AN ABSTRACT OF THE DISSERTATION OF

Paul W. Bradley for the degree of Doctor of Philosophy in Environmental Sciences presented on November 12, 2015.

Title: Disentangling the Biotic and Abiotic Drivers of the Amphibian Disease Chytridiomycosis.

Abstract approved:

______________________________________________________
Andrew R. Blaustein

The ongoing worldwide loss of biodiversity has been described as a “biodiversity crisis,” “the Anthropocene defaunation,” and alternatively “an extinction spasm.” More recently, many scientists have come to the conclusion that we are witnesses to Earth’s sixth major mass extinction event, which has the potential to fundamentally alter basic ecological functions on global scale. One of the many causes of population declines, species extirpations, and extinctions contributing to this profound loss of biodiversity are emerging infectious diseases. The occurrence and spread of infectious diseases can be assisted by numerous anthropogenic causes. For example, habitat fragmentation and access to formerly undeveloped areas can increase the rate and the risk of interaction between domestic and wild animals, leading to “spill-over” of pathogens from unaffected reservoir hosts to susceptible hosts. Alternatively, global trade can lead to “pathogen pollution” caused by the translocation of pathogens or parasites directly or via the translocation of invasive species that often act as reservoir hosts in invaded ranges. Moreover, an overall increase in animal stress associated with habitat degradation, invasive species, or
climate change can negatively affect the immune responses of an otherwise healthy host, which can transform benign infections into pathogenic infections.

One emerging infectious disease that is in part responsible for this great loss of biodiversity is chytridiomycosis, which has been associated with numerous amphibian population declines and extinctions. Chytridiomycosis is caused by the fungal pathogen *Batrachochytrium dendrobatidis* (Bd), which has been found on every continent where amphibians exist. Whereas Bd can infect a wide range of amphibians, there is also a wide range of heterogeneity of responses to infection. This heterogeneity exists at the species-level where some species can act as unaffected reservoirs of the disease; while at the other end of the spectrum, some species will die within days of exposure. This heterogeneity can exist at the population-level within a species; some populations survive with a persistent infection, while simultaneously mass mortality events can eliminate nearby populations of the same species. Furthermore, heterogeneity can exist within one population, where some amphibian life stages, or some individuals of the same life stage, survive after Bd-exposure while other life stages or individuals will not survive. Understanding the biotic and abiotic causes, of different responses to Bd is paramount to limiting further losses of amphibian biodiversity as well cascade effects of the loss of amphibians in ecosystems. This thesis elucidates some of the potential causes of these differences, specifically addressing heterogeneity among host species, host populations, host ages, and environmental temperature, a key environmental component that influences the biology of Bd.
Previous studies have investigated the relationship between climate and chytridiomycosis by comparing differences susceptibility or sensitivity as a function of mean temperature over time. However, in addition to the predicted general warming trends associated with anthropogenic climate change, many models also predict increases in both the magnitude and frequency of extreme weather events, which can result in unusual temperature shifts for a given habitat. In Chapter 2, I describe an experiment in which I investigated how temperature shifts may influence Bd infection intensity and survival in amphibian larvae. Consistent with the “lag effect” hypothesis, Bd abundance was higher in larval red legged frogs (*Rana aurora*) that experienced a shift in temperature from cold to warm compared to frogs exposed to a constant temperature. Similarly, Bd abundance was lower in larval western toads (*Anaxyrus boreas*) that experienced a shift in temperature from warm to cold, compared to larval toads exposed to a constant temperature. In Chapter 3, I discuss the ontogeny of susceptibility to Bd infection and report on an experiment I performed in two species of frog over the first nine months post-metamorphosis. The youngest frogs of both species were the least susceptible to chytridiomycosis. Increasing age was associated with an increase in likelihood of Bd infection, increased infection intensity, and increased risk of mortality after infection. In Chapter 4, I examine differences in response to Bd infection among 10 distinct populations, using wood frogs (*Lithobates sylvaticus*) collected as eggs and raised in a common garden environment through metamorphosis. I observed differences in survival after Bd-exposure among the populations, but did not observe differences in infection intensity among populations. These results suggest that populations of wood
frog share a similar level of resistance to Bd infection, but differ in levels of tolerance to infection of a given intensity.

