticosterone treatment, animals were exposed to Bd treatments three times during the subsequent 15 days. Previous studies have demonstrated immunosuppressive effects of corticosterone in vertebrates after as few as 5 days (Martin et al. 2005, Kiank et al. 2006). To culture Bd, we inoculated tryptone-agar plates with strain JEL 274 (originally isolated from *A. boreas* in Colorado). Ten days after inoculation, agar plates were flooded with 15mL distilled water to release zoospores and 2mL of this water was added to each larva's container. Bd-exposed larvae were given approximately $3x10^4$ zoospores/mL at each exposure (quantified by measuring a sample of the inoculum under a hemocytometer). Bd-control animals were given a similar treatment using sterile agar plates. Corticosterone treatments were maintained throughout this infection period with water changes every three days. Mortality was monitored daily and any dead animals were removed and preserved in 95% ethanol. The experiment was terminated after 30 days, two days after the last water change (timeline shown in Appendix A).

At the end of the experiment, 5 animals from each treatment were flash-frozen for corticosterone analysis after being immersed in MS-222 for less than 60 seconds. For *L. catesbeianus*, only four animals were tested for corticosterone in the water control, Bd-exposed treatment. Samples were stored in a -80ºC freezer prior to radioimmunoassay to determine whole-body levels of corticosterone. Radioimmunoassay followed the procedures of Belden et al. (2003), with some minor modifications. Samples were run in two assays, one consisting of the *R. cascadae* and *L. catesbeianus* samples (N=79) and one consisting of *A. boreas* samples (N=40). Each

individual sample was weighed and homogenized with a mass adjusted amount of distilled water (mass x 10ml + 0.5 ml rinse; minimum 1.5 ml and maximum 4 ml). For estimation of extraction efficiency (recoveries), each homogenized sample was equilibrated overnight with 2,000 cpm of tritiated corticosterone. Each sample was then extracted in 5 ml of dichloromethane. To break the emulsion, each sample was centrifuged at 2000 rpm for 15 minutes, and then the organic phase was removed and dried in a warm water bath, under a stream of nitrogen gas. The extracts were resuspended in 10% ethyl acetate in isooctane. To separate the steroid fractions from the neutral lipids, the samples were chromatographed through individual diatomaceous earth columns. The fractions were eluted using stepwise increasing proportions of ethyl acetate in isooctane and the purified eluates were dried and resuspended in 600 µl buffer (phosphate buffer saline with 0.1% gelatin) for the assay.

For the assay, individual sample recoveries were determined from 100 µl of the sample while 200 µl of the sample was allocated in duplicate for the assay. Serial dilutions for the standard curves were performed in triplicate. All samples, including serial dilutions and total bound, were incubated overnight with 100 µl of antibody and 100 µl of tritiated steroid. Unbound steroid was separated using dextran-coated charcoal and the bound steroid decanted into scintillation vials. Average intraassay variation was 10% and interassay variation was 25%. Average extraction efficiency was 59%. Levels off the standard curve (too little hormone in the sample or too much hormone in the sample) were set at the limit of detectability for analysis, which was 7.8 pg at the

low end and 1000 pg at the high end. However, whether we can determine the exact amount of corticosterone in a sample is also dependent on the extraction efficiency and mass of the individual sample (i.e., we can detect smaller amounts of corticosterone (as ng/g) in a larger individual). For each of the three species there were 1-2 individuals in the ethanol or water control treatments that were off the low end of the curve (too little hormone in sample), and for *A. boreas*, there was one individual in the low corticosterone treatment off the low end of the curve. For *A. boreas* and *R. cascadae* in the high corticosterone treatments, 10/10 and 9/10 samples, respectively, were off the top of the curve (too much hormone in the sample). The estimated values we report for those groups are likely underestimates of the true levels, although it is clear that the treatment did elevate the levels above both the control treatments and low corticosterone treatments (Figure 2.1). For *L. catesbeianus*, only a single individual in the high corticosterone treatment was off the top of the curve. All corticosterone levels were log-transformed prior to analysis.

Our corticosterone treatments successfully elevated whole-body levels of corticosterone (Figure 2.1). Corticosterone levels in our control and low corticosterone treatments were within normal ranges endogenously produced in larval amphibians (Belden et al. 2003, Crespi and Denver 2005, Belden et al. 2010). However, corticosterone levels in *A. boreas* and *R. cascadae* in the high corticosterone treatments may have been above normal levels. As almost all of those values are estimated, it is not possible to know how high they actually were.

The remaining 10-15 animals/treatment (depending on mortality) were euthanized in MS-222 and then weighed, measured (snout-vent length), staged (Gosner 1960) and preserved in 95% ethanol. We performed quantitative-PCR (qPCR) on all Bd-exposed animals and five randomly chosen Bd-control animals from each species. QPCR methods followed those by Boyle et al. (2004), except we used 60 µL Prepman Ultra (Applied Biosystems) in DNA extractions instead of 40 µL. Using qPCR allowed us to determine infection status of each animal (infected/uninfected) and the severity of Bd infection. To control for body size in our infection analysis, we recorded the quantity of nucleotides in each DNA extraction using a Nanodrop 1000 (Thermo Scientific) and calculated the genome equivalents of Bd per nanogram mouthpart DNA in each sample. Thus, our infection data was in the form of Bd concentration.

Mortality was compared among species and treatments with generalized linear models using a logit link. Animals that died before experimental take-down were not included in the other analyses. Corticosterone levels among all treatments and species were compared using analysis of variance (ANOVA) followed by a Tukey's HSD test. All other analyses were conducted separately for each species. Comparing among Bd treatment and corticosterone treatments, we analyzed mass, length and developmental stage using two-way ANOVA (including the interaction between Bd and corticosterone treatments) followed by Tukey's HSD tests. For infection status and level, we only used data from the Bd-exposed groups and compared among corticosterone treatments. Infection status (infected or uninfected) was analyzed using generalized linear models

and infection severity was analyzed using a Kruskal-Wallis rank sum test including only individuals testing positive for infection.

## Results

Infection status of the Bd-exposed animals was not affected by corticosterone treatment in any species (*A. boreas*: $X^2 = 0.59$, df = 3, p = 0.90; *R. cascadae*: $X^2 = 4.02$, df = 3, p = 0.26; *L. catesbeianus*: $X^2 = 2.64$, df = 3, p = 0.45). Additionally, corticosterone treatment did not affect infection severity (Kruskal-Wallis; *A. boreas*: $X^2 = 4.55$, df = 3, p = 0.21; *R. cascadae*: $X^2 = 4.53$, df = 3, p = 0.21; *L. catesbeianus*: $X^2 = 4.13$, df = 3, p = 0.25). We did not detect Bd on any of the tested Bd-control animals.

Although we found that exposure to corticosterone did not affect infection in any species, we did find differences among species in infection prevalence (percent infected) and whole-body levels of corticosterone. Infection prevalence varied by species with 50% of *A. boreas* infected, 52.8% of *R. cascadae* infected, and 85.5% of *L. catesbeianus* infected. Additionally, although all species were exposed to the same corticosterone exposure regime, they differed in the amount of corticosterone detected in their bodies ($F_{2,117} = 42.57$, $p < 0.01$; Figure 2.1). *Anaxyrus boreas* consistently had higher levels of corticosterone than the other two species (Tukey's HSD; $p < 0.01$ for both species). On average, *L. catesbeianus* had the lowest levels of corticosterone for all treatments, but these levels were not significantly different from those of the *R. cascadae* (Tukey's HSD; p = 0.59).

Our method of exposing larvae to corticosterone was successful. Corticosterone level was not affected by Bd treatment ($F_{1,118} = 0.26$, $p = 0.61$), but was affected by corticosterone treatment ($F_{3,116} = 59.24$, $p < 0.01$; Figure 2.1). There was no difference in corticosterone levels between the water and ethanol control treatments (Tukey's HSD; $p = 0.99$). Corticosterone levels were higher in the corticosterone-exposed treatments compared to the controls and the low corticosterone treatment had lower levels than the high corticosterone treatment (Tukey's HSD; $p < 0.01$ for these comparisons).

Mortality did not differ among species or treatments ($p>0.05$ for all models). *A. boreas*, exposed to corticosterone were smaller (mass) and less developed at the end of the experiment relative to non-exposed individuals, but length was not affected (ANOVA; mass: $F_{3,87} = 2.73$, $p = 0.05$; length: $F_{3,87} = 1.13$, $p = 0.34$; stage: $F_{3,87} = 5.60$, $p < 0.01$; Figure 2.2A,D,G). Similarly, for *R. cascadae*, corticosterone-exposed individuals were smaller (mass) and shorter at the end of the experiment relative to non-exposed individuals, but development was unaffected (mass: $F_{3,87} = 5.31$, $p < 0.01$; length: $F_{3,87} = 9.00$, $p < 0.01$; stage: $F_{3,87} = 0.79$, $p = 0.50$; Figure 2.2B,E,H). In contrast, mass, length and stage were unaffected by corticosterone treatment in *L. catesbeianus* (mass: $F_{3,99} = 0.13$, $p = 0.94$; length: $F_{3,99} = 0.12$, $p = 0.95$; stage: $F_{3,99} = 1.04$, $p = 0.38$; Figure 2.2C,F,I).

In all species, individuals exposed to Bd were longer compared to Bd-control individuals (ANOVA; *A. boreas*: $F_{1,87} = 3.99$, $p = 0.05$; *R. cascadae*: $F_{1,87} = 7.74$, $p < 0.01$; *L. catesbeianus*: $F_{1,99} = 4.14$, $p = 0.04$; Figure 2.2D,E,F). Individuals were heavier

when exposed to Bd compared to unexposed individuals in *R. cascadae* ($F_{1,87} = 3.92$, $p = 0.05$; Figure 2B), but mass was unaffected by Bd treatment in the two other species (*A. boreas*: $F_{1,87} = 0.86$, $p = 0.36$; *L. catesbeianus*: $F_{1,99} = 2.99$, $p = 0.09$; Figure 2.2A,C). Developmental stage was unaffected by Bd treatment, although *L. catesbeianus* tended to be more developed when exposed to Bd compared to the unexposed individuals (*A. boreas*: $F_{1,87} = 0.15$, $p = 0.70$; *R. cascadae*: $F_{1,87} = 1.74$, $p = 0.19$; *L. catesbeianus*: $F_{1,99} = 3.75$, $p = 0.06$; Figure 2.2G,H,I). The interaction between Bd treatment and corticosterone treatment was not significant for any parameter in any species (p-values $> 0.05$ for all models).

## Discussion

Our study demonstrated that exposure to exogenous corticosterone did not affect Bd infection prevalence or severity in the larvae of three species of amphibians. Even in our high-dose treatment of corticosterone, we did not find evidence for elevated Bd infection compared to the treatments without corticosterone. Although we did not directly measure immune responses, other investigations have shown that chronic exposure to glucocorticoids alters immune function in amphibians (Rollins-Smith and Blair 1993, Rollins-Smith et al. 1997, Belden and Kiesecker 2005) and other vertebrates (e.g. Auphan et al. 1995, Martin et al. 2005, Kiank et al. 2006), generally in ways that are immunosuppressive. In fact, immunosuppression is the major reason for which glucocorticoids are prescribed to human patients after undergoing transplants (reviewed in Franchimont 2004). Alterations to the immune system from glucocorticoids are

therefore expected to increase susceptibility to infectious diseases when at chronic levels. This has been demonstrated in amphibians where chronic exposure to exogenous corticosterone at the same levels used in this experiment, increased trematode infection in larval amphibians (Belden and Kiesecker 2005). Therefore, since our corticosterone levels were at the high end of the physiological range (possibly into pharmacological levels), and we exposed animals to corticosterone at chronic levels, we expected to see an increase in Bd infection when animals were exposed to corticosterone compared to the unexposed controls.

Since we did not find an increase in Bd infection with exposure to corticosterone, our study may have implications for the immune response of larval amphibians to Bd. For example, the larval immune system may not launch an effective immune response to Bd. Little is known about the larval immune response to Bd, but infection can cause an increase in circulating neutrophils and a decrease in eosinophils (Davis et al. 2010). However, if the immune response of larval amphibians is ineffective at reducing infection, then suppression of the response from elevated corticosterone would have little to no effect on infection severity. Alternatively, larval amphibians may launch an effective immune response to Bd, but it could involve an aspect of the immune system that is not suppressed by corticosterone exposure. Different aspects of the immune system can be important in fighting a given pathogen, and not all aspects may be suppressed by exposure to corticosterone (Franchimont 2004, Martin et al. 2006).

Even though all species were exposed to the same levels of corticosterone, we found differences in whole-body concentrations of corticosterone among species. *Anaxyrus boreas* had the highest whole-body levels of corticosterone in all treatments while *L. catesbeianus* had the lowest levels. In another study with chronic exposure to exogenous corticosterone, Belden et al. (2010) found that Eastern spadefoot toads (*Scaphiopus holbrooki*) on average had higher baseline levels of corticosterone than two other species (*Rana sylvatica* and *Ambystoma jeffersonianum*). Thus, it appears that toads may elevate their corticosterone levels to a greater degree when exposed to exogenous corticosterone, compared to other amphibian species. There may be physiological or behavioral differences between species that influences an amphibian's response to stress, its ability to absorb corticosterone exogenously, or to process and break down corticosterone. Further studies are necessary to understand how these differences may impact an animal's response to stressful conditions.

We found that corticosterone exposure decreased mass and developmental stage in *A. boreas* and decreased mass and length in *R. cascadae*. This effect has been demonstrated in other amphibian species, where elevated levels of corticosterone decreased rates of growth and development (Hayes et al. 1993, Glennemeier and Denver 2002b). Thus, even if elevated corticosterone does not directly alter infection, it may indirectly affect infection by reducing body size and altering time to metamorphosis, which could increase the amount of time they are in contact with infectious particles. In one species, *L. catesbeianus*, growth and development was unaffected by corticosterone, but this species also had the lowest concentrations of

corticosterone compared to the two other species. Thus, *L. catesbeianus* may be more resistant to stress than the other two species and is able to prevent the negative effects of corticosterone on growth and development.

Although corticosterone levels were low in *L. catesbeianus*, this species had higher infection prevalence than the other two species, even though all species were exposed to the same amount of Bd. This may indicate that *L. catesbeianus* acquire Bd infection more easily than the other two species. At the larval and postmetamorphic stages, *L. catebeianus* are seemingly unaffected by Bd and can survive with high levels of infection (Daszak et al. 2004, Blaustein et al. 2005b). This combination of high tolerance and ease at acquiring Bd indicates that *L. catebeianus* may be particularly competent hosts and act as reservoirs for Bd, facilitating its spread around the world. For example, *L. catesbeianus* have been introduced to many continents for commercial farming, and they are often infected with Bd these locations (Hanselmann et al. 2004, Garner et al. 2006, Fisher and Garner 2007). Thus, as *L. catesbeianus* are moved to new locations for farming, they may introduce Bd to new locations and naïve hosts.

In all species, individuals exposed to Bd were longer at the end of the experiment, and exposed *R. cascadae* individuals also weighed more than unexposed individuals. Since we did not see a difference in mortality with Bd infection, this finding is not the result of Bd killing the smallest, weakest larvae and leaving only the larger animals in the Bd-exposed treatments. When larval amphibians are infected with Bd, their mouthparts are affected, causing reduced feeding rates in some species (Venesky et al. 2010). Therefore, it is generally believed that Bd infection will slow

growth and development in larval amphibians. However, larvae may increase growth rates in response to zoospore presence as an attempt to speed development and exit dangerous water bodies. Amphibian larvae can alter developmental rates in response to many factors including pond drying and predation (Werner 1986, Semlitsch and Wilbur 1988) and embryonic amphibians can speed development in response to pathogens (Warkentin et al. 2001, Gomez-Mestre et al. 2006). Therefore, even though we did not see an increase in developmental rate with Bd exposure, increasing body size may be a strategy to prepare for a reduced time to metamorphosis to exit locations where pathogens are present.

Based on our results, stressors that chronically elevate corticosterone may not influence larval susceptibility to Bd. However, responses of the immune system and levels of corticosterone may vary through development (Jaffe 1981, Rollins-Smith et al. 1997, Crespi and Denver 2005), so more studies are necessary to determine if similar trends are found at different developmental stages. Even though physiological stress did not increase infection in larvae, stress may still play a role in the global increase of Bd by affecting post-metamorphic susceptibility or other aspects of the amphibian-Bd relationship such as transmission, reproductive rates, recovery, and host mortality. Future studies are necessary to fully understand the influence of stress on global Bd prevalence and severity.

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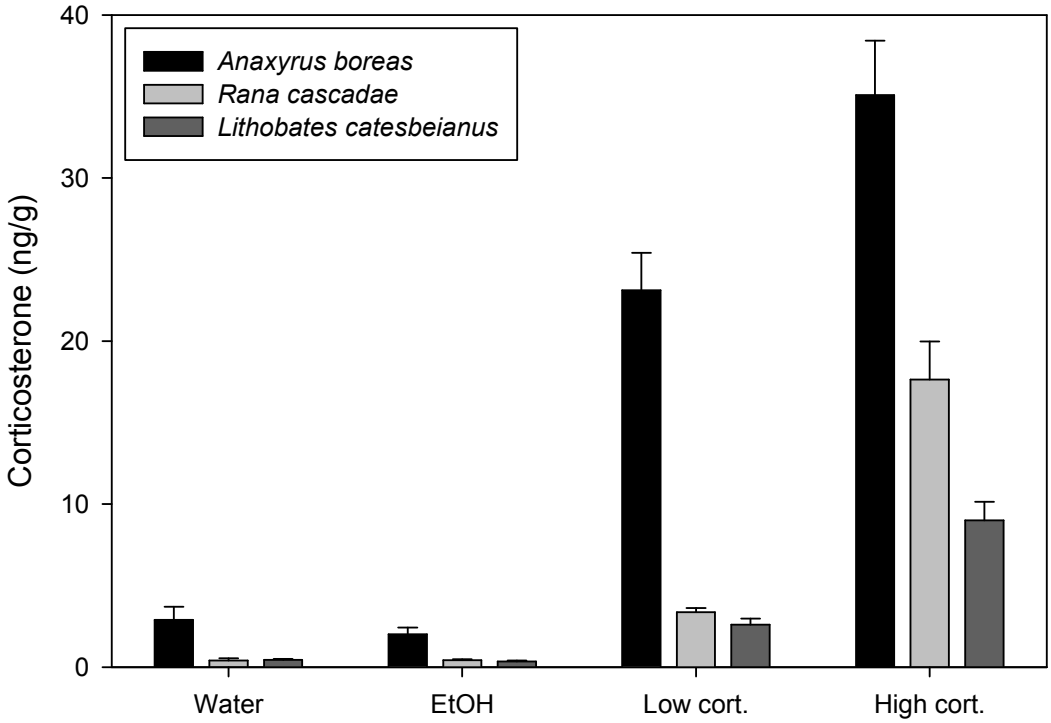


**Figure 2.1:** Whole-body corticosterone levels for all species (+SE). Treatments are shown on the x-axis with both water and ethanol controls (Water, EtOH), low corticosterone (Low cort.; 0.01 μM solution) and high corticosterone (High cort.; 0.1 μM solution). Each bar represents animals pooled from both exposed to *Batrachochytrium dendrobatidis* (Bd) and Bd-control groups (unexposed). Corticosterone treatments successfully elevated whole-body levels of corticosterone. Additionally, we consistently found higher levels of corticosterone in *Anaxyrus boreas* compared to the other two species.

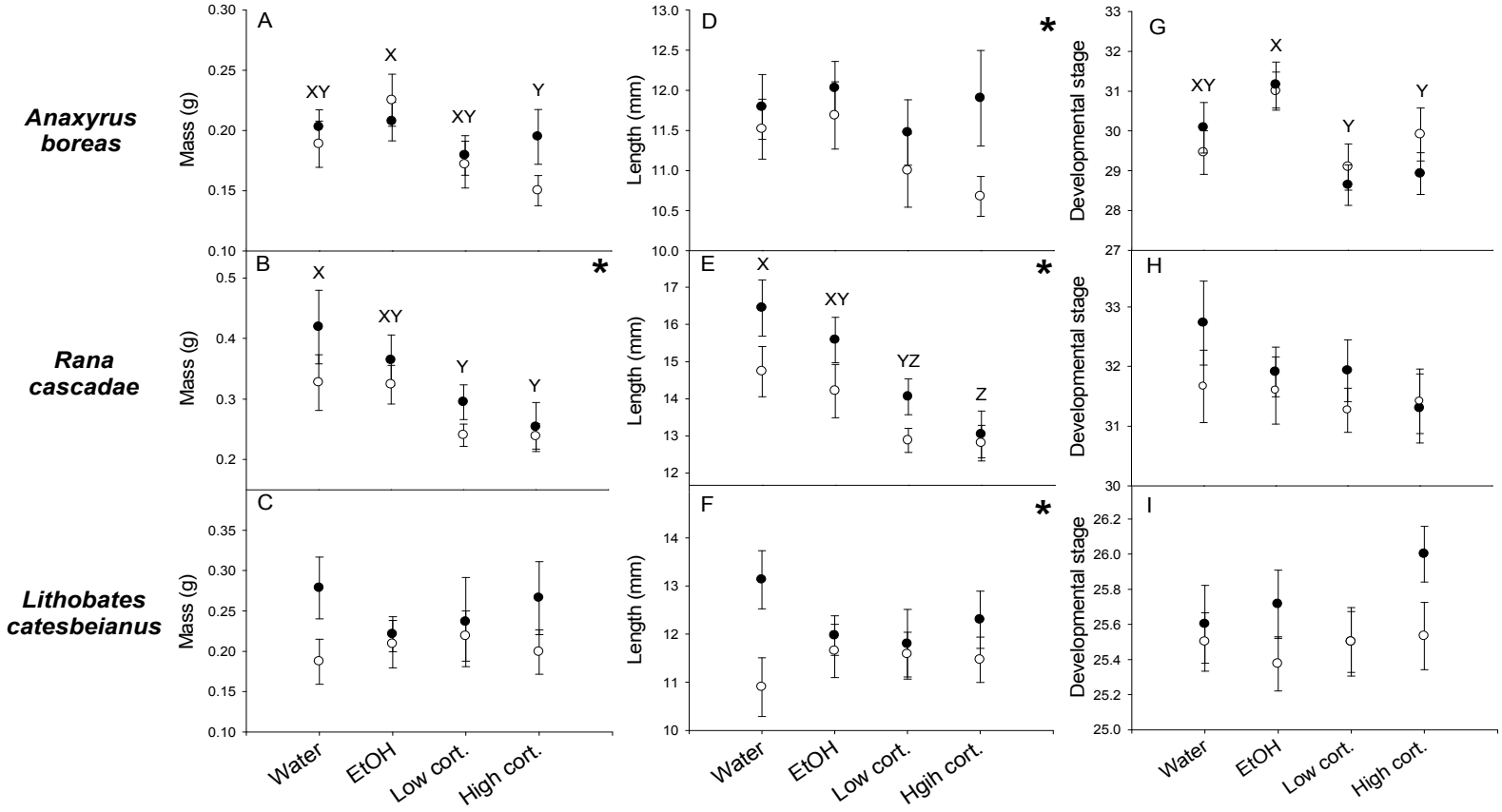


**Figure 2.2:** Effects of treatment on A) *Anaxyrus boreas* mass, B) *Rana cascadae* mass, C) *Lithobates catesbeianus* mass, D) *A. boreas* length, E) *R. cascadae* length, F) *L. catesbeianus* length, G) *A. boreas* developmental stage, H) *R. cascadae* developmental stage, and I) *L. catesbeianus* developmental stage. Open circles represent *Batrachochytrium dendrobatidis* (Bd) control treatments while closed circles represent Bd-exposed treatments. Stars indicate a significant effect of Bd treatment.

# CHAPTER 3

## Experimental examination of the effects of ultraviolet-B radiation in combination with other stressors in frog larvae

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**Abstract**

Ultraviolet-B radiation (UVB) is a ubiquitous stressor with negative effects on many aquatic organisms. In amphibians, ambient levels of UVB can result in impaired growth, slowed development, malformations, altered behavior and mortality. UVB can also interact with other environmental stressors to amplify these negative effects on individuals. In outdoor mesocosm and laboratory experiments we studied potential synergistic effects of UVB, a pathogenic fungus, *Batrachochytrium dendrobatidis* (Bd), and varying temperatures on larval Cascades frogs (*Rana cascadae*). First, we compared survivorship, growth and development in two mesocosm experiments with UVB and Bd exposure treatments. We then investigated the effects of UVB on larvae in the laboratory under two temperature regimes, monitoring survival and behavior. We found reduced survival of *R. cascadae* larvae with exposure to UVB radiation in all experiments. In the mesocosm experiments, growth and development were not affected in either treatment, and no effect of Bd was found. In the laboratory experiment, larvae exposed to UVB demonstrated decreased activity levels. We also found a trend towards reduced survival when UVB and cold temperatures were combined. Our results show that amphibian larvae can suffer both lethal and sublethal effects when exposed to UVB radiation.

## Introduction

In the past 50 years, humans have altered ecosystems more rapidly than during any other time in history (Millennium Ecosystem Assessment 2005) creating environmental conditions that threaten many ecosystems. For example, recent anthropogenic emissions into the atmosphere have reduced stratospheric ozone from historic levels. This depletion of the ozone layer allows more harmful ultraviolet-B radiation (UVB; 280-315 nm) to reach the earth's surface. While the success of the 1987 Montreal Protocol on Substances that Deplete the Ozone Layer has greatly reduced production of ozone-depleting chemicals (Solomon 2004), some estimates suggest that full recovery is not expected to occur until 2068 (Newman et al. 2006). Thus, the impacts of increased UVB exposure will continue for some time.

In aquatic systems, exposure to UVB can kill sensitive organisms or induce sublethal effects (Bancroft et al. 2007, Häder et al. 2007) that could potentially have large impacts on populations. In addition to population effects, UVB can profoundly affect community structure in experimental aquatic systems (Chatila et al. 1999, Danilov and Ekelund 2000). For example, Danilov and Ekelund (2000) found that marine epilithic communities dominated by diatoms in microcosms quickly shifted to a community dominated by cyanobacteria after only 7 days exposure to UVB radiation. Another example is that of Bothwell et al. (1994) who showed that greater amounts of algae accumulate in UV-exposed habitats than in UV-protected environments. UVB radiation inhibits algal consumers (Diptera: Chironomidae) since larval chironomids are

more sensitive to UVB than sympatric algae. Differential sensitivity to UVB between algae and herbivores contributes to counterintuitive increases in algae in habitats exposed to UVB.

The ubiquitous nature of UVB radiation may result in wide-reaching effects on many organisms. Amphibians are one group of organisms for which there are well-documented negative effects of exposure to ambient levels of UVB radiation (reviewed in Croteau et al. 2008). UVB exposure in amphibians can reduce hatching success (eg. Lizana and Pedraza 1998, Häkkinen et al. 2001), cause developmental and physiological deformities (eg. Hays et al. 1996, Starnes et al. 2000, Pahkala et al. 2001, Ankley 2002), alter growth rates (eg. Smith et al. 2000, Pahkala et al. 2003a, Pahkala et al. 2003b), increase pigmentation (Langhelle et al. 1999), alter behavior (eg. Kats et al. 2000) and decrease survival (eg. Nagl and Hofer 1997, Tietge et al. 2001, Blaustein et al. 2005a). However, these effects are species specific, vary with life history stage, and may be context-dependent.

Many studies of the effects of UVB exposure have focused on amphibian embryos assuming them to be the most susceptible life stage. However, a recent meta-analysis by Bancroft et al. (2008a) demonstrates that amphibian larvae are more sensitive to UVB exposure than embryos. Limited data on the effects of UVB radiation on post-hatching stages of amphibians have restricted our ability to ascertain the overall importance of UVB on amphibian populations. Mortality at post-hatching stages may impact the population more significantly than mortality at the embryonic stage (Vonesh

and De la Cruz 2002). Therefore, understanding the effects of UVB on amphibian larvae is an important step in understanding population level processes.

Although exposure to UVB radiation alone can be harmful to amphibians, it may be especially harmful in combination with other stressors. Pollutants (e.g. Hatch and Blaustein 2003, Macías et al. 2007), pathogens (e.g. Kiesecker and Blaustein 1995), and low pH (e.g. Long et al. 1995, Pahkala et al. 2002), can enhance the toxic effects of UVB exposure. Additionally, UVB radiation may increase the negative effects of other stressors. For example, exposure to ambient UVB radiation can increase the occurrence and pathogenic effects of an oomycete (*Saprolegnia ferax*) in amphibian embryos (Kiesecker and Blaustein 1995). Complex interactions involving El Niño events and exposure to UVB radiation may influence the emergence of this pathogen (Kiesecker et al. 2001). Exposure to UVB radiation may make hosts more susceptible to pathogens by compromising immune systems or inducing other stress effects (Tevini et al. 1993). While pathogens and UVB can interact to impact amphibian embryonic development and survival, no studies have examined this interaction in larvae. This is surprising because amphibian larvae are hosts to several emerging pathogens such as water molds (Kiesecker and Blaustein 1995), trematode parasites (Johnson et al. 2002) and the fungus, *Batrachochytrium dendrobatidis* (Longcore et al. 1999), and the forces stimulating the emergence of these pathogens are unknown.

*Batrachochytrium dendrobatidis* (Bd) is an emerging infectious pathogen associated with mass mortality events of amphibians around the world (Lips et al. 2006, Rachowicz et al. 2006, Skerratt et al. 2007). It affects both larval and metamorphosed

amphibians by infecting their mouthparts and keratinized epidermal cells, respectively (Longcore 1999, Piotrowski et al. 2004). Infection by Bd can kill larvae and post-metamorphic stages of amphibians (Berger et al. 2005a, Blaustein et al. 2005b, Carey et al. 2006) and can cause sublethal damage (Parris and Cornelius 2004). Susceptibility to Bd may be context-dependent where changes in temperature (Andre et al. 2008), exposure to water contaminants (Parris and Baud 2004), climate change (Pounds et al. 2006, Bosch et al. 2007), differences in host species (Blaustein et al. 2005b) and differences in Bd strain (Berger et al. 2005a) may alter host-pathogen dynamics. Thus, the combined detrimental effects of both Bd and UVB radiation on amphibian larvae may not be as straightforward as the effects of either stressor acting alone. There have been no investigations of the combined effects of UVB and Bd on larval amphibians which are important in understanding disease dynamics in amphibian populations.

UVB effects on amphibians can also be influenced by abiotic factors such as temperature. Van Uitregt et al. (2007) show an increase in the negative effects of UVB when the embryos and larvae of one species of amphibian are exposed to colder temperatures. If this occurs in other species, larvae in colder habitats or larvae that choose colder temperatures may be at greater risk than those in warmer temperatures. However, many amphibians seek warmer temperatures (Lucas and Reynolds 1967, Brattstrom 1979, Dupré and Petranka 1985, Wollmuth et al. 1987) to speed their development. Warmer temperatures preferred by amphibian larvae are often located in shallow areas of aquatic habitats where levels of UVB are higher (Kirk 1994, Bancroft et al. 2008b). Thus, larval preference to choose warmer temperatures may also expose

them to higher levels of UVB, but this temperature choice may influence the degree to which they are affected.

We conducted a series of experiments to examine the combined effects of UVB radiation and Bd infection, and the combined effects of UVB and temperature on the larvae of Cascades frogs (*Rana cascadae*). *Rana cascadae* are found in the Cascade Mountains of the Pacific Northwest, USA, where they serve an important ecological role as herbivores at the larval stage and carnivores as adults (Jones et al. 2005). Population declines of *R. cascadae* have been observed in some parts of their range (Jones et al. 2005). In nature, *R. cascadae* are continually exposed to a combination of UVB radiation (Blaustein et al. 1994), pathogens (Kiesecker and Blaustein 1995, Fellers et al. 2008) and other biotic and abiotic stressors. Exposure of *R. cascadae* to UVB radiation as embryos decreased hatching success (Blaustein et al. 1994), and as larvae reduced survival (Belden et al. 2003), decreased growth, and increased deformities (Romansic et al. 2009). In laboratory experiments, juvenile *R. cascadae* show reduced survival when exposed to Bd (Garcia et al. 2006). In the only experimental study of the effects of Bd on larval *R. cascadae*, Bd-exposed individuals had malformed mouthparts but did not show greater mortality than control larvae (Blaustein et al. 2005b). These stressors have been examined in isolation, but it is unclear if they have synergistic effects on *R. cascadae*.

Because of the sensitivity of *R. cascadae* to both UVB radiation and Bd, we hypothesized that Bd and UVB may act synergistically to harm *R. cascadae* larvae. To test this hypothesis, we performed experiments in outdoor mesocosms. Outdoor

mesocosms allow animals to be exposed to natural changes in light and temperature to best mimic natural conditions. In addition, we performed a laboratory experiment where we artificially controlled temperature and UVB to determine how temperature interacts with UVB to affect survival and activity in *R. cascadae* larvae.

## Materials and Methods

*Animal husbandry*

We collected *R. cascadae* as larvae (mesocosm experiment 1) or embryos (mesocosm experiment 2 and the laboratory experiment) and reared them in the laboratory in 37.8 L aquaria filled with dechlorinated water. As embryos, animals were maintained with one egg mass per aquarium. Larvae were kept at a density of approximately 200 animals per aquarium and fed a mixture of ground rabbit chow and Tetramin fish food (3:1 ratio by volume). Animals were given weekly water changes in a controlled laboratory environment with a 12:12 light:dark photoperiod at 14.5-15.5ºC.

*Mesocosm experiment 1*

This experiment was conducted from 12 September to 19 November. It consisted of a 2x3 design with larvae exposed to two levels of UVB radiation (shielded and exposed [ambient]) and three levels of Bd (strain JEL197, strain JEL215, and unexposed control) conducted in outdoor mesocosms near Corvallis (Benton County, Oregon; elevation: 71 m). Each treatment was replicated in five randomly assigned mesocosms. We filled 30 mesocosms (1.9 m in diameter) with water to a depth of 52

cm to contain ~368 L of water. Into each mesocosm, we added dried leaves, phytoplankton, rabbit chow, and zooplankton to mimic a natural environment. Mesocosms were left undisturbed for 2 weeks prior to the addition of larvae and application of treatments to allow phytoplankton and zooplankton to establish. At the beginning of the experiment, we measured nitrate (<2mg/L), dissolved oxygen (6.4-8.4 mg/L), and pH (6.2-6.8) of the mesocosm water.

*Rana cascadae* larvae were collected from Potholes wetland (Deschutes County, Oregon; elevation: 2300 m) in August, 2000. Eight larvae (Gosner (1960) stages 29-39) were randomly assigned and added to each of the 30 mesocosms. We controlled UVB exposure by covering mesocosms with transparent plastic filters that either shield or transmit UVB (mylar and acetate, respectively; Hillcor Plastics; 0.127mm thickness). Mylar filters transmit 5% of ambient UVB radiation while acetate filters transmit 80% of ambient UVB radiation though both allow equal levels of other wavelengths to pass through (Blaustein et al. 1994). Filters were attached to the top of each mesocosm. Temperature loggers were placed in three UVB shielded and three UVB exposed mesocosms to record water temperature every 6 hours for the duration of the experiment. Temperatures in the mesocosms ranged from 1-27 ºC during the experiment.

Bd was cultured in the laboratory using standard protocol (Longcore et al. 1999). One day after adding larvae into the mesocosms, we added a single 10cm Petri dish containing JEL197, JEL215 or a Bd-free control of nutrient agar to each mesocosm. Upon visual confirmation of zoospores using a light microscope, each Petri

dish was flooded with 3 mL of mesocosm water and allowed to sit for 3 minutes to allow zoospores to discharge from zoosporangia (Longcore et al. 1999). After 3 minutes, 0.5 mL of the zoospore suspension was collected and analyzed under a hemocytometer to estimate zoospore density of each dish. Petri dishes were then placed in mesh bags approximately 2 cm beneath the surface of the mesocosm water and attached to the sides of the mesocosms with metal clips. Water samples were collected from the mesocosms for the following two days and observed under a light microscope to estimate zoospore densities in the mesocosms.

Three weeks after the addition of larvae to the mesocosms, we counted the number of larvae in each mesocosm to determine larval mortality (percent survival at week 3). We also collected a subsample of 3 larvae from each mesocosm, which were sent to the Zoological Society of San Diego to determine presence of Bd infection in mouthparts through histological examination.

During the experiment, any larva reaching metamorphosis (Gosner (1960) stage 43) was removed and recorded (time to metamorphosis). To determine if treatments had a carry-over effect into post-metamorphic stages, we fed and maintained these animals in the laboratory for 30 days post-metamorphosis to observe survivorship. These animals were placed in individual 15cm Petri dishes with moistened paper towels at 14.5-15.5ºC. Paper towels were changed once a week and animals were fed pinhead crickets approximately twice a week. After 30 days in the laboratory, animals were euthanized in MS-222 and preserved in 70% ethanol.

Survival to week 3, time to metamorphosis, and survival at 30 days past metamorphosis were analyzed with a generalized linear mixed model implemented in R statistical software environment. Mortality data was analyzed using a logit link while time to metamorphosis used an identity link. We nested individuals by mesocosm and tested UVB effects, Bd effects and the interaction of the two variables.