This thesis describes and helps disentangle the biotic drivers (the ontogeny of susceptibility and population-level variation in susceptibility) and a key abiotic parameter (temperature) of chytridiomycosis in amphibians. The information provided may assist conservationists and population managers to focus conservation efforts and mitigate the losses of these ecologically important creatures caused by this disease.
Disentangling the Biotic and Abiotic Drivers of the Amphibian Disease
Chytridiomycosis

by
Paul W. Bradley

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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.

__________________________________________________________________________

Paul W. Bradley, Author
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CHAPTER 1 – INTRODUCTION

Paul W. Bradley
Introduction

The ongoing worldwide loss of biodiversity represents one of the paramount issues facing humankind. The International Union for the Conservation of Nature and Natural Resources (IUCN) monitors the conservation status of species across the globe and has determined that one-fifth of all vertebrate species are currently threatened with extinction. Furthermore, the pace of species extinction is increasing. Over 50 species of mammals, birds, and amphibians move one step closer to extinction each year (IUCN 2012). This rate and magnitude of species loss is unprecedented in modern times, eclipsing the background expected rate of extinctions by orders of magnitude (Pimm et al. 1995; May 2010). The ecological consequences of this crisis are not limited to the numerical loss of individual populations. Widespread declines in the size of populations are also occurring. On average, populations of vertebrate species are approximately half the size they were just 40 years ago (WWF 2014). An increasing number of scientists now claim that we are witnesses to the Earth’s sixth mass extinction event (Wake & Vredenburg 2008; Kolbert 2009; Barnosky et al. 2011; Ceballos et al. 2015).

One group facing an elevated risk of extirpations and extinctions is the amphibians (Alford & Richards 1999; Houlanan et al. 2000; Stuart et al. 2004; Hof et al. 2011; Dirzo et al. 2014), with an estimated extinction rate over 200 times greater than one would predict based upon the background rate (McCallum 2007). In addition to the many amphibian species that have already been lost, nearly one-third of amphibian species are currently classified as being in threat of extinction (Hoffmann et al. 2010; IUCN 2012). The observed population declines, range reductions, extirpations, and extinctions of amphibians have numerous causes, such as habitat loss, invasive species,
climate change, enhanced UV-B radiation, and pollution (Blaustein et al. 1995; Pounds et al. 1999; Blaustein et al. 2003; Kats & Ferrer 2003; Relyea et al. 2005; Cushman 2006; Hayes et al. 2010; Blaustein et al. 2011; Blaustein et al. 2012; Bucciarelli et al. 2014). Complicating matters further, the causes of many declines are context dependent and may differ from region to region (Kiesecker et al. 2001; Blaustein & Kiesecker 2002; Blaustein et al. 2010; Blaustein et al. 2011; Doddington et al. 2013). However, one cause that has been associated with numerous recent amphibian losses across taxonomic groups and geographic ranges is infectious disease (Daszak et al. 1999; Blaustein et al. 2011; Hoverman et al. 2012; Price et al. 2014).

One disease that has been associated with many amphibian population declines across the globe is chytridiomycosis (Green et al. 2002; Daszak et al. 2003; Stuart et al. 2004; Crawford et al. 2010). This disease is caused by the aquatic fungal pathogen \textit{Batrachochytrium dendrobatidis} (Bd), which often leads to the death of individual amphibians (Berger et al. 1998; Longcore et al. 1999) and has been associated with numerous amphibian species extinctions (Skerratt et al. 2007). Since this disease was first identified in 1998, scientists have documented over 500 species of frogs and salamanders as being susceptible to Bd infection, and found infected individuals on every continent where amphibians exist (Olson et al. 2013), as well as throughout Oregon (Pearl et al. 2007; Pearl et al. 2009; Chestnut et al. 2014).

Bd is a member of the Phylum Chytridiomycota, the most ancestral lineage of fungi, and the Class Chytridiomycetes (Longcore et al. 1999). Whereas Bd can cause chytridiomycosis in both frogs and salamanders, the disease can also be caused by a closely related sister species, \textit{Batrachochytrium salamandrivorans} (Bsal), which can
infect salamanders (Martel et al. 2013; Martel et al. 2014; Yap et al. 2015). Together, these two pathogens represent the only known members of Chytridiomycota to parasitize vertebrate hosts.

Bd has a complex lifecycle with an infective stage consisting of free-living flagellated aquatic zoospores and a substrate-dependent, non-motile reproductive stage represented by a zoosporangia (Longcore et al. 1999). The infection is limited to keratinized structures of the host (Longcore et al. 1999; Berger et al. 2005c; Brutyn et al. 2012; Greenspan et al. 2012b), and the zoospore can exhibit chemotaxis towards such tissue (Moss et al. 2008) where it can settle and encyst to initiate an infection. The encysted zoospore then forms a zoosporangium where asexual reproduction and the creation of additional zoospores occurs (Berger et al. 2005a). Newly formed zoospores are then released into the aquatic environment where they can re-infect the same host or may be transmitted to another host. Outside of host tissue, the pathogen can survive for over seven weeks in lake water, and up to four weeks in deionized water lacking nutrients (Johnson & Speare 2003). The pathogen is an abundant member of the microbial community in some ponds (Chestnut et al. 2014) and some have suggested that Bd can form a resting stage or undergo sexual reproduction in unfavorable environments lacking nutrients or susceptible hosts (Longcore et al. 1999; Di Rosa et al. 2007; Morgan et al. 2007; Schloegel et al. 2012).

For many amphibian species, the negative effects of infection may differ between life-stages, and are often are more pronounced in the post-metamorphic stage (Rachowicz & Vredenburg 2004; Kilpatrick et al. 2010). Keratinized tissue is generally restricted to the epidermal surface of post-metamorphic amphibians (Heatwole & Barthalmus 1994;
Haslam et al. 2013). However this limitation still allows for the entire skin surface of a post-metamorphic amphibian to be colonized by Bd, potentially resulting in death (Olsen et al. 2004; Rachowicz & Vredenburg 2004; Baitchman & Pessier 2013). In tadpoles, Bd infection can also cause host mortality (Blaustein et al. 2005; Garner et al. 2009; Gervasi et al. 2013a; Searle et al. 2013), however as keratin is localized in the mouthparts (Berger et al. 1998; Marantelli et al. 2004; Rollins-Smith et al. 2011) the infection is generally limited in scope, often resulting in sublethal effects (Rachowicz & Vredenburg 2004; Gervasi et al. 2013a). These effects can include inhibited foraging capacity, reduced growth and development, altered predator avoidance, or changes to other behaviors (Parris et al. 2004; Parris & Beaudoin 2004; Parris & Cornelius 2004; Parris et al. 2006; Han et al. 2008; Venesky et al. 2010; Venesky et al. 2011; Buck et al. 2012).

Additionally, sublethal effects in tadpoles can lead to poor body condition at metamorphosis, leading to a decreased size or vigor in the post-metamorphic stage even if the infection is lost prior to metamorphosis (Buskirk & Saxer 2001; Alvarez & Nicieza 2002; Relyea & Hoverman 2003; Van Allen et al. 2010; Bouchard et al. 2015). Larval individuals can shed the infection, as they near metamorphic climax and shed their mouthparts (Marantelli et al. 2004; Searle et al. 2013). However, in some species of amphibians, keratin may be present in both mouthparts and skin during late larval stages (Marantelli et al. 2004), allowing the infection to be maintained through metamorphosis (McMahon & Rohr 2015).

Clinical signs of chytridiomycosis in post-metamorphic amphibians include lethargy, abnormal posture, anorexia, skin sloughing, a loss of righting reflex, and death (Berger et al. 1998; Daszak et al. 1999; Pessier et al. 1999; Nichols et al. 2001). Some
species will display only sublethal physiological effects, such as inflammation of the skin, increases in stress hormones, or deviation from normal metabolic rate (Pessier et al. 1999; Peterson et al. 2013). Evidence suggests that host mortality is caused by the disruption of host skin integrity (Brutyn et al. 2012), and in turn the disruption of physiological functions through the loss of electrolytes across the epidermis (Voyles et al. 2007; Campbell et al. 2012), ultimately leading to cardiac arrest (Voyles et al. 2009).

Alternatively, prior to infection or in the process of infection, amphibian tissue can be damaged by proteases released by Bd, resulting in disruption of intercellular junctions of epidermal cells (Brutyn et al. 2012). Such observations support the hypothesis that Bd zoospores release a toxin that causes mortality for some host species in the absence of infection (Blaustein et al. 2005; Rosenblum et al. 2008; McMahon et al. 2012), further complicating the ability to accurately model infection and disease dynamics.