*Mesocosm experiment 2*

This experiment was conducted from 14 July to 31 July. Mesocosm experiment 2 differs from experiment 1 by using a different strain of Bd (JEL274), modifying the inoculation method, and increasing our sample size (in the number of mesocosms and the number of larvae per mesocosm). This experiment is also shorter in duration than mesocosm experiment 1 to determine the short-term effects of UVB and Bd exposure. We used a 2x2 design with 2 levels of UVB (shielded and exposed [ambient]) and 2 levels of Bd (strain JEL274, and unexposed control). Each treatment was replicated in six randomly chosen mesocosms. Mesocosms were filled as described in experiment 1 and left for 3 weeks before initiation of the experiment. We placed a temperature logger into 3 UVB shielded and 3 UVB exposed mesocosms to record temperature every hour. Temperatures in the mesocosm ranged from 19.1-34.2ºC.

We created UVB exposed and UVB shielded treatments using filters as described in experiment 1. UVB levels were measured using a hand-held Solar Light meter with a UVB attachment (meter: PMA21100, probe: 2102; Solar Light Company, Philadelphia, PA) every five days between 11:00 and 13:00. Ambient UVB levels at

midday ranged from 16.6-19.4 μW/cm$^2$. Mesocosms covered with mylar had UVB levels from 1.3-1.9 μW/cm$^2$ at the top of mesocosm water, attenuating to 0.1-0.5 μW/cm$^2$ at 10cm below the water's surface. Mesocosms covered with acetate had UVB levels from 9.6-13.5 μW/cm$^2$ at the top of mesocosm water, attenuating to 0.3-2.0 μW/cm$^2$ at 10cm below the water's surface.

In June, 2006, three egg masses of *R. cascadae* and three egg masses of Pacific treefrogs (*Psuedacris regilla)* were collected from Susan's pond (Deschutes County, Oregon; elevation: 1954 m). We applied Bd treatments by placing either Bd exposed or Bd control *P. regilla* larvae into the mesocosms. Therefore, *P. regilla* acted as our stimulus animals while *R. cascadae* remained our focal animals. To inoculate *P. regilla* larvae, we divided them into 6, 37.8 L aquaria with approximately 80 animals per aquarium and each aquarium was randomly assigned to Bd or control treatments. We used 8 Petri dishes containing Bd strain JEL274 to inoculate the *P. regilla* in each Bd aquaria and 8 sterile agar dishes for each control aquaria. Each dish was flooded with 15 ml distilled water for 20 minutes and this wash was then poured into the aquaria. Wash from three additional Bd dishes were flooded in the same manner and used to determine zoospore densities on a hemocytometer. Stimulus Bd treatment *P. regilla* larvae were exposed to an average of 1,020 zoospores of Bd per liter in their exposure aquaria. Similar methods have been shown to successfully infect both larval amphibians and adults (Berger et al. 2005a, Blaustein et al. 2005b, Carey et al. 2006, Han et al. 2008). We allowed Bd infection to develop for 3 weeks after inoculation before using the *P. regilla* in our experiment.

On 13 July, we added 10 *R. cascadae* larvae (Gosner (1960) stage 24-25) into each mesocosm. The following day, 12 *P. regilla* larvae (Gosner (1960) stage 25-27) confined within mesh bags were added to each mesocosm. Control mesocosms were given 12 unexposed *P. regilla* while Bd mesocosms were given 12 Bd-exposed *P. regilla*. Because Bd is transmitted through water (Longcore et al. 1999), infected *P. regilla* can shed zoospores that pass out of the bags to infect the *R. cascadae*. After 18 days, all animals were euthanized in MS-222 and preserved in 70% ethanol. Length (snout-vent) and weight was measured for each animal. To test for Bd infection, we randomly selected three *R. cascadae* from each mesocosm (if three animals were available) to analyze with quantitative-PCR (qPCR). Additionally, six randomly chosen *P. regilla* were also tested for Bd infection using qPCR. Mouthparts were removed from each animal and DNA was extracted using Prepman Ultra (Applied Biosystems) methods described by Boyle et al. (2004). Extractions were diluted to a 10% solution. Using the methods described by Boyle et al. (2004), we performed qPCR on each sample using an ABI PRISM 7500 Sequence Detection System. Each sample was run in triplicate and any value above 0.1 genome equivalents was considered to be infected.

Length, weight, and survival data were analyzed as described in mesocosm experiment 1. In one UVB-shielded, Bd exposed mesocosm, *P. regilla* larvae escaped from their mesh bags so this mesocosm was excluded from analysis.

*Laboratory Experiment*

To examine the combined effect of UVB radiation and temperature on larvae, we used a 2x2 design in a controlled laboratory environment with 2 levels of UVB (shielded and exposed) and 2 levels of temperature (hot and cold). Twenty water baths were created and randomly assigned a hot or cold treatment. An aquarium heater was placed into the center of each hot treatment bath and turned on at 09:00 and off at 17:00 each day. This created an increase in water temperature during the middle of the day in the hot treatments, and a return to similar temperatures as the cold treatments at night. Into each water bath, we placed 6, 1 L containers filled with 800mL of dechlorinated water to a depth of 11.5cm. A digital thermometer (Fisher Scientific, Traceable Thermometer) was used to measure temperature every 2 hours at the center of the water bath. Containers in the cold water baths averaged low temperatures of 15.2ºC (SD=0.46) and high temperatures of 16.0ºC (SD=0.60) throughout the day. Containers in the hot treatments averaged low temperatures of 15.6ºC (SD=0.36) and high temperatures of 30.5ºC (SD=1.87). Temperatures within the water baths are shown in Appendix B.

Animals from six egg masses of *R. cascadae* were collected from Parish pond (Linn County, Oregon; elevation: 1,130 m) in April, 2007. One larva (Gosner (1960) stages 25-26) was added to each container. UV-B treatments were applied by placing larvae under a mixture of UVB (Q-Panel UVB 313; Q-Panel, Cleveland, OH) and full-spectrum bulbs (Vita-Light; Durotest Corporation, Fairfield, NJ). Lights were turned on from 08:00 to 18:00 each day. In each water bath, 3 animals were randomly assigned a

UVB exposed treatment and 3 were assigned a UVB shielded treatment. Treatments were applied by covering each container with either a mylar or acetate filter as in the previous experiments. UVB levels at the top of the water below acetate filters ranged from 11.8-15.7 $\mu W/cm^2$, whereas UVB levels below mylar filters ranged from 0.2-0.7 $\mu W/cm^2$. These are within UVB levels found in the Cascade Mountains, where *R. cascadae* naturally occur (Bancroft et al. 2008b).

Treatments were applied for five consecutive days. Each day, the containers within each water bath were randomly rearranged to remove possible effects of orientation. At 18:00 on day 5, survival was recorded for all individuals. Fourteen surviving individuals from each treatment were randomly chosen as a subsample of animals for a behavior trial measuring activity levels. Activity levels are closely associated with growth rates, which are extremely important for development to metamorphosis (Werner and Anholt 1993). Thus, if UVB or temperature treatments influence larval activity, this could have profound effects on individuals. The behavior trial was conducted from 09:00 to 13:00 on day 6 after allowing treatments to acclimate overnight to the behavioral arena's temperature of 15ºC. Each larva was placed into a plastic container (20cm long by 30cm wide) filled with water to a depth of 5cm over a grid containing 5x5cm squares. Containers were placed randomly within an observation arena surrounded by black sheets with small openings to allow observers to record tadpole movement without disturbing the animals. After a one hour acclimation period in the new containers, activity levels were recorded by counting the number of

lines crossed by each animal over a total of 4 minutes. Two observers recorded activity levels of each larva 8 times for 30 seconds with 30 minutes between observations.

We analyzed both survival and behavior using generalized linear mixed models to determine the effects of UVB, temperature and the interaction of the two variables. Survival was analyzed with a logit link function while activity levels used an identity link. Individuals were nested by water bath.

## Results

*Mesocosm experiment 1*

After 3 weeks, larvae exposed to UVB had lower survival rates than shielded larvae ($X^2 = 9.27$, df = 1, p = 0.002; Figure 3.1). On average, percent survival to 3 weeks in UVB-exposed mesocosms was 90.0% (SE = 3.0) while those shielded from UVB had an average survival rate of 99.2% (SE = 0.8; Figure 3.1). There was no difference in survival at 3 weeks between Bd treatments ($X^2 = 0.19$, df = 1, p = 0.663) and no interaction between the two variables ($X^2 = 1.99$, df = 1, p = 0.158). Time to metamorphosis and survival to 30 days post-metamorphosis were not affected by either treatment. Active zoospores were seen on all plates when placed into the mesocosm. Few zoospores were seen on water collected from mesocosms on day 2, and none were found on day 3. Histological examination of larval mouthparts (by A. Pessier) indicated that larvae did not develop visible infections in our mesocosms.

*Mesocosm experiment 2*

Exposure to UVB reduced survival in *R. cascadae* larvae ($X^2 = 7.76$, df = 1, p = 0.005; Figure 3.2). On average, 20.0% of animals survived in UVB exposed mesocosms (SE = 9.2) while 56.4% of animals shielded from UVB survived (SE = 10.9). There was no effect of Bd on survival ($X^2 = 0.96$, df = 1, p = 0.327) and there was no interaction between Bd and UVB ($X^2 = 2.47$, df = 1, p = 0.116). Of the animals that survived to the end of the experiment, growth and development were not affected by either treatment, or the interaction between treatments. In our qPCR analysis, all animals tested below the threshold for positive Bd infection.

*Laboratory experiment*

Exposure to UVB reduced survival in *R. cascadae* larvae ($X^2 = 27.99$, df = 1, p < 0.001; Figure 3.3a). Additionally, individuals exposed to UVB experienced reduced activity levels ($F_{1,16} = 41.64$, p < 0.001; Figure 3.3b). Temperature did not have a significant effect on survival or activity levels alone, but there was a trend for an interaction between UVB and cold temperatures on survival ($X^2 = 3.31$, df = 1, p = 0.069).

## Discussion

Exposure to ambient levels of UVB radiation reduced survival in larval *R. cascadae*. In all experiments, *R. cascadae* exposed to UVB radiation had significantly lower survival than those shielded from UVB. This is one of the few experimental

studies to unequivocally demonstrate mortality of amphibian larvae when exposed to ambient levels of UVB radiation (Nagl and Hofer 1997, Tietge et al. 2001, Belden et al. 2003).

Furthermore, in our mesocosm experiments, larvae were exposed to ambient levels of UVB radiation in a close approximation of natural habitat (O'Hara and Blaustein 1985, Hokit and Blaustein 1997). Reduced survival in these conditions suggests that UVB exposure can cause mortality of amphibian larvae in the wild. In mesocosms, as in many natural habitats, larvae had the opportunity to behaviorally reduce their UVB exposure by choosing deeper waters or hiding under leaves. While we did not directly record behavior, we often observed *R. cascadae* larvae near the surface of the mesocosm water where UVB levels were highest. Additionally, mortality in the UVB-exposed treatments suggests that animals did not behaviorally limit their exposure to UVB radiation. This adds to the growing evidence that some species of larval amphibians do not behaviorally avoid UVB radiation, even when it causes them harm (Wollmuth et al. 1987, van de Mortel and Buttemer 1998, Belden et al. 2003, Bancroft et al. 2008b).

Direct comparisons between the mesocosm experiments cannot be made, however, overall survival in mesocosm experiment 1 was much greater than survival in mesocosm experiment 2. These differences could be explained in a number of ways. For example, the experiments were conducted in different years, different months, different durations and with different protocols. Also, larvae in mesocosm experiment 2 were exposed at earlier developmental stages than those in the first mesocosm

experiment. However, UVB exposure increased mortality in both mesocosm experiments.

Although we exposed our animals to Bd in mesocosm experiments, no effect of Bd was found. In experiment 1, animals did not show signs of Bd infection, even though appropriate levels for infection (Carey et al. 2006) were found inside the mesocosm water. It is possible that under our conditions the virulence of Bd was compromised. This could be due to a number of factors including competition with other microorganisms in the mesocosm, changes in temperature or the relative resistance of the host species. Another possibility is that larvae may have carried low levels of Bd infection that were undetectable by histological examination in mesocosm experiment 1. In experiment 2, temperatures in the mesocosms reached over 30 ºC for eight consecutive days (experimental days 7-14) which is high enough to kill Bd in culture (Piotrowski et al. 2004). Therefore, our negative qPCR results may demonstrate the ability of high temperatures to cure Bd infection in aquatic systems (Woodhams et al. 2003). *Rana cascadae* were exposed to Bd in the first 6 days of the experiment, but previous studies by Blaustein et al. (2005b) found no mortality of *R. cascadae* larvae with exposure to Bd, so this species may not be susceptible to Bd infection at this life stage. In a study on juvenile *R. cascadae*, Garcia et al. (2006) tested the combined effects of UVB radiation and Bd, and found increased mortality with exposure to Bd, but no UVB effect or interaction between treatments. However, Bd infection can vary greatly between life stages due to changes in keratinized structures. While larval amphibians are only infected in their mouthparts, juveniles have keratinized epidermal

tissue which makes them susceptible to infection over most of their skin. Varying responses to the separate and combined effects of UVB and Bd infection between life stages of *R. cascadae* highlights the complexity underlying amphibian population declines.

In the laboratory, we found both lethal and sublethal effects on *R. cascadae* when exposed to UVB radiation. While lethal effects can dramatically alter populations, sublethal effects such as reduced activity can also have a substantial impact on larvae in the wild. Reduced activity levels can decrease feeding rates and lead to smaller size at metamorphosis (Skelly and Werner 1990). Lower activity levels may prevent predators from finding larvae (Werner and Anholt 1993), but if pursued by a predator, lethargic larvae may be unable to escape. Therefore, these sublethal effects of UVB may indirectly reduce larval survival and thus reduce population size.

Additionally, we found reduced survival of animals exposed to UVB radiation in colder temperatures. This complements the findings of van Uitregt et al. (2007) who observed similar trends with reduced survival, growth and performance when striped marsh frog (*Limnodynastes peronii*) larvae were exposed to UVB at colder temperatures. Cold temperatures could increase the negative effects of UVB by physiologically reducing a larva's ability to prevent damage caused by UVB. For example, repair enzymes are essential in fixing DNA damage caused by UVB radiation (Pang and Hays 1991). These enzymes work faster at warmer temperatures, so cold conditions may slow an organism's ability to recover after exposure to UVB. Additionally, changes in temperature may influence larval behavior and therefore alter

their exposure to UVB radiation. Amphibian larvae at high elevations are exposed to high levels of UVB and colder conditions compared to those at lower elevations. This combination of environmental factors may act together and could contribute to amphibian population declines at high elevations. UVB can cause mortality in cold temperatures, but even surviving animals may have a decreased ability to cope with additional stressors. Therefore, any UVB-sensitive species occurring in cold climates may be at risk. However, within a habitat, many larval amphibians chose regions with warmer temperatures. While larval preference for warmer temperatures may help mitigate the effects of UVB, warmer regions of water bodies often have higher levels of UVB. Therefore, larval amphibians are forced to choose between high levels of UVB in warm conditions where they are more able to prevent negative effects of UVB, or lower levels of UVB in cold regions where they are less able to cope with UVB. This complicated pattern makes it difficult to predict the threat of UVB in larval amphibians in the wild.

Our study shows that larval amphibians can suffer both lethal and sublethal negative effects of UVB radiation. Future work should continue to investigate the impacts of UVB on amphibian populations by studying all life stages to fully understand its effects on populations. While we did not directly study the effects of UVB at the population level, our results suggest that UVB radiation can impact *R. cascadae* at a life stage that is critical to amphibian populations (Vonesh and De la Cruz 2002). Therefore, investigating only the embryonic or adult stages may underestimate the negative effects of UVB. The cumulative effects of embryonic, larval and adult

mortality from UVB could have larger impacts on populations than predicted by studying one stage alone. The ubiquitous increase of UVB radiation across the globe makes it a potentially major factor contributing to amphibian declines. Therefore, future work should focus on the effects of UVB to better understand how degradation of the ozone layer has affected, and continues to affect aquatic organisms.

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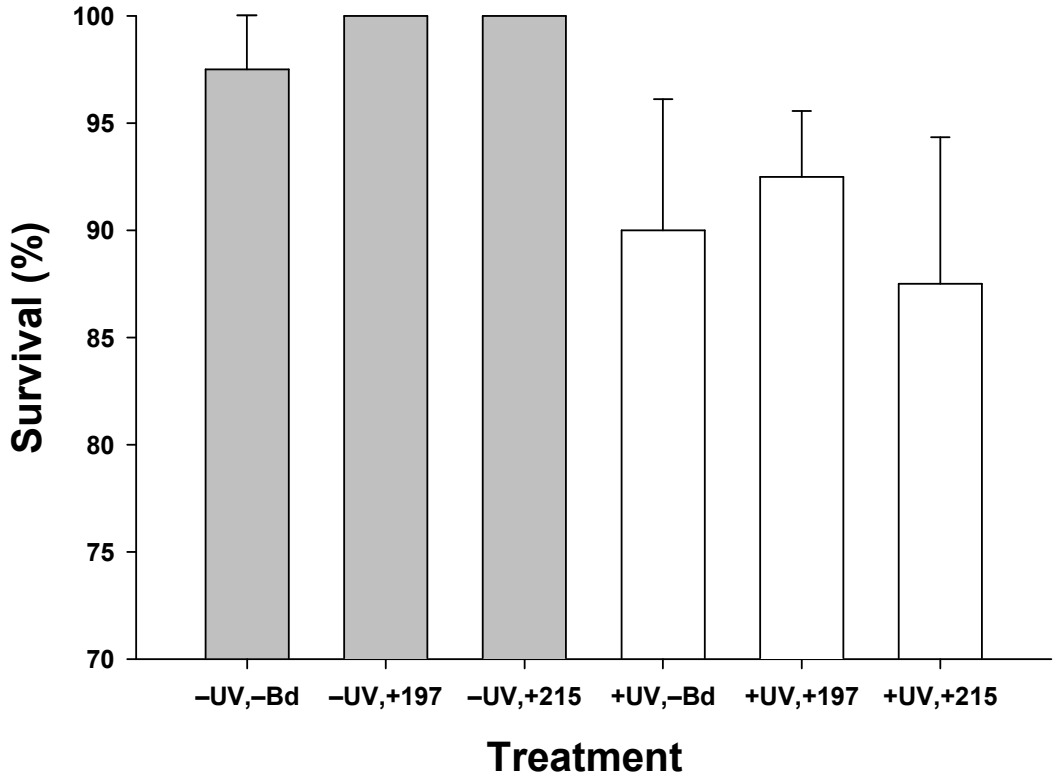


**Figure 3.1:** Percent survival per mesocosm of *Rana cascadae* larvae in mesocosm experiment 1 (mean + SE) shielded (-UV, dark bars) or exposed (+UV, light bars) to UVB. *Batrachochytrium dendrobatidis* treatments are indicated as control (-Bd), JEL strain 197 (+197) or JEL strain 215 (+215). In treatments -UV,+197 and –UV,+215, all mesocosms experienced 100% survival.