While Bd has been identified relatively recently as the etiological agent of chytridiomycosis (Longcore et al. 1999), the pathogen appears to have had a cosmopolitan distribution for decades if not longer. Retrospective Bd-positive amphibian samples, obtained from museums and other collections, have been discovered with collection dates as far back as 1928 (California, USA), 1933 (Cameroon), 1933 (China), 1934 (Kenya), 1938 (South Africa), 1961 (Canada), 1978 (Australia), and 1977 (Mexico) (Berger et al. 1999; Rollins-Smith et al. 2002; Weldon et al. 2004; Ouellet et al. 2005; Soto-Azat et al. 2009; Vredenburg et al. 2013; Huss et al. 2014; Zhu et al. 2014).

However, Bd strains differ among regions (Annis et al. 2004; Morgan et al. 2007; Fisher et al. 2009), and virulence among Bd strains is not homogeneous (Berger et al. 2005b; Retallick & Miera 2007; Farrer et al. 2011; Gervasi et al. 2013b). These traits make it
difficult to distinguish movement of the pathogen against movement of the disease across the landscape. Because Bd strains vary in virulence, the presence of Bd-DNA on museum samples from early in the 20th century does not necessarily mean that chytridiomycosis was endemic in the regions where these samples were collected. The hypothesis that some strains of Bd have had a worldwide distribution for decades is not incompatible with the hypothesis that a more virulent strain associated with chytridiomycosis has spread across the globe more recently. Many have embraced the “out of Africa” hypothesis (Weldon et al. 2004; Rachowicz et al. 2005; Soto-Azat et al. 2009), noting the prevalence of infected individuals among samples from Africa at earlier dates, as well as the lack of pathogenicity observed in Bd-infected African clawed frogs (*Xenopus laevis*). As a model vertebrate species used in laboratories worldwide, these frogs have been frequently transported intercontinentally since the 1930s (Rachowicz et al. 2005; Kilpatrick et al. 2010). It has been hypothesized that *Xenopus* is an asymptomatic carrier, spreading a virulent strain of Bd beyond its historical range, and in turn introduced the disease chytridiomycosis to naïve and susceptible amphibian species (Weldon et al. 2004; Solís et al. 2009). More recently, genetic analysis of Bd has provided support for the hypothesis of repeated novel introductions combined with genetic recombination in strains associated with epizootic events (Farrer et al. 2011; Schloegel et al. 2012; Bataille et al. 2013). This suggests that while some Bd strains may have had a cosmopolitan distribution historically, some virulent strains associated with chytridiomycosis may have spread worldwide more recently. The distribution and spread of Bd strains is further complicated because there is heterogeneity in species susceptibility among the many Bd strains.
Amphibian species identity and susceptibility to chytridiomycosis

Not all species of amphibians respond similarly to exposure to Bd, in turn complicating efforts to manage chytridiomycosis outbreaks and protect amphibian populations. Mass die-offs in the field of natural amphibian populations infected by Bd have been observed, while other sympatric amphibians survive and lack chronic signs of disease (Retallick et al. 2004; Reeder et al. 2012). In laboratory studies, some highly susceptible species succumb to death rapidly after exposure (Blaustein et al. 2005; Gahl et al. 2011a; Searle et al. 2011), whereas other species are asymptomatic infected carriers (Daszak et al. 2004; Reeder et al. 2012). These infected survivors have the potential to act as a reservoir, maintaining pathogen persistence across a landscape (Hanselmann et al. 2004; Simoncelli et al. 2005; Briggs et al. 2010; Reeder et al. 2012; Narayan et al. 2014). Additionally, there are species that are resistant to infection in varying degrees, ranging from unsusceptible to infection (Van Rooij et al. 2012; Pasmans et al. 2013) to being able to clear an infection (Kriger & Hero 2006; Brannelly et al. 2012; Ellison et al. 2014; Gervasi et al. 2014). This level of variation in response to infection highlights the difference between host susceptibility to Bd infection versus host susceptibility to the disease chytridiomycosis. Despite susceptibility to Bd infection, some Bd infected species/populations/individuals do not display signs of the disease and are not susceptible to the disease chytridiomycosis (Briggs et al. 2005; Forrest & Schlaepfer 2011; Searle et al. 2011).