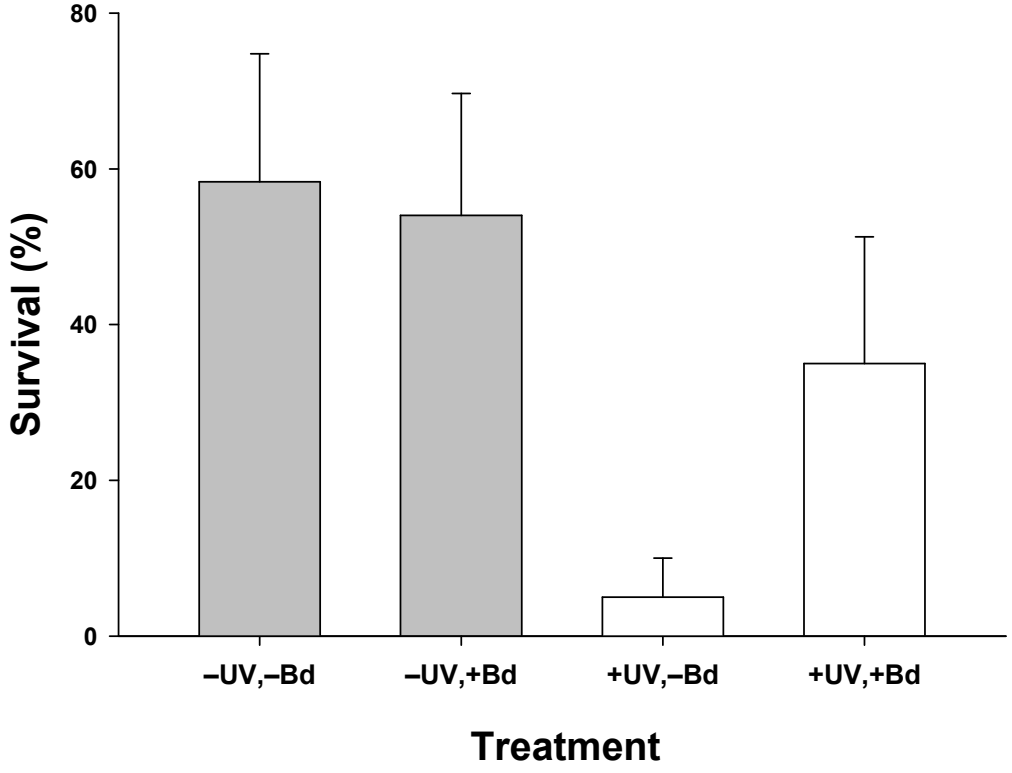


**Figure 3.2:** Percent survival per mesocosm of *Rana cascadae* larvae in mesocosm experiment 2 (mean + SE) shielded (-UV, dark bars), or exposed (+UV, white bars) to UVB. *Batrachochytrium dendrobatidis* treatments are control (-Bd), or exposed with JEL strain 274 (+Bd)

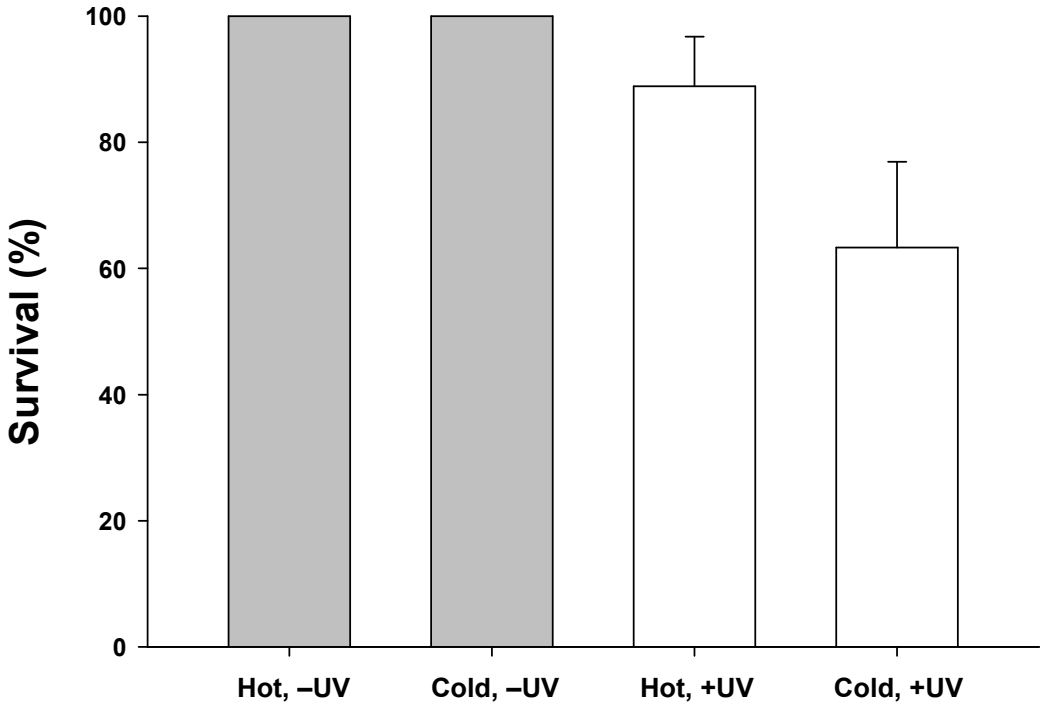


**Figure 3.3a**: a) Percent survival per replicate of *Rana cascadae* larvae after 5 days shielded (-UV, dark bars) or exposed (+UV, white bars) to UVB in hot or cold water in the laboratory (mean + SE). Survival was 100% in the UVB-shielded treatments.

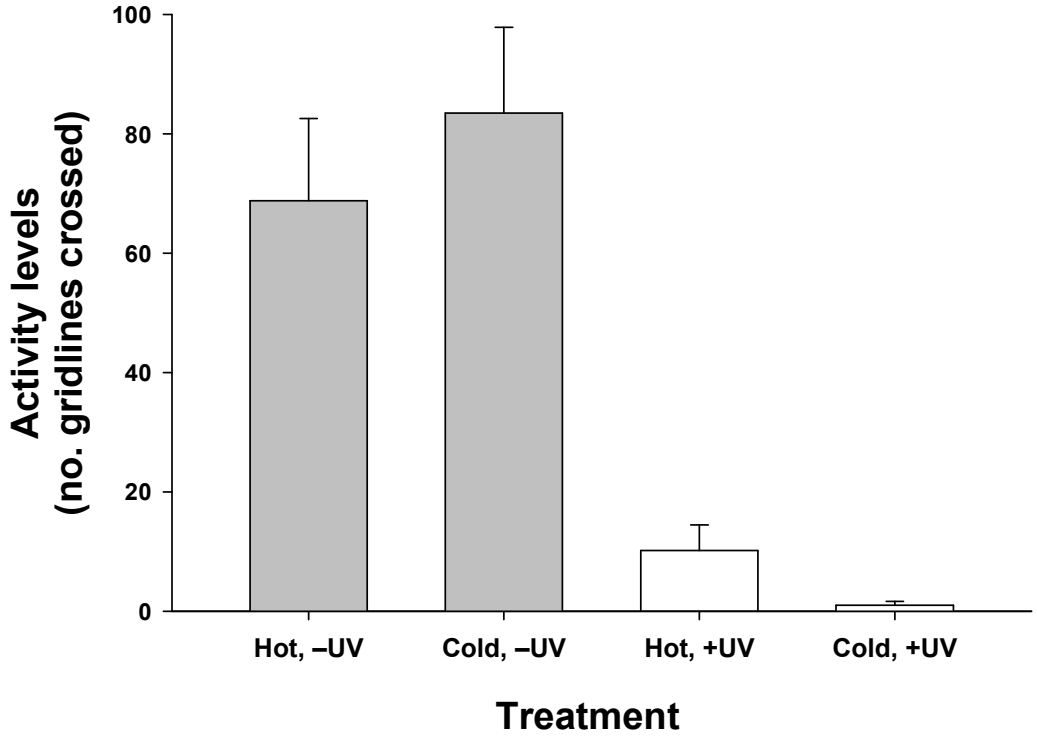


**Figure 3.3b**: Activity levels from behavior trials after 5 days shielded (-UV, dark bars) or exposed (+UV, white bars) to UVB in hot or cold water in the laboratory (mean + SE). Animals were monitored for 30 second intervals eight times. The total number of gridlines each animal crossed during the 4 minute observation time equals its activity level.

# CHAPTER 4

## Differential host susceptibility to *Batrachochytrium dendrobatidis*, an emerging amphibian pathogen

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**Abstract**

The amphibian fungal pathogen, *Batrachochytrium dendrobatidis* (Bd), has received considerable attention due to its role in amphibian population declines around the world. While many amphibian species appear to be affected by Bd, there is little information on species-specific differences in susceptibility to this pathogen. We used a comparative experimental approach to examine Bd susceptibility in six amphibian species from the United States. We exposed post-metamorphic animals to Bd for 30 days and monitored mortality, feeding rates, and infection levels. In all species tested, Bd-exposed animals had higher rates of mortality than unexposed (control) animals. However, we found differences in mortality rates among species, even though species did not differ in the amount of Bd detected on their bodies. Of the species tested, *Anaxyrus terrestris* and *Lithobates sylvaticus* had the highest rates of Bd-related mortality. Within species, we detected lower levels of Bd on individuals that survived longer and found that the relationship between body size and infection levels differed by species.

## Introduction

Understanding patterns of host-pathogen dynamics is essential for mitigating the negative effects of infectious diseases. In many cases, infectious diseases are studied in a one-pathogen, one-host system, with little attention to how pathogens may affect different hosts. However, most pathogens can infect more than one host species (Woolhouse et al. 2001), and hosts may differ in susceptibility to a given pathogen. Differences in host susceptibility can drive important ecological phenomena such as pathogen dilution or pathogen amplification (Schmidt & Ostfeld 2001; LoGiudice et al. 2003; Keesing et al. 2006). Additionally, some hosts may act as reservoirs for a disease. In the absence of a reservoir host, transmission of directly-transmitted pathogens is generally density-dependent (Nokes 1992). Reservoir hosts, however, allow a pathogen to persist even when the density of susceptible hosts is low, exacerbating the severity of disease epidemics (Haydon et al. 2002). Thus, understanding how different host species respond to infection by a pathogen is essential for predicting the effects of a pathogen and identifying species of conservation concern. It is clear that disease poses a substantial threat to biodiversity as exemplified by global amphibian population declines (Stuart et al. 2004).

Global population declines of amphibians have been attributed to multiple factors including infectious diseases (Houlahan et al. 2000; Blaustein and Kiesecker 2002; Stuart et al. 2004). In particular, the emerging fungal pathogen, *Batrachochytrium dendrobatidis* (Bd), has been associated with amphibian population declines throughout the globe (e.g. Lips et al. 2006; Skerratt et al. 2007; Vredenburg et

al. 2010). This fungus infects numerous amphibian species with over 400 infected species documented to be infected (www.parcplace.org/BdMappingUpdate-July2010.html; www.spatialepidemiology.net/Bd-maps). *Batrachochytrium dendrobatidis* infects keratinized structures of amphibians such that the mouthparts of larvae are infected, but post-metamorphic amphibians can be infected over their entire epidermis (Marantelli et al. 2004; Berger et al. 2005b). Therefore, rates of mortality from Bd appear to be more pronounced at the post-metamorphic stage (Parris & Cornelius 2004; Berger et al. 2005a; Carey et al. 2006) when Bd disrupts various physiological functions (Voyles et al. 2009). The effects of Bd on amphibian populations in the wild appear to vary depending on the species and location. For example, mass die-offs associated with Bd have occurred in California and Colorado, USA (Muths et al. 2003; Vredenburg et al. 2010), Queensland, Australia (Berger et al. 1998) and throughout Panama (Lips et al. 2006). In contrast, in other locations including South Carolina, USA (Daszak et al. 2005) and some species in Queensland (Kriger & Hero 2006), populations appear to be persisting in the presence of Bd.

Differential susceptibility to Bd observed in the field may be due to multiple factors including host physiology, host life history, environmental conditions, community structure, or a combination of these factors. For example, in other organisms, body size has been shown to be an important influence on rates of pathogen prevalence and diversity in hosts. Larger animals are predicted to have higher pathogen loads since they are easier targets for a pathogen and usually are older with more time to acquire pathogens (Kuris et al. 1980; Poulin 1995). In amphibians, Kriger et al. (2007)

found that smaller post-metamorphic animals actually had higher levels of Bd than larger animals, but the opposite relationship was found in amphibian larvae in two other studies (Smith et al. 2007; Symonds et al. 2007). Seasonal changes in temperature can alter growth rates of pathogens and host responses creating cyclical patterns in pathogen prevalence (Dowell 2001; Raffel et al. 2006). This may be particularly relevant for Bd that is known to be a cold-associated pathogen (Longcore et al. 1999; Piotrowski et al. 2004). In a field survey, Berger et al. (2004) found a greater incidence of Bd in winter months compared to the summer, and experimentally infecting animals at colder temperatures increased the negative effects of Bd. Life history traits may also influence susceptibility as certain life history strategies may increase rates of transmission or favor pathogen growth. Kriger and Hero (2007) performed a field survey and found that stream-breeding amphibians were more likely to be infected with Bd than pond-breeders and almost all infected animals were found in permanent water bodies. Bielby et al. (2008) analyzed correlates of Bd-associated declines and found that high-altitude species with restricted ranges were most likely to undergo Bd-related declines. Whereas these patterns provide valuable information for predicting future declines, it is difficult to consider all factors in one study. Our laboratory study investigates the fundamental question of which species are negatively affected by Bd in an experimental framework, allowing us to address causation and not just correlation.

The few studies that have comparatively tested amphibians for susceptibility to Bd have done so with few species or using animals in the larval stage. Exposing larvae of four species of anurans (frogs and toads) to Bd resulted in differences in mortality

and mouthpart pigmentation (Blaustein et al. 2005b). Studies that have tested susceptibility to Bd at post-metamorphic stages have only examined three or fewer species (Nichols et al. 2001; Daszak et al. 2004; Garcia et al. 2006). Our study contributes baseline experimental data by testing the susceptibility to Bd of six anuran species shortly after metamorphosis. We monitored mortality, infection levels and feeding rates to determine baseline differences in how these species responded to Bd exposure. Our study provides critical information to help understand the effects of Bd on amphibian individuals and species.

## Materials and Methods

*Animal husbandry*

Animals were collected as eggs from the eastern United States. Upland chorus frogs (*Pseudacris feriarum*) were collected from Seven Island Wildlife Refuge, Tennessee (35ºN, 83ºW). Wood frogs (*Lithobates sylvaticus*) and western chorus frogs (*Pseudacris triseriata*) were collected from the Edwin S. George Reserve, Michigan (42ºN, 84ºW). Gray tree frogs (*Hyla versicolor*) and northern leopard frogs (*Rana pipiens*) were collected from Pymatuning Laboratory of Ecology farm site and Mallard Pond, respectively, Pennsylvania (both 41ºN, 80ºW). Southern toads (*Anaxyrus terrestris*) were collected from Orange County, Florida (29ºN, 81ºW). Eggs were transported to the Pymatuning Laboratory of Ecology in Pennsylvania where they were hatched in 200 L wading pools containing aged well water and fed rabbit chow *ad libitum*. Once the tadpoles achieved the free-swimming stage, they were raised in 100 L

wading pools filled with 90 L of well water, aliquots of pond water, 5 g rabbit chow and 100 g of dried oak leaves (*Quercus* spp.). The pools were allowed to sit for at least 15 days to allow the algal community to develop. Larvae were raised at a density of approximately 25 tadpoles per pool. All pools were covered with 60% shade cloth to exclude predators and prevent animals from escaping. As animals reached metamorphosis, Gosner (1960) stages 42-44, they were removed from wading pools and housed in 1 L containers lined with sphagnum moss until full tail absorption. Post-metamorphic animals were fed pinhead crickets *ad libitum* for 1 to 2 weeks before overnight shipping to Oregon State University (OSU), Corvallis, Oregon.

Upon arrival at OSU, animals were placed in glass terraria in a laboratory maintained at a temperature of 21.5-23.3ºC with a 13:11 light:dark photoperiod. Animals were allowed to acclimate for 24 hours before initiation of the experiment. For each species, individuals were randomly assigned to either Bd-exposed or unexposed (control) treatments. We used a total of 50 individuals for each species (25 per treatment) except for *L. sylvaticus* where 44 animals were used (22 per treatment). Upon initiation of the experiment, individuals were weighed, measured (snout-vent length) and placed into large Petri dishes (140 x 30 mm) with holes in the lid to allow air flow. Petri dishes had a thin film of water covering the bottom of the dish. Animals were able to partially climb the walls of the Petri dish, but could not completely lift themselves off the bottom, keeping them in constant contact with the water. Animals were kept in these dishes for the duration of the experiment (30 days) and fed twice a week with an established number of pinhead crickets based on the average size of the

species (one cricket for every 0.1 g body mass). Due to differences in breeding phenology, we were unable to test all species simultaneously, but all species were treated with identical methods in the same laboratory.

*Experimental procedure*

Animals were exposed to their experimental treatment when they were transferred to individual Petri dishes. We used Bd strain JEL 274 (originally isolated from an *Anaxyrus boreas* in Colorado; Annis et al. 2004) cultured on 1% tryptone agar plates that were made 5-7 days prior to inoculation and held at approximately 22ºC. To harvest Bd from agar, we flooded plates with 15mL dechlorinated water for 5 min. To standardize inoculation dose among exposed animals, we pooled water from at least 10 Bd-inoculated plates for each inoculation and quantified the number of zoospores in the inoculum using a hemocytometer. After quantifying zoospores, we diluted inoculum to create a concentration of $1.0 \times 10^5$ zoospores per mL inoculum. Then, we added 15 mL inoculum to each Petri dish. Thus, Bd-exposed animals were exposed to approximately $1.5 \times 10^6$ total zoospores. Control animals were given a similar treatment except sterile agar plates were used. All species were exposed to the same Bd dose at each inoculation and each inoculation during the experiment was uniformly conducted. We placed an additional 10 ml of water into each Petri dish, which combined with the inoculum was sufficient to completely cover the bottom of the dish with a thin film of water. Animals therefore remained in direct contact with water on their ventral side, but never submerged.

We monitored mortality daily. Dead animals were removed from their dishes and preserved in 95% ethanol. We monitored feeding rates by recording the number of crickets left in each dish 24 h after each feeding. Water in Petri dishes was changed every seven days, with re-inoculation occurring simultaneously.

After 30 days, all surviving animals were weighed, measured, then euthanized in MS-222 and preserved in 95% ethanol. We then determined Bd-infection levels using quantitative-PCR (qPCR), analyzing all Bd-exposed animals and four randomly selected control individuals from each species. Using a sterile fine-tip swab (Medical Wire and Equipment) we swabbed the left ventral surface of each animal ten times over to cover the legs, feet, and drink patch, and placed each swab into a sterile vial. Swabbing was conducted after the animals had been preserved, so both the animals that died during the experiment and those that were euthanized after 30 days were tested for Bd. To extract DNA, we added 60µL Prepman Ultra (Applied Biosystems), heated the vial to 100ºC for 10 min, cooled for 2 min, then extracted the supernatant. Before conducting qPCR, each sample was diluted to a 10% solution. We conducted qPCR on an ABI PRISM 7500 (Applied Biosystems) according to methods by Boyle et al. (2004). Each sample was analyzed in triplicate and the average number of genome equivalents per individual was calculated. If a sample tested positive in only one or two replicates, the sample was re-analyzed. An individual was considered Bd-positive if 3 out of 3 samples (run once) were positive, or if 4 out of 6 samples (run twice) were positive. Additionally, any Bd-exposed animal testing negative for infection was swabbed a second time on their right side (not previously swabbed) and re-analyzed.

*Statistical analysis*

Statistical analyses were performed in *R* statistical computing environment, version 2.9.0, using the *survival* package for survival analyses. To test for differences in size between species, we performed one-way analysis of variance (ANOVA) followed by a Tukey's HSD test on initial mass and length. These comparisons provided baseline information on how initial sizes of species differed.

We used a Cox's proportional hazards model to compare rates of survival among species and treatments. This is a method of survival analysis that allows us to compare the probability of mortality from multiple factors by comparing differences in survival curves (Cox 1972; Parmar & Machin 1995). Cox proportional hazards model provides a hazard ratio which indicates the effect of a factor on the probability of mortality. A hazard ratio greater than one indicates an increase in the probability of mortality, while a value less than one indicates a decrease. Our initial among-species model included Bd treatment, species, and the interaction between these factors. We then selected the model with the lowest Akaike's information criterion (AIC). Additionally, we performed Cox's proportional hazard models on individual species so we could accurately estimate hazard functions for Bd treatment, mass and length within each species.

Infection levels were transformed (log average genome equivalents per individual +1), and then used in an among-species general linear model using predictors of species, mass, length, days to death, and all two-way interactions between these factors. We then selected the model with the lowest AIC. Additionally, to understand

within-species patterns, we performed general linear models on individual species testing for the effects of mass, length and time-to-death on infection levels.

Feeding rates were determined for each animal by calculating the average number of crickets eaten in the 24 h periods after feeding. We used a two-tailed Mann-Whitney U test to compare differences in feeding between control and Bd-exposed animals within each species. We did not compare feeding rates among species because not all species were fed the same number of crickets.

## Results

*Among-species comparisons*

Initial body mass and length differed among species (one-way ANOVA; mass: $F_{5,288} = 580.9$, $p < 0.01$; length: $F_{5,288} = 844.0$, $p < 0.01$; Table 4.1). A Tukey's HSD test revealed that *H. versicolor* and *L. sylvaticus* did not differ in mass or length. Additionally, *P. triseriata*, *P. feriarum* and *A. terrestris* did not differ in mass and *P. feriarum* and *P. triseriata* did not differ in length. All other comparisons among species showed differences in these parameters ($p < 0.05$).

There was greater mortality of animals in the Bd-treatments compared to the controls (Fig. 4.1), but the effects of Bd differed by species. After using AIC to select a Cox's proportional model for survival, our model included Bd treatment and the Bd treatment by species interaction (Appendix C). Including species in the model yielded a similar but slightly higher AIC value. Exposure to Bd increased the hazard of mortality across species by a factor of 3.63 (95% confidence interval: 1.99-6.63, $p < 0.01$).

However, the effects of Bd differed by species (Bd-treatment by species interaction; $p < 0.01$).

When analyzing levels of infection, we found that both time-to-death and mass were associated with Bd infection. We did not detect Bd on any control animals. In contrast, all but two Bd-exposed animals (both *P. triseriata*) tested positive for Bd infection. When these two uninfected animals in the Bd-exposed treatment were re-analyzed a second time, we still failed to detect Bd (i.e. Bd was not detected in any of the 6 qPCR wells of these two individuals). Our all-species general linear model included predictors of species, mass, time to death, species by mass interaction and species by time to death interaction (Appendix C). Animals that survived longer had lower infection levels than those that died earlier in the experiment ($F_{1,289} = 42.94$, $p < 0.01$) and heavier animals had lower levels of infection ($F_{1,289} = 4.25$, $p = 0.04$). There was also an interaction between species and mass ($F_{1,289} = 4.09$, $p = 0.05$; Fig. 4.2), but species did not significantly explain variance of infection levels ($F_{1,289} = 1.43$, $p = 0.23$) and was excluded in a candidate model with a similar AIC value. Average infection levels by species (in genome equivalents Bd [ge], ± standard error) were 174.1 ge (± 31.2) for *A. terrestris*, 133.3 ge (± 31.9) for *L. sylvaticus*, 72.6 ge (± 23.5) for *P. triseriata*, 98.6 ge (± 18.2) for *R. pipiens*, 281.3 ge (± 54.8) for *P. feriarum*, and 57.4 ge (± 14.6) for *H. versicolor*.

*Within-species comparisons*

When we performed Cox's proportional model to compare survival on individual species (Fig. 4.1), all species showed a significant increase in the probability of mortality with exposure to Bd, but with different hazard ratios for each species (*A. terrestris*: hazard ratio = 171.27, $p < 0.01$; *L. sylvaticus*: hazard ratio = 77.80, $p < 0.01$; *P. triseriata*: hazard ratio = 23.18, $p < 0.01$; *R. pipiens*: hazard ratio = 16.69, $p < 0.01$; *P. feriarum*: hazard ratio= 4.92, $p < 0.01$; *H. versicolor*: hazard ratio = 2.99, $p = 0.01$). Thus, *A. terrestris* had the greatest hazard ratio while *H. versicolor* had the lowest (Table 4.1). Four species showed a relationship between mortality and mass where increased mass decreased the hazard of mortality (*A. terrestris*: hazard ratio = 0.98, $p = 0.03$; *L. sylvaticus*: hazard ratio = 0.98, $p = 0.01$; *P. triseriata*: hazard ratio = 0.97, $p = 0.01$, *R. pipiens*: hazard ratio = 0.99, $p < 0.01$). The other two species showed a non-significant effect in this direction (*P. feriarum*: hazard ratio = 0.98, $p = 0.09$; *H. versicolor*: hazard ratio = 0.99, $p = 0.49$). Length did not explain variance in mortality for any species.

Time-to-death explained variance in observed levels of Bd infection in all species, where animals that survived longer had lower levels of infection. (*A. terrestris*: $F_{1,22} = 5.69$, $p = 0.03$; *L. sylvaticus*: $F_{1,19} = 4.75$, $p = 0.03$; *P. triseriata*: $F_{1,22} = 5.22$, $p = 0.03$; *R. pipiens*: $F_{1,22} = 19.68$, $p < 0.01$; *P. feriarum*: $F_{1,22} = 17.26$, $p < 0.01$; *H. versicolor*: $F_{1,22} = 11.54$, $p < 0.01$). Mass explained variance in infection in only two species (*R. pipiens*: $F_{1,22} = 21.35$, $p < 0.01$; *H. versicolor*: $F_{1,22} = 10.79$, $p < 0.01$) with larger animals having lower infection levels in *R. pipiens* and the opposite effect in *H.*

*versicolor* (Fig. 4.2). Body length explained variance in observed infection for *L. sylvaticus* ($F_{1,19}$ =7.30, p = 0.03), where longer animals had lower infection levels. Mass and length were not significant for any other species (p>0.05).

In all species, Bd-exposed animals on average had reduced feeding rates compared to the control animals (Fig. 4.3). However, this effect was only significant in *P. triseriata* (Mann-Whitney: p < 0.01) with trend toward reduced feeding rates in *P. feriarum* (Mann-Whitney: p = 0.06).

## Discussion

Our study represents a step in understanding interspecific differences in amphibian susceptibility to Bd. In all six species we examined, exposure to Bd increased mortality, but the magnitude of this effect differed among species. Species did not differ in infection levels, suggesting physiological or immunological difference in how these species acquire or respond to Bd infection. When an organism is infected by a pathogen, there are two strategies the host can employ to defend itself; resistance or tolerance (Schneider & Ayres 2008; Raberg et al. 2009). Resistance is the ability to limit pathogen burden while tolerance is the ability to limit harm caused by the pathogen (Raberg et al. 2009). In our study, we found that species had similar Bd infection levels but differences in mortality, suggesting that species have similar resistance but dissimilar tolerance to Bd. Since we did not measure infection throughout the experiment it is also possible that species differed in infection levels at different times during the experimental period. Additionally, we found two Bd-exposed

*P. triseriata* that were not infected with Bd even though these individuals lived through the entire experiment and were inoculated with Bd four separate times. These individuals may represent a genotype that is resistant to acquiring Bd or can quickly clear infection. In previous experimental studies, the American bullfrog (*Lithobates catesbeianus*) was unaffected by Bd infection (Daszak et al. 2004) and field studies have identified some species that appear to survive in the wild with Bd infections (Daszak et al. 2005; Kriger & Hero 2006, Goka et al. 2009). Thus, there may be multiple mechanisms by which amphibians can prevent mortality from Bd.

*Anaxyrus terrestris* and *L. sylvaticus* had the highest rates of Bd-related mortality, whereas *P. feriarum* and *H. versicolor* experienced the lowest. Other studies have suggested that toads such as *A. terrestris* may be more susceptible to Bd than other species (Blaustein et al. 2005b; Garner et al. 2009), but these conclusions are not possible without comparative studies where species are tested under the same experimental conditions. Comparative studies allow us to identify conservation priorities and predict which species are likely to suffer most from Bd. As the study of Bd has progressed in the last ten years, researchers have been able to develop models to predict sites of Bd epidemics and declines (Ron 2005; Bielby et al. 2008; Rödder et al. 2009). However, these models have generally focused on environmental factors that affect Bd growth, not host characteristics that lead to declines. Bielby et al. (2008) and Rödder et al. (2009) included life history characteristics of amphibian hosts, but did not incorporate differences in species susceptibility to Bd, likely because these data do not exist for many species.

Within species we found that animals with higher levels of Bd died sooner than those with lower infection levels. This indicates that individuals with higher initial infections died sooner, or that individuals able to reduce their infections survived longer. Bd infection in post-metamorphic amphibians covers the skin and reduces an amphibian's ability to osmoregulate, leading to electrolyte imbalance and death (Voyles et al. 2009). With higher Bd loads, more skin is covered by the pathogen which could lead to electrolyte imbalance occurring faster. Additionally, animals may reach a threshold level of infection where their physiological functions that normally prevent or clear infection are overwhelmed, causing mortality. If animals with higher Bd loads die faster in the field, this could have implications for Bd dynamics. For example, Vredenburg et al. (2010) suggested that mass die-offs of amphibians occur when infection intensity reaches a threshold. If highly infected animals die quickly, it may take longer to achieve this threshold. Thus, understanding levels of infection, rather than simple presence/absence, is important when studying Bd dynamics in the laboratory and field.

The size of a host can have a dramatic effects on the degree to which it is infected by a pathogen (Kuris et al. 1980; Poulin 1995). We found that the relationship between mass and infection levels varied depending on the species; the relationship was positive in some species but negative in others. However, we did not find a relationship between infection load and length, even though length may be an indicator of the surface area available for Bd to infect. Larger animals could acquire greater amounts of Bd since they have more surface area for Bd to infect. In contrast, larger animals may

also be healthier and have more resources available to fight infection which could lead to lower infection levels and longer survival. In previous studies with different species than those used in our study, both positive and negative relationships have been found between body size and Bd infection (Smith et al. 2007; Symonds et al. 2007; Kriger et al. 2007). In our study we found a positive correlation between mass and infection levels in *H. versicolor*, but the other species showed either no effect or a negative relationship between these two factors. Thus, there may be species-specific differences in how size affects infection levels. We also found that exposure to Bd reduced feeding rates in *P. triseriata* with a trend for the same relationship in *P. feriarum*. Reduced feeding could lead to smaller animals which may change how they respond to Bd infection. In the field, amphibian size may be reduced from stressors such as climate change and habitat alteration (Karraker & Welsh 2006; Reading 2006). This could lead to a chain of events where environmental stressors and Bd alter behavior (such as feeding rates), which in turn can affect body size and Bd infection rates.

Exposing amphibians to Bd under identical conditions is essential for understanding basic differences in species susceptibility. However, there are limitations for interpreting the results of our controlled laboratory experiments. For example, we could not account for habitat or behavioral differences of species that may influence susceptibility of amphibians to Bd in nature. Thus, Bd had the greatest negative effect on *A. boreas* compared to the other species under laboratory conditions. However, in nature, *A. boreas* are often found in dry habitats that are suboptimal for Bd growth (Piotrowski et al. 2004), which may decrease their susceptibility to Bd. Additionally,

habitats of these species may differ in humidity and temperature, also affecting Bd growth. Therefore, when incorporating the ability of species to exhibit habitat choice, the effect of Bd on each species may change. Additionally, other factors such as bacteria living on amphibian skin in nature but not in our mesocosm-reared test animals may provide protection from Bd (Woodhams et al. 2006; Becker & Harris 2010). We also only used one strain of Bd to inoculate all species, but species may show differential sensitivity to different Bd strains (Retallick & Miera 2007). It is important to consider these variables when predicting species susceptibility to Bd, but investigating all these factors simultaneously in models or experiments is difficult.

*Batrachochytrium dendrobatidis* has been found in the field on all of the species we tested except for *P. feriarum* (Longcore et al. 2007; Rothermel et al. 2008; Rizkalla 2010). Thus, Bd has been found on the species that were most affected by Bd in our study, indicating that Bd is likely affecting these species in the field. Expanding our study to compare susceptibility among large numbers of amphibians could identify reservoir hosts and help predict patterns of Bd prevalence in different amphibian communities. Additionally, experimental exposures could elucidate patterns of susceptibility that can be predicted by taxonomic classification, body size or geographic location. Although other studies have attempted to do this using field surveys and predictive modeling, experimental manipulation of Bd infections are the only reliable tests of differences in susceptibility. On a large scale, comparative susceptibility studies can identify factors correlated with infection patterns and allow us to better shape conservation efforts instead of managing on a species-by-species basis. Susceptibility

data generated from controlled experiments compliment field surveys, correlational studies and modeling efforts so that conservation strategies can be optimized. Together, these studies provide a complete overview for management and conservation efforts of threatened species.

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**Table 4.1:** Summary information for each amphibian species tested in order of descending hazard ratio, indicating the effect of *Batrachochytrium dendrobatidis* infection on probability of mortality.

| **Species** | **Average mass** (mg: SE)* | **Average length** (mm: SE)* | **Hazard ratio** (SE) |
|---|---|---|---|
| *Anaxyrus terrestris* | 146.8 (2.2) | 11.45 (0.08) | 171.27 (1.11) |
| *Lithobates sylvaticus* | 303.7 (9.4) | 15.16 (0.13) | 77.80 (0.74) |
| *Pseudacris triseriata* | 173.1 (3.8) | 13.20 (0.12) | 23.18 (0.66) |
| *Rana pipiens* | 997.2 (30.4) | 23.39 (0.26) | 16.69 (0.51) |
| *Pseudacris feriarum* | 199.1 (3.8) | 13.14 (0.11) | 4.92 (0.36) |
| *Hyla versicolor* | 267.1 (5.5) | 14.61 (0.11) | 2.99 (0.32) |

*Average mass and length were determined using initial measurements recorded before initiation of the experiment.

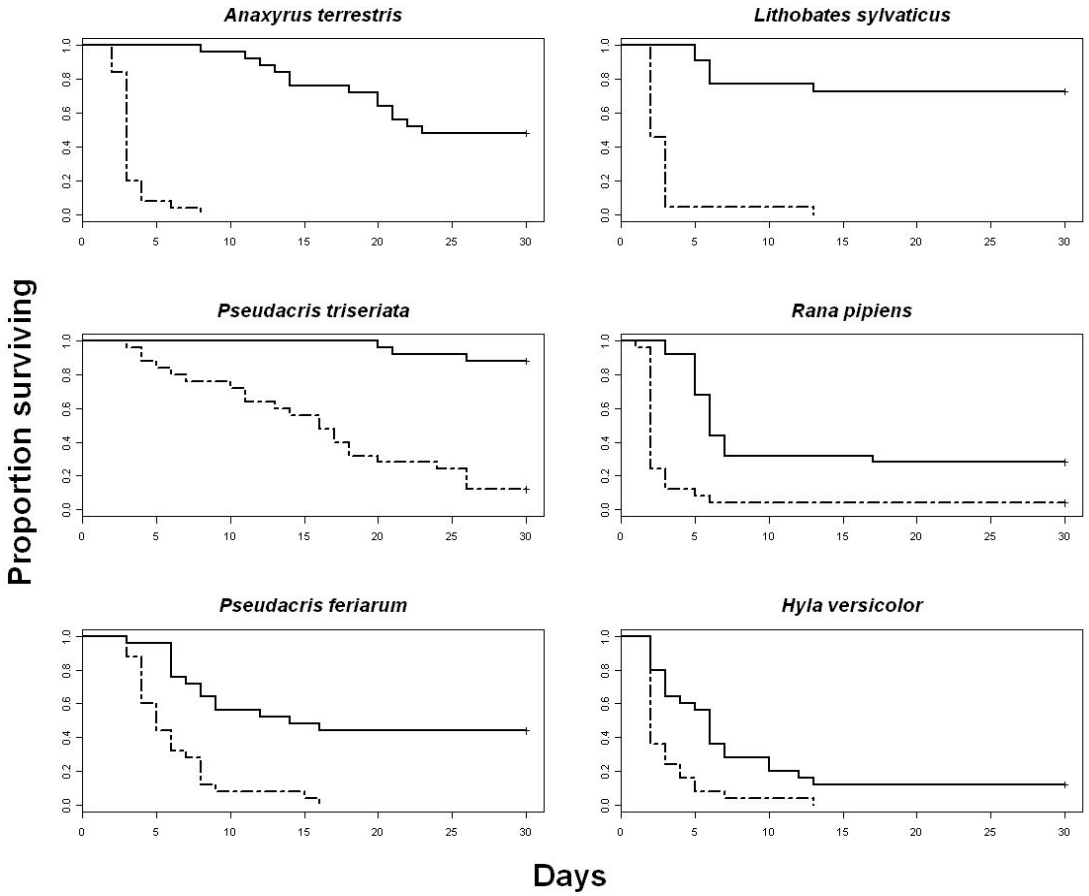


**Figure 4.1:** Survival of *Batrachochytrium dendrobatidis* (Bd)-exposed and control groups for each amphibian species tested. Solid lines represent unexposed animals and dashed lines represent Bd-exposed animals. For each species, half the animals were exposed to Bd while the other remained unexposed. The experiment was conducted for 30 days with mortality monitored daily.