Individuals capable of maintaining a Bd infection without showing clinical signs of disease are considered to be tolerant (Schneider & Ayres 2008; Medzhitov et al. 2012; Venesky et al. 2012). Of tolerant individuals, those with a high level of host competence
(e.g., those with an elevated ability to maintain and transmit infectious zoospores to the environment or directly to other individuals) may act as reservoirs for chytridiomycosis (Schmidt & Ostfeld 2001; Venesky et al. 2013). In particular, one Bd tolerant amphibian species is commonly cited as having a preponderance of responsibility for maintaining, translocating, and spreading the pathogen across the landscape: the American bullfrog (*Lithobates catesbeiana*). As discussed below, the significance of bullfrogs as carriers of Bd is complex.

American bullfrogs are native to the central and southeastern parts of the United States. As early as the late 1800s, bullfrogs have been actively transported to many regions in an attempt to farm them for human food (Hayes & Jennings 1986; Kupferberg 1997; Blaustein & Kiesecker 2002; Kats & Ferrer 2003; Bucciarelli et al. 2014). This practice continues to this day, often moving populations of bullfrog from one introduced range to another (Hanselmann et al. 2004; Ficetola et al. 2007; Akmentins & Cardozo 2009; Schloegel et al. 2009; Luja & Rodríguez-Estrella 2010). Bullfrogs are often bred and maintained in high densities (Rodriguez-Serna et al. 1996; Mazzoni et al. 2003), with the potential of increasing susceptibility to infection and subsequent rates of pathogen transmission (Hudson et al. 2002). Once established, these farms often ship live bullfrogs elsewhere for the restaurant trade, with the potential to further spread Bd to locations far from the farms. The U.S. Fish and Wildlife Service estimates that over one million bullfrogs are imported into the United States every year from South America alone (Cunningham et al. 2003). While bullfrogs are often implicated as important contributors to “pathogen pollution” (Cunningham et al. 2003; Rachowicz et al. 2005; Fisher & Garner 2007; Strauss et al. 2012; Peterson & McKenzie 2014), the situation is complex.
Bullfrogs can harbor Bd infections without displaying outward signs of disease (Daszak et al. 2004; Garner et al. 2006), and they can transmit this infection to other species (Greenspan et al. 2012a). However, the ability of bullfrogs to tolerate Bd infections may be Bd strain-specific and may be bullfrog population-specific (Gervasi et al. 2013b). Investigating Bd susceptibility in American bullfrogs collected as eggs from their invaded range in Oregon, Gervasi et al. (2013b) observed an elevated risk of bullfrog mortality for those individuals exposed to a novel strain of Bd isolated from a western toad in Colorado. The risk of mortality for bullfrogs exposed to a stain isolated from a bullfrog in Oregon, however, was not elevated. While these results do not provide evidence against the hypothesis that bullfrogs can act as reservoirs of Bd, they do highlight the complexity of susceptibility to this disease.

Host variation in infection tolerance and competence may result in complicated disease patterns (Haydon et al. 2002; Mitchell et al. 2008). A population of individuals with elevated infection tolerance has the potential to produce and shed more infectious propagules into the environment. Alternatively, individuals may maintain normal health and behavior, leading to a longer period of shedding propagules or host-host contact rate. Either of these possibilities may allow persistence of chytridiomycosis in the habitat, potentially increasing the risk to sympatric species or populations less tolerant of Bd (Venesky et al. 2012). To investigate the heterogeneity in susceptibility to chytridiomycosis at the species level, I performed several controlled experiments on several amphibian host species.
Population-level heterogeneity in susceptibility to chytridiomycosis

Even within one species, not all populations respond similarly to Bd exposure (Briggs et al. 2005; Brem & Lips 2008; Forrest & Schlaepfer 2011). However, distinguishing the causes of this population-level heterogeneity can be difficult. Differences in local environmental conditions, such as water temperature or chemistry, hydroperiod, or presence of contaminants, and many other factors, can all affect the outcome of Bd exposure (Parris et al. 2004; Davidson et al. 2007; Schlaepfer et al. 2007; Sodhi et al. 2008; Boisvert & Davidson 2011; Gahl et al. 2011b; Paetow et al. 2012; Doddington et al. 2013; McMahon et al. 2013; Schlaepfer et al. 2011). These environmental effects on the dynamics of chytridiomycosis can act by way of altering either the pathogen virulence or host susceptibility (Carey et al. 2006; Raffel et al. 2006; Andre et al. 2008; Richards-Zawacki 2010; Geiger et al. 2011; Becker et al. 2012). In addition to different environmental conditions among habitats of different amphibian populations (McDiarmid & Altig 1999; Dodd 2010; Sparling 2010), differences in anti-microbial peptides, skin microbiota, MHC genotypes, and host behavior influence Bd susceptibility, and can vary among populations (Rollins-Smith & Conlon 2005; Woodhams et al. 2006; Rowley & Alford 2007; Harris et al. 2009; Lam et al. 2009; Woodhams et al. 2010; May et al. 2011; Savage & Zamudio 2011; Venesky et al. 2011; Hossack et al. 2013).