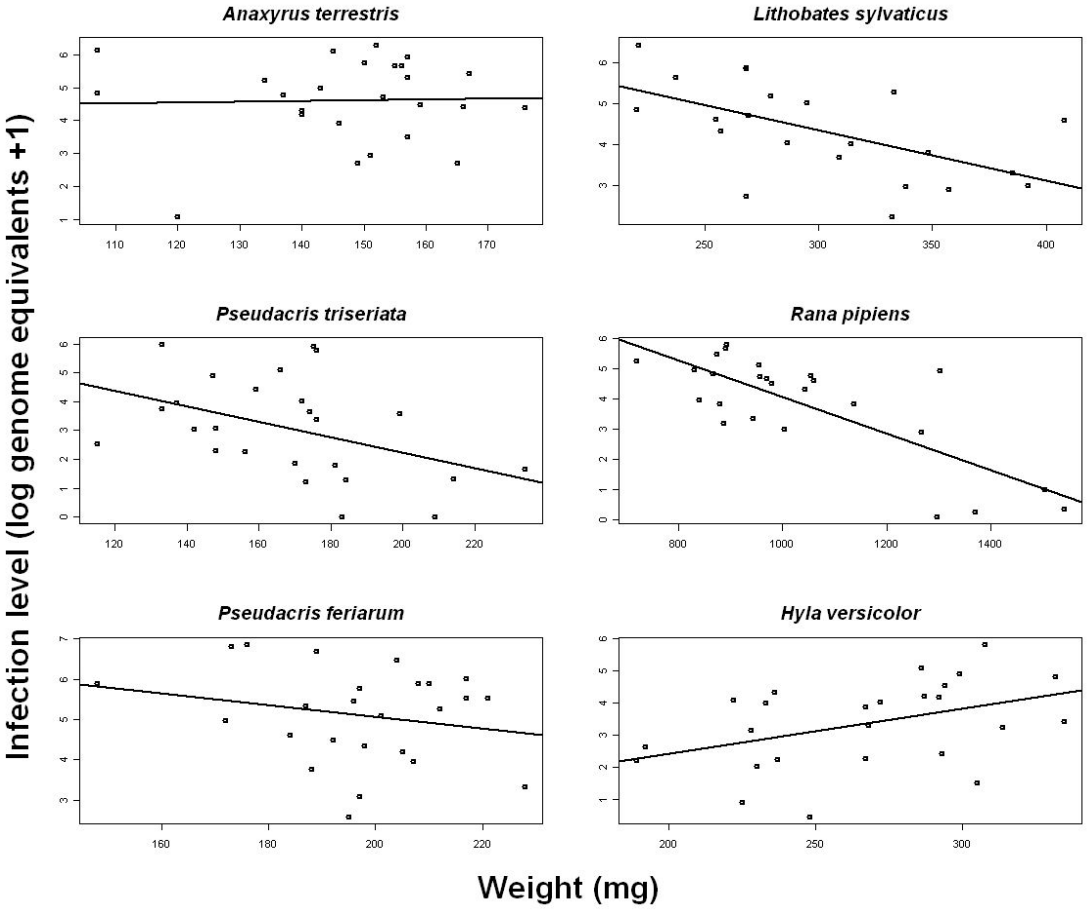


**Figure 4.2:** *Batrachochytrium dendrobatidis* infection severity (log average genome equivalents per individual + 1) of all amphibian species in relation to body mass (mg). Within species, mass was a significant predictor of infection level in *Rana pipiens* and *Hyla versicolor*. *Rana pipiens* exhibited a negative relationship whereas *H. versicolor* exhibited a positive relationship.

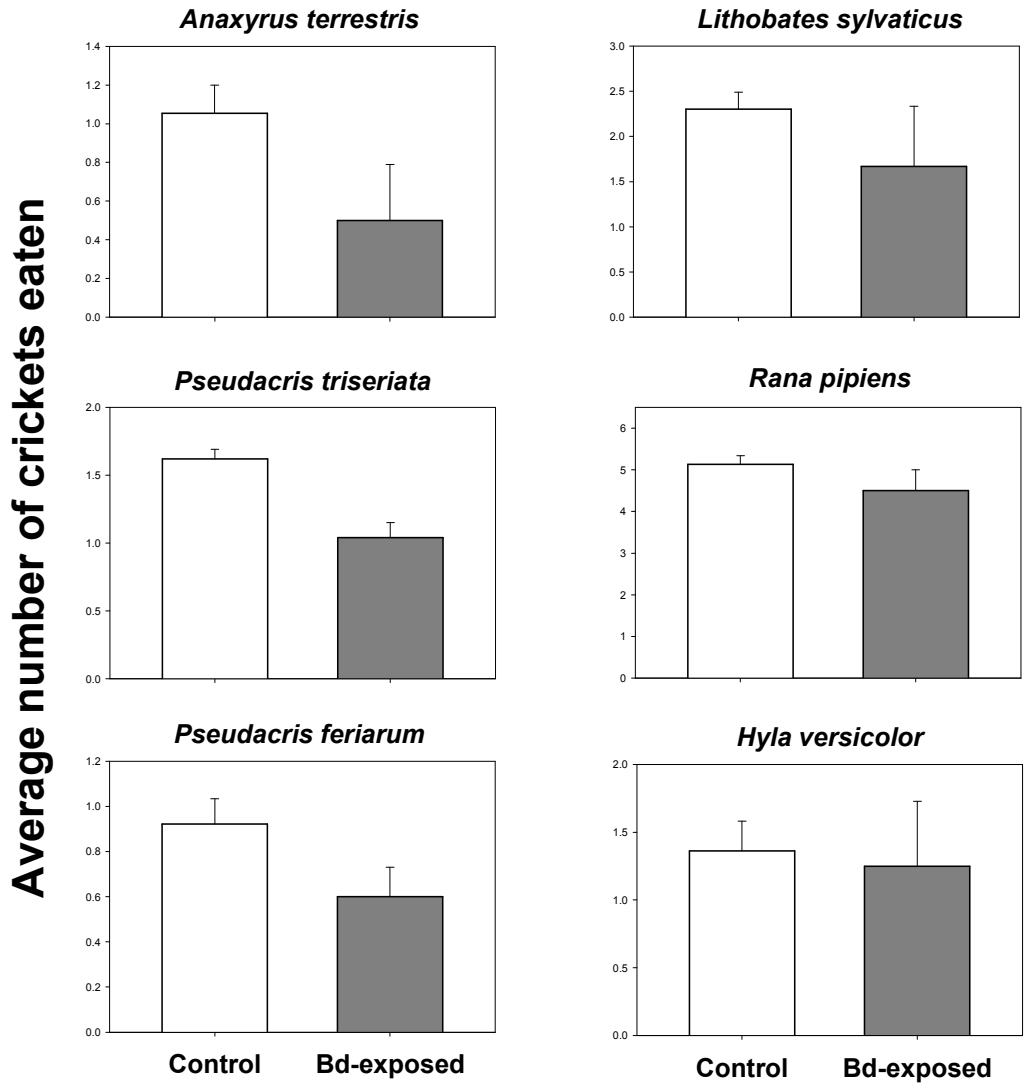


**Figure 4.3:** Average number of crickets eaten at each feeding event for each species. Open bars represent control animals (mean + SE) while solid bars represent Bd-exposed animals (mean + SE).

# CHAPTER 5

## Evidence for a dilution effect in an emerging amphibian pathogen, *Batrachochytrium dendrobatidis*

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## Abstract

The relationship between biodiversity and disease risk is complex and multi-faceted. An emerging infectious disease, chytridiomycosis, caused by the fungal pathogen *Batrachochytrium dendrobatidis* (Bd) is responsible for many amphibian declines worldwide. Despite the continued spread of Bd, its relationship with host diversity is unknown. Yet, this aspect of Bd dynamics is crucial for understanding the emergence and maintenance of the pathogen in ecosystems that differ in biodiversity. We experimentally manipulated host species richness (a measure of biodiversity) and the density of a focal species, the western toad (*Anaxyrus boreas*), in the presence of Bd. We then quantified disease risk in five ways and found a dilution effect where increased species richness decreased disease risk in 4 of the 5 measures. These results demonstrate the importance of incorporating community structure into studies of disease dynamics.

## Introduction

Many scientists argue that we are experiencing the sixth great mass extinction event in earth's history (Wake & Vredenburg 2008). Estimates suggest that extinction rates are between 100 and 1,000 times historic rates (Pimm et al. 1995). Although numerous factors appear to be contributing to the loss of biodiversity, infectious disease plays a prominent role (Daszak et al. 2001; Jones et al. 2008). The relationship between biodiversity and infectious disease is dynamic and often complex, especially in multi-host pathogen systems where host species vary in their ability to acquire pathogens, defend themselves against pathogens, and transmit pathogens to others. Thus, changes in biodiversity that affect host species composition can dramatically alter disease dynamics within a community (Johnson & Thieltges 2010; Keesing et al. 2010).

Changes in biodiversity within a community can have several outcomes on disease risk, which can be defined in many ways including density of infected hosts and prevalence of infection in hosts (Keesing et al. 2006). The dilution effect occurs when biodiversity and disease risk are negatively correlated. This can occur through several pathways including reduced encounter rates between susceptible and infectious individuals, reduced transmission rates, reduced host density, increased mortality of infected individuals, or accelerated recovery of infected individuals (Keesing et al. 2006). The dilution effect has primarily been studied in vector-transmitted pathogens (Schmidt & Ostfeld 2001; Johnson et al. 2008; Thieltges et al. 2008; Allan et al. 2008

but see Hall et al. 2009; Dizney 2009), but the theoretical models predicting dilution effects apply to all pathogen types.

The amplification effect, in contrast, occurs when there is a positive relationship between biodiversity and disease risk. This occurs when low-diversity systems contain poorly competent hosts, and adding species to the community increases the number of hosts that acquire and transmit the pathogen easily. Examples of the amplification effect often involve pathogen spillover where epidemics are driven by transmission from a reservoir population (e.g. Purcell & Saunders 1999; Power & Mitchell 2004). A comparative study in a grassland ecosystem showed the dilution effect to be more common than the amplification effect (Mitchell et al. 2002). Additionally, ecological models predict the dilution effect to occur, and not the amplification effect, if species diversity is non-randomly manipulated (Ostfeld & LoGiudice 2003).

A dilution effect can occur if communities with higher biodiversity contain more competitors or predators, lowering the density of susceptible individuals. Fewer susceptible hosts lead to reduced pathogen transmission and a lower disease risk. In field studies, it is difficult to determine whether dilution effects occur simply from changes in host density or from biodiversity changes. Diversity *per se* does not always affect disease risk, but changes in host density as a result of altered diversity can create the observed patterns (Mitchell et al. 2002). Thus, it is essential to account for host density in addition to biodiversity to understand the effects that each factor has on disease risk. Studies accounting for both are rare.

*Batrachochytrium dendrobatidis* (Bd), is an emerging amphibian pathogen responsible for numerous population declines around the world (e.g. Lips et al. 2006; Skerratt et al. 2007; Vredenburg et al. 2010). It has recently been stated that "The impact of chytridiomycosis on frogs is the most spectacular loss of vertebrate biodiversity due to disease in recorded history" (Skerratt et al. 2007). Although there has been extensive rise in the number of studies, publications, and citations regarding Bd (Ohmer & Bishop 2010) since it was first described (Berger et al. 1998; Longcore et al. 1999), we know very little about its biology in a community context (but see Parris & Beaudoin 2004; Parris & Cornelius 2004). Understanding the relationships between Bd, host diversity, and host density is essential for a better understanding of Bd dynamics and transmission, which is crucial for effective conservation efforts.

We tested the effects of host density and species richness (taxonomic diversity) on disease risk from Bd. Bd infects keratinized cells of amphibians (larval mouthparts and post-metamorphic skin) and appears to cause higher mortality at the post-metamorphic stage (Parris & Cornelius 2004; Berger et al. 2005a; Carey et al. 2006). However, larval amphibians can suffer both lethal and sublethal effects of infection and may act as important reservoirs for Bd, maintaining persistence of Bd even in the absence of post-metamorphic hosts (Rachowicz & Vredenburg 2004; Briggs et al. 2010). We focused our study on western toads (*Anaxyrus boreas*), which are particularly susceptible to Bd at both the larval and post-metamorphic stages (Blaustein

et al. 2005b; Carey et al. 2006). In nature, populations of *A. boreas* have suffered declines associated with Bd (Muths et al. 2003).

In the Cascade Mountain Range of Oregon, USA, *A. boreas* is sympatric with several other potential Bd hosts including the Cascades frog (*Rana cascadae*) and Pacific treefrog (*Pseudacris regilla*; Nussbaum et al. 1983). However, they can also be found in habitats where other amphibians are rare or absent (Nussbaum et al. 1983). *Anaxyrus boreas* larvae aggregate at high densities often in contact with larvae of *P. regilla* and *R. cascadae* (O'Hara & Blaustein 1982; Nussbaum et al. 1983; Bancroft et al. 2008b). Thus, they represent an ideal system to test the effects of biodiversity and density on disease risk.

## Materials and Methods

*Collection and rearing*

To ensure that animals were not infected with Bd, we collected all species as eggs from sites in the Cascade Mountains, OR (USA). Western toad (*Anaxyrus boreas*) and Pacific treefrog (*Pseudacris regilla*) eggs were collected from Little Three Creeks Lake (Deschutes county; elevation 2093m) and Cascades frog (*Rana cascadae*) eggs were collected from Parrish Pond (Linn County; elevation 1130m). Eggs were brought to the laboratory for rearing in 38L aquaria filled with dechlorinated water. Conditions in the laboratory are maintained at 13.5-15.0ºC with a light regime that simulates natural conditions. Upon hatching, animals were kept at densities of approximately 200

larvae per aquaria and fed a 3:1 ratio (by volume) of rabbit chow to fish food (Tetramin).

*Experimental procedure*

Our experimental unit was a rectangular plastic container (8.5 x 20 x 30cm) filled with 2.5L dechlorinated water. We manipulated both the density of *A. boreas* and the number of species present in each container. Our density treatments were containers with two, three, or six *A. boreas*. Species richness was manipulated keeping density constant at six individuals and combining *A. boreas* with either *P. regilla* (3 *A. boreas* and 3 *P. regilla*), *R. cascadae* (3 *A. boreas* and 3 *R. cascadae*), or both (2 *A. boreas*, 2 *P. regilla* and 2 *R. cascadae*). Additionally, each container was assigned randomly either to be exposed to Bd or control (unexposed). Therefore, we had 12 total treatments that were each replicated nine times.

Animals were placed into their experimental containers and given one hour to acclimate before application of their Bd treatment. We cultured Bd (JEL strain 274, originally isolated from *A. boreas* in Colorado) on 1% tryptone agar Petri plates that had been kept at approximately 14ºC for 15 days. We harvested Bd by flooding plates with distilled water and waiting for 30 minutes for zoospores to release into the water. We then pooled inoculum from 20 plates and quantified zoospores with a hemocytometer. This inoculum was then diluted 1:10 and 20mL was added to each experimental container. Five days later, before any mortality had occurred, the animals

were exposed again in the same manner. Combined, these two inoculations added 2.47 x $10^4$ zoospores to each Bd-exposed container. Control animals were inoculated under a similar regime using sterile agar plates.

We monitored containers daily for mortality and dead animals were immediately removed and preserved individually in 95% ethanol. Animals were fed *ad libitum* and water was changed every two weeks. Thirty-five days after initiation of the experiment, we euthanized all animals in MS-222 and preserved them in 95% ethanol. We measured both mass (to the nearest 0.001 g) and length (snout-vent length; to the nearest 0.1 mm) for all individuals. We then extracted mouthparts from all Bd-exposed animals and ten randomly-selected control animals for infection analysis with quantitative-PCR (qPCR). Our qPCR methods followed those by Boyle et al. (2004) except we used 60µL Prepman Ultra (Applied Biosystems) instead of 40 mL in DNA extractions. Extractions were diluted 1:10 and processed in an ABI PRISM 7500 (Applied Biosystems). Each sample was analyzed in triplicate and the average number of genome equivalents Bd per animal was calculated. If a sample tested positive for Bd in only one of three replicates, it was re-analyzed. A sample was considered positive if at least 2 of the 3 replicates were positive. Additionally, each sample was analyzed in a nanodrop ND-1000 UV-vis spectrophotometer (Thermo Scientific) to determine the concentration of DNA in each sample. In this way, we calculated the concentration of Bd in each animal (genome equivalents Bd per nanogram DNA; referred to as "infection severity").

*Statistical analysis*

All infection statistics were preformed using data from Bd-exposed containers only. Our first analyses focused on the effects of density and species richness on our focal species, *A. boreas* using generalized linear models (GLM). Our model analyzed log-transformed toad infection severity with the following predictors: number of *A. boreas*, number of species, and total number of individuals. Individuals were nested by container. We analyzed toad infection severity using all Bd-exposed individuals, and again using only Bd-positive individuals. Reported statistics and graphs include all Bd-exposed animals. We also performed a GLM on the percentage of *A. boreas* infected (toad infection prevalence) using the same predictors.

In addition to infection in our focal species, *A. boreas*, we also determined infection patterns across all species. We tested 3 measurements of disease risk: infection severity for all species, infection prevalence for all species, and the total amount of Bd infection in a container (referred to as "total infection"). We performed analyses on infection severity including all Bd-exposed individuals, and again using only those that tested positive for infection. The statistics and graphs we report include all Bd-exposed animals. To determine total infection, we used the sum of the Bd quantity of all individuals in a replicate (not corrected for mouthpart size). If a replicate contained an individual that died and was not recovered (presumably due to cannibalism), this replicate was not included in the analysis. For these analyses, we performed GLM's with predictors of total number of individuals, number of *A. boreas*

individuals, and number of species present. Individuals were nested by container for analyses of infection severity and prevalence.

We tested the effect of Bd treatment, density/richness treatment, and species identity on mortality using logistic regression nested by container. Mass, length and infection severity among species was compared using a GLM with individuals nested by container. To compare mass and length of *A. boreas* among treatments, we performed a GLM using predictors for number of *A. boreas*, number of species, number of individuals, and Bd treatment (exposed or unexposed). For *R. cascadae* and *P. regilla*, we tested the effects of Bd-treatment and number of species (2 or 3) treatment on average mass and length using a two-way analysis of variance (ANOVA).

## Results

Increased species richness significantly reduced the severity of toad infection ($F_{1,50} = 18.31$, $p < 0.01$; Figure 5.1A, Table 5.1). Neither the number of *A. boreas* individuals nor the total number of individuals were significant predictors of infection. These findings were the same regardless of whether we included Bd-exposed *A. boreas* that tested negative for infection (i.e. with an infection severity of zero) or if we excluded these individuals in the analyses. Additionally, species richness significantly decreased toad infection prevalence ($F_{1,52} = 22.67$, $p < 0.01$; Figure 5.1C, Table 5.1), but it was not affected by the number of *A. boreas* or the total number of individuals.

When incorporating all species, infection severity showed a negative relationship with species richness ($F_{1,50} = 9.86$, $p < 0.01$; Figure 5.1B, Table 5.1), while neither the total number of animals nor the number of *A. boreas* were significant predictors. Again, these findings were the same whether or not we included Bd-negative individuals in the analyses. Infection prevalence was predicted by both species richness and the density of hosts (richness: $F_{1,52} = 22.37$, $p < 0.01$, density: $F_{1,52} = 4.43$, $p = 0.04$; Figure 5.1D, Table 5.1), where more animals and more species led to a reduction in the percent of individuals infected. Total infection, however, followed a different trend. Species richness and total density were not significant predictors of total infection (richness: $F_{1,40} = 0.03$, $p = 0.86$; density: $F_{1,40} = 0.86$, $p = 0.36$). However, containers with fewer *A. boreas* had significantly less Bd ($F_{1,40} = 7.81$, $p = 0.01$; Figure 5.2, Table 5.1).

Species did not significantly differ in infection severity ($F_{2,192} = 1.12$, $p = 0.33$), even though *A. boreas* and *P. regilla* had high Bd loads compared with *R. cascadae* (mean genome equivalents per ng DNA; *A. boreas* = 0.44 [SD = 1.26], *P. regilla* = 0.40 [SD = 0.64], *R. cascadae* = 0.08 [SD = 0.28]). All tested Bd-control individuals (unexposed) were negative for Bd infection.

Mortality did not differ by species ($X^2 = 1.12$, df = 2, $p = 0.57$), Bd treatment ($X^2 = 0.16$, df = 1, $p = 0.69$) or density/richness treatment ($X^2 = 9.23$, df = 5, $p = 0.10$). Mass and length of *A. boreas* was not predicted by Bd treatment or species richness. However, *A. boreas* were heavier and longer when fewer total animals were present

(mass: $F_{2,224} = 16.70$, $p < 0.01$; length: $F_{2,224} = 7.08$, $p < 0.01$) and when there were fewer *A. boreas* (mass: $F_{1,104} = 19.18$, $p < 0.01$; length: $F_{1,104} = 15.10$, $p < 0.01$). For *R. cascadae* and *P. regilla*, mass and length did not differ by treatment. Among-species comparisons revealed that both mass and length differed by species (mass: $F_{2,387}=136.97$, $p > 0.01$, length: $F_{2,387}=151.69$, $p>0.01$). On average, *A. boreas* were the smallest of the three species (mean mass = 65.1mg [SD = 35.2], length = 7.9mm [SD = 1.5]) with *R. cascadae* being the largest (mean mass = 210.7mg [SD = 140.4], length = 12.0mm [SD = 2.6]) and *P. regilla* intermediate (mean mass = 120.5mg [SD = 85.8], length =9.1mm [SD = 2.4]).

## Discussion

Our results demonstrate a dilution effect in Bd. The majority of our disease risk measurements found that increased species richness, and not host density, decreased disease risk in larval amphibians. None of our measures indicated an amplification effect and this system generally appears to follow trends predicted with the dilution effect.

Increased species richness caused a decrease in disease risk for 4 of the 5 measures tested. Specifically, both Bd prevalence and infection severity were reduced in treatments with greater species richness, whether we focused on *A. boreas* alone or all species together. Infection prevalence is an important measure of disease risk, as it may indicate the likelihood of a susceptible host acquiring the pathogens. If fewer

infected individuals are present in a system (as we found in treatments with high species richness), the encounter rate between susceptible and infected individuals is expected to be reduced. Infection severity, on the other hand, may be a good predictor of how strong an infection will become for an individual in a given system. Thus, since both measures of disease risk were negatively correlated with species richness, high-diversity systems may contain fewer infected individuals and those infected will have lower pathogen loads. This relationship between biodiversity and disease risk has been found in other systems (e.g. Schmidt & Ostfeld 2001; Thieltges et al. 2008; Dizney 2009) and there are multiple possible mechanisms that lead to this pattern. However, this pattern has not been reported previously regarding amphibians and Bd, and has rarely been demonstrated in directly-transmitted pathogens (but see Hall et al. 2009; Dizney 2009).

Differential rates of transmission among species may be one mechanism that drives the dilution effect we found. For example, if low-diversity systems contain individuals that are particularly good at transmitting pathogens, a dilution effect will occur. In our study, *A. boreas* may shed more infectious particles (zoospores) than the other species, driving the pattern where containers with only *A. boreas* had higher infection prevalence and severity than containers with more species. In this scenario, *P. regilla* and *R. cascadae* would essentially act as pathogen sinks (absorbing more Bd than they transmit), while *A. boreas* act as pathogen sources (transmitting more Bd than they absorb). Few studies have attempted to quantify transmission rates of Bd among individuals, and none have done so in a comparative framework with multiple species

(Rachowicz & Vredenburg 2004; Rachowicz & Briggs 2007). Thus, more information on transmission rates of Bd among species is necessary to understand how transmission rates can be affected by species richness.

The observed dilution effect could also be caused by changes in behavior or physiology in response to density of conspecifics or the presence of other species. Larval amphibians alter their behavior in response to their biotic and abiotic environment (e.g. Bridges 1997; Relyea 2001). Different densities of conspecifics or presence of other species may alter behaviors such as schooling or foraging, which could change their likelihood of encountering Bd or transmitting it to other individuals. Additionally, density and species richness may affect stress in *A. boreas*, altering their susceptibility to Bd. Stress hormones such as corticosterone affect amphibian immune function (Rollins-Smith & Blair 1993; Rollins-Smith et al. 1997; Belden & Kiesecker 2005) and can be elevated in response to high larval density (Glennemeier & Denver 2002b) and presumably in the presence of interspecific competition. Thus, our manipulations of larval density and species richness could have altered stress hormones subsequently altering *A. boreas* susceptibility to Bd.

If our findings had been driven by changes in host density, we would expect to see a positive relationship between density and disease risk. However, there was no clear pattern between these two factors. Infection prevalence was negatively correlated with host density, but only when focusing on all species. Thus, treatments with more individuals had lower infection prevalence, which was also negatively correlated with

species richness. In contrast, containers with more *A. boreas* had higher levels of total infection, but this was not affected by species richness or total number of individuals. This finding may indicate that there is an upper limit to the amount of pathogen that a single *A. boreas* can harbor and that individuals in our study may have been reaching that limit. Thus, more *A. boreas* hosts allow for more pathogen to be present in the ecosystem. Other species may not exhibit similar upper-limits on infection, which could explain why total number of individuals did not predict total infection.

Across species and treatments, there was no difference in mortality. It is possible that higher doses of Bd or a longer exposure period can cause lethal or sublethal effects. Larval amphibians can suffer mortality from Bd-exposure in some cases (Blaustein et al. 2005b), and can alter behavior and rates of growth and development (Parris & Beaudoin 2004; Garner et al. 2009; Venesky et al. 2010). Additionally, since tadpoles can act as important reservoirs for Bd (Rachowicz & Vredenburg 2004; Briggs et al. 2010), it is essential to understand Bd dynamics in larvae so that we may better-predict dynamics in communities. *Anaxyrus boreas* in lower-density treatments had a higher mass than treatments with more individuals, but this did not affect infection severity. Specifically, *A. boreas* were larger when fewer total animals were present and when fewer *A. boreas* were present, suggesting inter- and intraspecific competition even though animals were fed *ad libitum*.

Our study focused on *A. boreas*, which has suffered population declines throughout much of its range, in some cases correlated with the presence of Bd (Wente

et al. 2005; Fisher & Shaffer 1996; Muths et al. 2003). The results of our study indicate that *A. boreas* may experience the lowest risk from Bd in high-diversity systems. Thus, in developing a conservation plan for *A. boreas* and other species suffering Bd-related declines, it is essential to consider the presence of other amphibian species in predicting the prevalence and severity of Bd infection.

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**Table 5.1**: Summary information for the relationships between disease risk, focal species density (*Anaxyrus boreas*), total species density and species richness.

| Measurement of disease risk | Definition of disease risk | Relationship with all-species density | Relationship with focal species density | Relationship with species richness |
|---|---|---|---|---|
| Infection prevalence (focal species) | Percent of *A. boreas* infected in each container | — | — | Negative |
| Infection prevalence (all species) | Percent of all individuals infected in each container | Negative | — | Negative |
| Infection severity (focal species) | Average infection concentration of *A. boreas* | — | — | Negative |
| Infection severity (all species) | Average infection concentration of all individuals | — | — | Negative |
| Total infection | Sum of Bd infection in each container | — | Positive | — |

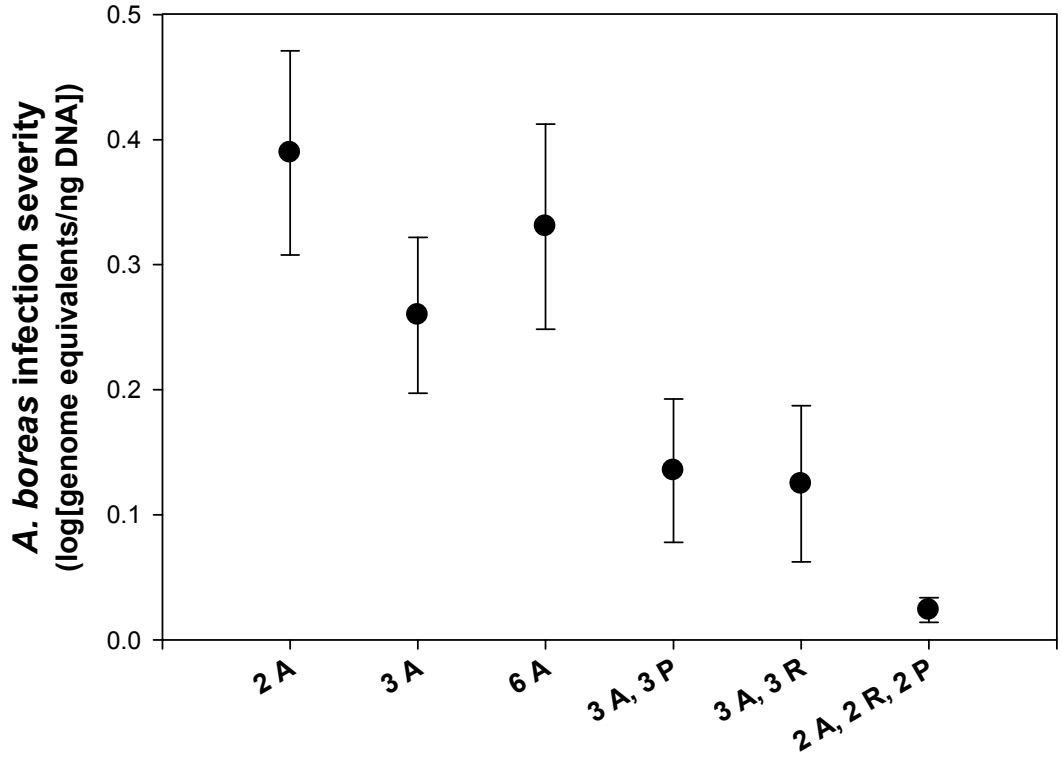


**Figure 5.1a:** Average infection severity of *Batrachochytrium dendrobatidis* (Bd) with varying host density and species richness. There were three *Anaxyrus boreas*-only treatments with 2, 3 or 6 *A. boreas* individuals and three mixed-species combinations with 6 individuals and either 2 or 3 species. Treatments are labeled on the x-axis representing the number of individuals of each species ("A" for *A. boreas*, "P" for *Pseudacris regilla* and "R" for *Rana cascadae*). Points represent the average Bd infection for our focal species, A. boreas (±SE).

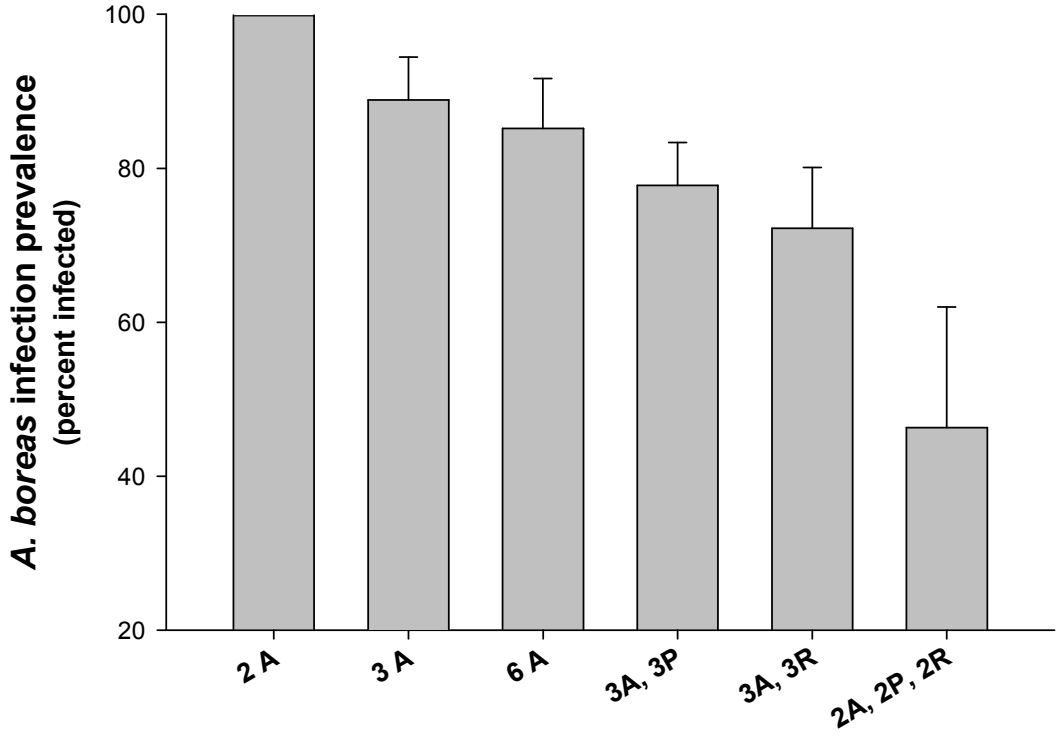


**Figure 5.1b:** Average infection prevalence of *Batrachochytrium dendrobatidis* (Bd) with varying host density and species richness. There were three *Anaxyrus boreas*-only treatments with 2, 3 or 6 *A. boreas* individuals and three mixed-species combinations with 6 individuals and either 2 or 3 species. Treatments are labeled on the x-axis representing the number of individuals of each species ("A" for *A. boreas*, "P" for *Pseudacris regilla* and "R" for *Rana cascadae*). Bars represent the percent *of A. boreas* testing positive for infection (+SE).

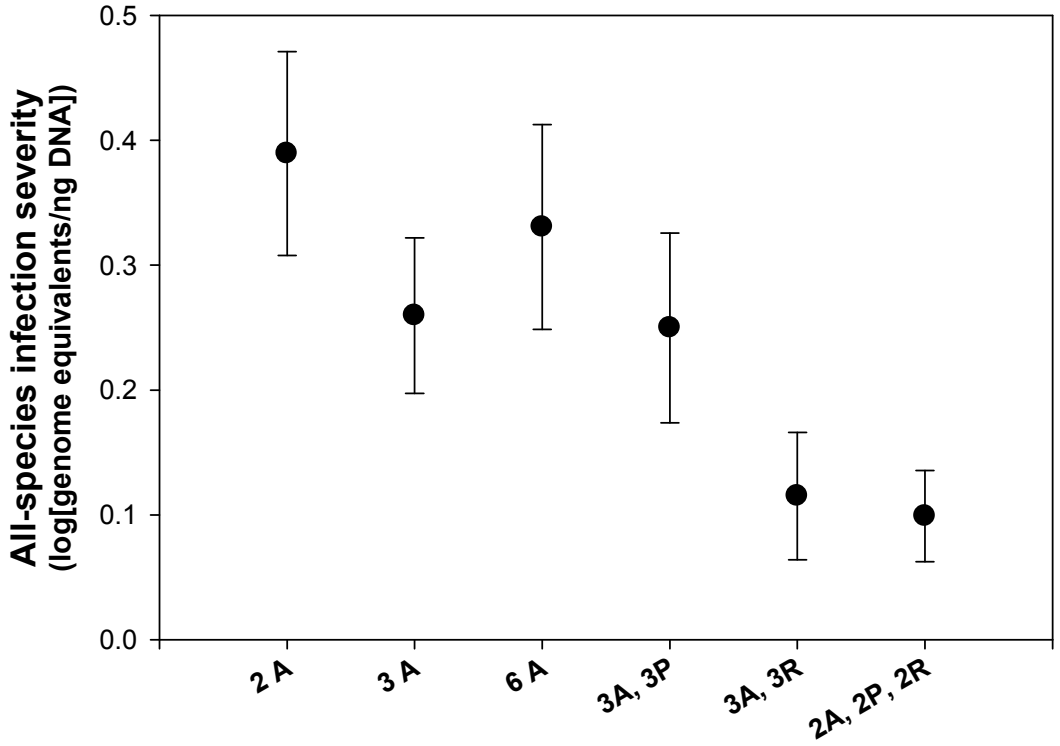


**Figure 5.1c:** Average infection severity of *Batrachochytrium dendrobatidis* (Bd) with varying host density and species richness. There were three *Anaxyrus boreas*-only treatments with 2, 3 or 6 *A. boreas* individuals and three mixed-species combinations with 6 individuals and either 2 or 3 species. Treatments are labeled on the x-axis representing the number of individuals of each species ("A" for *A. boreas*, "P" for *Pseudacris regilla* and "R" for *Rana cascadae*). Points represent the average Bd infection for all species present (±SE).

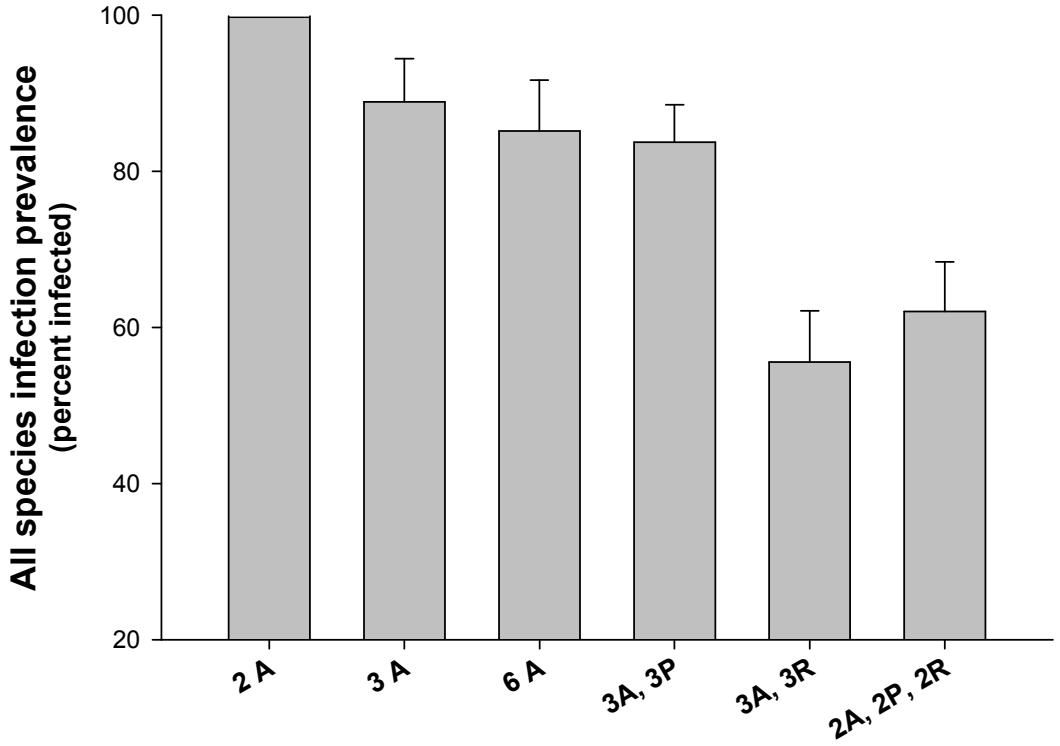


**Figure 5.1d:** Average infection prevalence of *Batrachochytrium dendrobatidis* (Bd) with varying host density and species richness. There were three *Anaxyrus boreas*-only treatments with 2, 3 or 6 *A. boreas* individuals and three mixed-species combinations with 6 individuals and either 2 or 3 species. Treatments are labeled on the x-axis representing the number of individuals of each species ("A" for *A. boreas*, "P" for *Pseudacris regilla* and "R" for *Rana cascadae*). Bars represent the percent infection for all species (+SE).

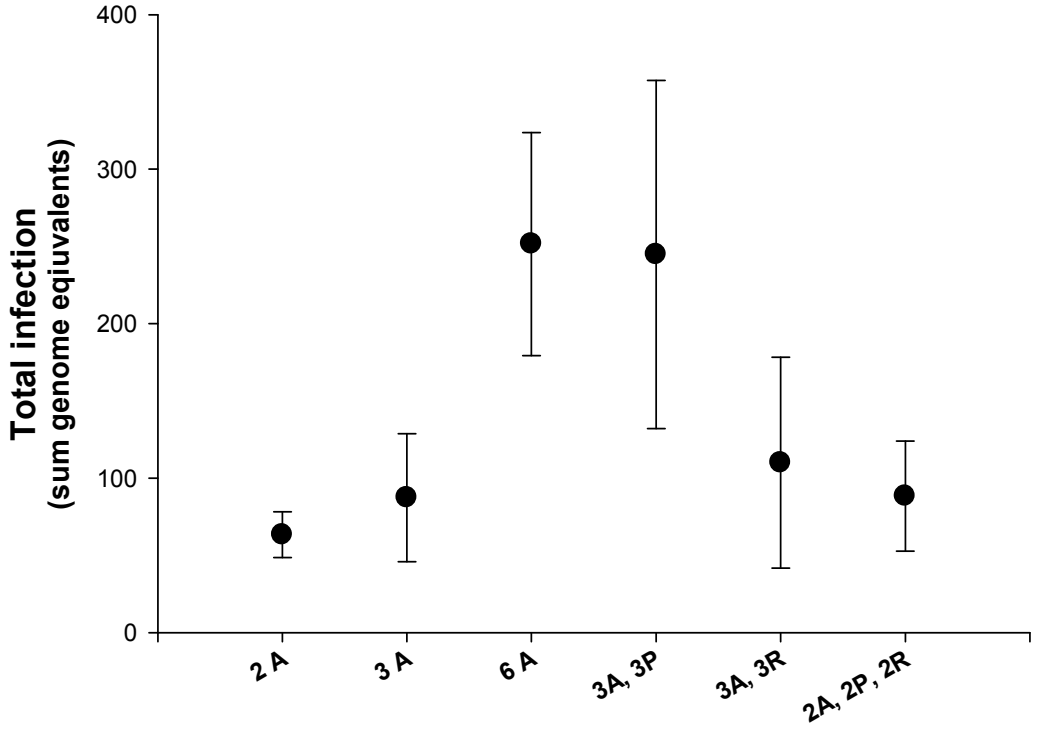


**Figure 5.2:** Average total *Batrachochytrium dendrobatidis* (Bd) infection by treatment. There were three *Anaxyrus boreas*- only treatments with 2, 3 or 6 *A. boreas* individuals and three mixed-species combinations with 6 individuals and either 2 or 3 species. Treatments are labeled on the x-axis representing the number of individuals of each species indicated as "A" for *A. boreas*, "P" for *Pseudacris regilla* and "R" for *Rana cascadae*. Points represent average total infection for Bd-exposed treatments (±SE). Only Bd-exposed treatments were used in calculating averages and each treatment was replicated 9 times.

# CHAPTER 6: CONCLUSIONS

My dissertation explored factors that influence amphibian susceptibility to the emerging infectious disease, *Batrachochytrium dendrobatidis* (Bd). Bd has received considerable attention due to its association with amphibian population declines around the world (e.g. Lips et al. 2006, Rachowicz et al. 2006, Vredenburg et al. 2010). Globally, instances of population declines and die-offs from Bd have increased in frequency and magnitude (Fisher and Garner 2007, Skerratt et al. 2007). It is likely that both anthropogenic introduction of Bd into naïve host populations and environmental changes that shift host-pathogen dynamics are contributing to the increase in Bd-associated population declines. Additionally, there may be species-specific and context dependent effects of Bd on amphibian populations. This has been demonstrated by the observation that populations and species in some locations experience mass die-offs from Bd (Lips et al. 2006, Rachowicz et al. 2006, Vredenburg et al. 2010), while species and populations in other locations persist in the presence of Bd without any identifiable negative effects (Daszak et al. 2005, Kriger and Hero 2006). My dissertation investigated the factors that influence susceptibility to Bd to better understand the impacts of global changes on Bd dynamics.

Although it has been hypothesized that environmental stressors may make hosts most susceptible to Bd (Fisher et al. 2009, Rachowicz et al. 2005), my research showed no evidence for this hypothesis. In Chapters 2 and 3, I tested the effects of stress (hormonal and ultraviolet-B radiation [UVB]) on Bd infection and did not find any

interactive effects of these stressors and Bd. In Chapter 2, I demonstrated that exposure to the stress hormone, corticosterone, did not increase Bd infection in larval amphibians. This result was consistently found in three species of amphibians, which varied in their susceptibility to Bd. In Chapter 3, I tested the effects of the environmental stressor, UVB, on Bd infection and again found that stress did to amplify the negative effects of Bd. However, although there was no interaction between stress and Bd infection, both Chapters 2 and 3 demonstrated that stress (either hormonal or in the form of UVB) can have negative effects on larval growth, development, and survival. Therefore, environmental stressors are likely to be contributing to amphibian population declines, even if they do not increase their susceptibility to Bd. Additionally, stressors may affect Bd dynamics by altering other factors such as transmission rates, recovery rates, and growth rates of the host and pathogen, which may not have been detected in laboratory or mesocosm settings. Thus, even though we found no evidence for an interaction between Bd and environmental stressors, anthropogenic changes that create physiological stress have the potential to affect amphibian-Bd dynamics and contribute to amphibian population declines.

My research also showed that species identity and changes to community structure can dramatically affect Bd infection. In Chapter 4, I demonstrated that Bd reduces survival at the post-metamorphic stage, but species differ in the degree to which Bd affects their survival. Many studies have hypothesized that susceptibility to Bd varies by species, but the majority of these studies have used field surveys which are confounded by differences in habitat choice and life history traits among species. My

study, comparing susceptibility under identical laboratory conditions, demonstrated that physiological differences among species may cause variable susceptibility in the field. Additionally, the relationship between body size and infection varied by species; sometimes the relationship was positive and other times it was negative. This further indicates that species vary in their relationship with Bd and how they respond to infection. In Chapter 5, I found a dilution effect in Bd where increased species richness led to a decrease in disease risk, even when taking host density into account. My focal species for this study, Western toads (*Anaxyrus boreas*), a declining amphibian species, has been identified as a species that is particularly susceptible to the negative effects of Bd infection (Blaustein et al. 2005b, Carey et al. 2006). My research indicates that, in the laboratory, *A. boreas* experience lower Bd risk in communities that contain other host species compared to single-species systems. Thus, maintaining diverse ecosystems may be a key management strategy to mitigate the negative effects of Bd on *A. boreas* and other species in the wild. This study also highlights the need to study emerging infectious diseases in a community context.

Comparing the effects of Bd across studies, I found that Bd appears to have a greater effect on survival at post-metamorphic stages compared to larval stages. In Chapters 2, 3 and 5, where I used amphibians at the larval stage, I did not find evidence that Bd reduced survival. However, in Chapter 4, where I used post-metamorphic amphibians, all species experienced higher mortality in individuals that were exposed to Bd compared to unexposed individuals. Previous studies have demonstrated that Bd can cause mortality at the larval stage (Mendelson et al. 2004, Blaustein et al. 2005b),

but this may only occur with high doses of Bd or under particular environmental conditions. However, it is still important to understand infection at the larval stage, as larvae can carry their infections through metamorphosis and transmit Bd to post-metamorphic amphibians (Rachowicz and Vredenburg 2004, Briggs et al. 2010).

In multiple chapters, I found that species differed in their responses to stressors and their relationship with Bd. In Chapter 2, species varied in the amount of corticosterone in their bodies and how their growth and development was affected by Bd infection. In Chapter 4, comparing among seven amphibian species, I found large differences in the susceptibility of post-metamorphic amphibians to Bd. Some species suffered high mortality from Bd while others were more tolerant of infection. This study also identified that species varied in their relationship between body size and infection. Finally, in Chapter 4, I found that the presence of other species altered infection in larval toads, even when density was held constant, indicating differences in the way that species acquire or transmit Bd. This variation among species found throughout my dissertation indicates physiological or behavioral differences in how species respond to their environment and to pathogen infection. This highlights the need for comparative studies to thoroughly understand of the ecology of amphibians and their pathogens.

The ecology of infectious diseases is often multifaceted and context-dependent. A pathogen that causes mortality in one species may have little to no effect on another species. Additionally, the effects of a pathogen in one location may vary from effects in other locations. Thus, understanding the complex relationships between infectious

diseases, amphibian hosts and their environment is essential for a complete picture of host-pathogen dynamics. My dissertation illustrated that biotic factors (including species identity and community structure) can be important in determining disease risk for a species and community. As humans increasingly alter our natural systems, we may continue to introduce novel pathogens to naïve hosts and alter communities in ways that affect disease dynamics. Thus, it is increasingly important to understand host-pathogen relationships and how our actions impact these systems. Studying infectious diseases at the community and ecosystem level is essential for understanding the dynamics of emerging infectious diseases and for developing policies to mitigate their negative effects.

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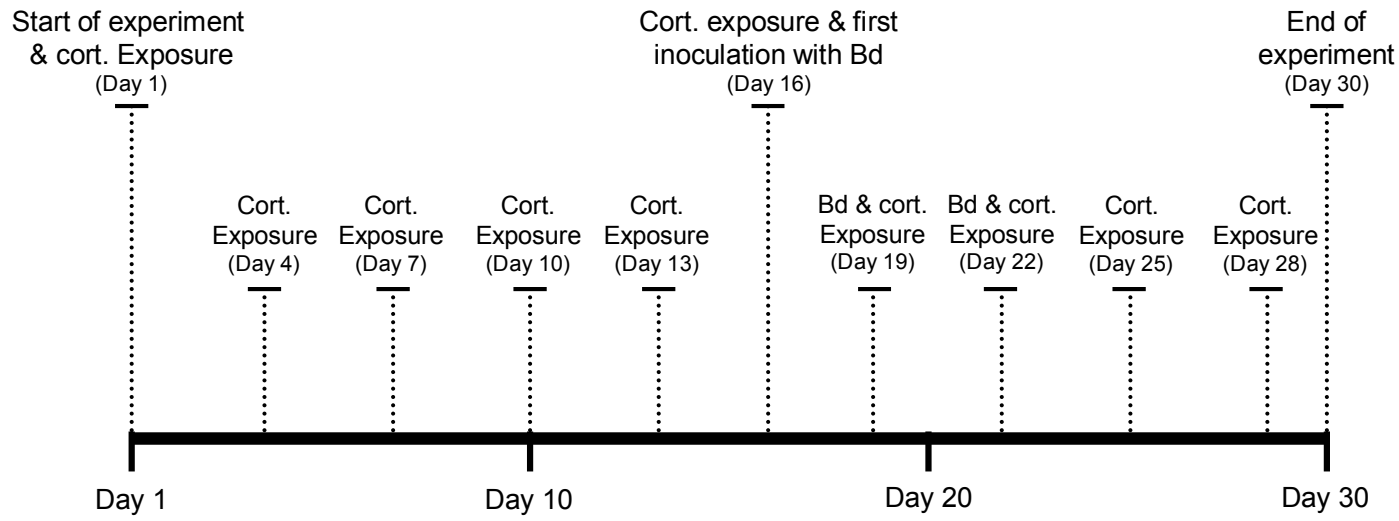
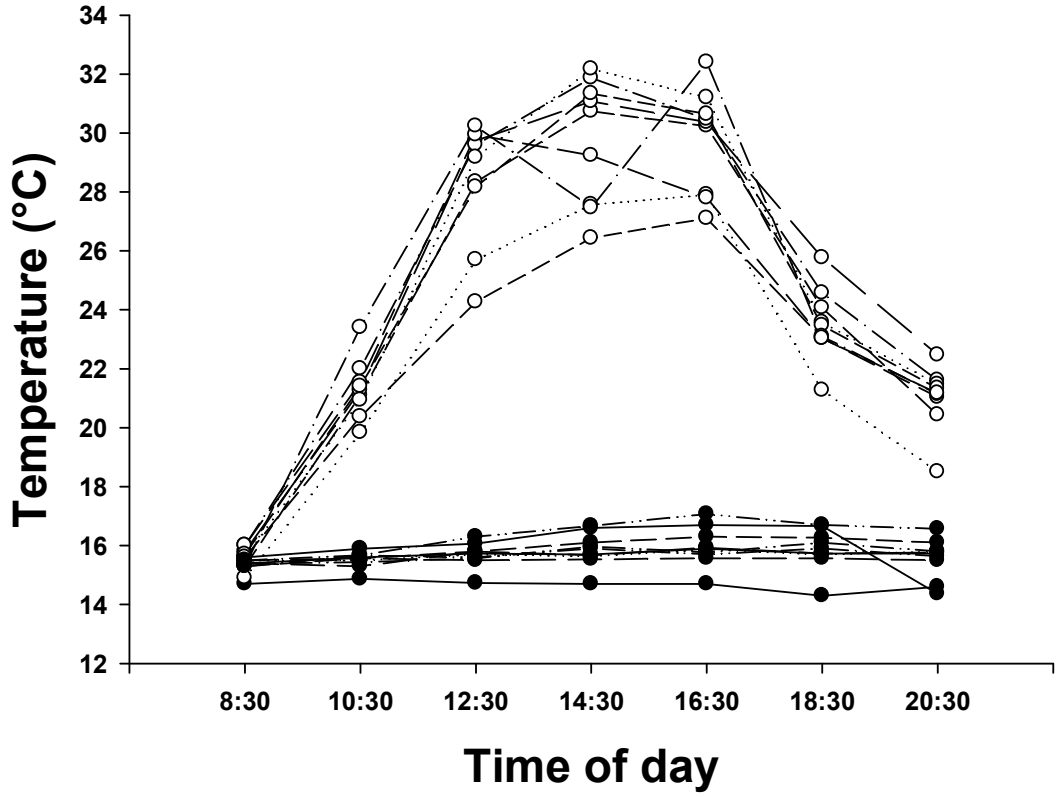
# APPENDICES




**Appendix A**: Timeline of corticosterone and *Batrachochytrium dendrobatidis* (Bd) exposure treatments. Animals were exposed to corticosterone ("cort.") treatments every three days throughout the experiment. Bd treatments were administered three times, starting on day 16 of the experiment.




**Appendix B:** Temperatures from the middle of the water baths on day 4 of the laboratory experiment (n=20) in Chapter 3. Open circles represent water baths in the hot treatments and closed circles represent cold treatments. Heaters were turned on at 9:00 each morning and off at 17:00 each day.

**Appendix C:** Candidate models to explain a) mortality among species and b) infection among species.

| **Model predictors**[a] | **AIC** | **$\Delta_i$**[c] |
|---|---|---|
| a) Among species mortality | | |
| Bd + species +Bd *species | 2121.0 | 0.5 |
| Bd + species | 2121.5 | 1.0 |
| **Bd + Bd *species** | **2120.5** | **0.0** |
| Species + Bd *species | 2137.1 | 16.6 |
| Bd | 2134.5 | 14.0 |
| Species | 2255.4 | 134.9 |
| Bd*species | 2137.1 | 16.6 |
| b) Among species infection[b] | | |
| Species + mass + length + days + species*mass + species*length +species*days | 522.6 | 2.3 |
| Species + mass + length + days + species*mass + species*days | 522.5 | 2.2 |
| Species + mass +days + species*mass + species*days + mass*days | 521.8 | 1.5 |
| **Species + mass + days + species*mass + species*days** | **520.3** | **0.0** |
| Mass + days + species*mass + species*days | 521.6 | 1.3 |
| Mass + days +species*mass | 522.0 | 1.7 |
| Mass + days + species*days | 522.0 | 1.7 |

[a] "Bd" indicates Bd treatment (exposed or unexposed); "species" indicates the species identity; "mass" and "length" indicate measurements recorded at the initiation of the experiment, and "days" indicates the number of days until death for each individual.
[b] Only models with the seven lowest AIC values for infection are shown in this table
[c] $\Delta_i$ indicates the differences in AIC values between the selected model and the candidate model. Smaller values indicate models that are more similar to our chosen model.