There is a paucity of experimental research investigating how amphibian populations differ in susceptibility to chytridiomycosis when controlling for environmental differences between habitats. Further, if population-level heterogeneity exists and is heritable, it suggests that natural selection may lead to the evolution of increased resistance or tolerance to Bd infection (Roy & Kirchner 2000; Vander Wal et
al. 2013). Such pathogen-driven host evolution has been observed in other amphibian disease systems (Tennesen & Blouin 2008; Teacher et al. 2009). Potential evolution of decreased susceptibility could thus lead to species persistence with some populations declining or undergoing extirpation, but other populations persisting to serve as a source for recolonization. In Chapter 4 I examine the degree of variance in infection dynamics and survival among 10 populations of wood frogs (*Lithobates sylvaticus*), all of which were collected as eggs and raised under common conditions through metamorphosis.

**Amphibian age and susceptibility to chytridiomycosis**

Little is known of the ontogeny of the amphibian immune system, but it has been hypothesized that the immune system is down-regulated during metamorphosis (Rollins-Smith 1998; Rollins-Smith & Woodhams 2011), and matures as individuals recover from the stress of metamorphic climax (Rollins-Smith et al. 2011). Several studies have suggested that the development and maturity after metamorphosis decreases frog susceptibility to chytridiomycosis (Briggs et al. 2010; Russell et al. 2010; Walker et al. 2010). This has led some researchers to hypothesize that the recently-post-metamorphic life stage is most at risk to chytridiomycosis (Gahl et al. 2011a; Tobler & Schmidt 2011; Pask et al. 2013; Narayan et al. 2014). Both field and laboratory studies have provided support for this hypothesis. Bd-related mass mortality events have been recorded for recently-post-metamorphic frogs, while sympatric larval individuals survived (Berger et al. 1998; Bosch et al. 2001; Bradley et al. 2002). Similarly, laboratory studies have also documented the survival of infected larvae despite mortality observed in post-
metamorphic individuals (Rachowicz & Vredenburg 2004; Rachowicz et al. 2006; Garner et al. 2009).

Studies comparing frogs after metamorphosis provide further evidence of a potential heightened susceptibility to chytridiomycosis in recently-post-metamorphic amphibians. Field studies quantifying Bd infection intensities on Bd-infected hosts found an association between increases in age and decreases in Bd infection prevalence and intensity (Briggs et al. 2010; Russell et al. 2010; Walker et al. 2010). Lamirande and Nichols (2002) observed 100% mortality in Bd-exposed recently-post-metamorphic blue-and-yellow poison dart frogs (*Dendrobates tinctorius*), whereas all of the sub-adult and adult frogs survived following exposure. Ortiz-Santaliestra et al. (2013) reported similar results with American toads (*Anaxyrus americanus*), which had a decreased risk of mortality when exposed to Bd 20 days after metamorphosis, compared to those exposed immediately after metamorphosis.

Development and maturation of the amphibian innate immune system after metamorphosis could be responsible for varying responses after exposure to Bd between recently post-metamorphic frogs and older frogs. The suite of antimicrobial peptides (AMPs) produced by an amphibian and the commensal microbial community on amphibian skin both can act as a barrier between microbes and host, and both can change as the amphibian matures (Kohl et al. 2013; Kueneman et al. 2014). Additionally, the frog immune system is not fully mature until well after metamorphosis (Flajnik et al. 1987; Pasquier et al. 1989; Rollins-Smith 1998; Rollins-Smith & Woodhams 2011; Holden et al. 2015). Furthermore, metamorphic climax is both energetically costly (Heatwole & Barthalmus 1994) and associated with the release of the hormone
corticosterone (Denver 1997; Hayes 1997), which has immunosuppressive properties (Rollins-Smith & Blair 1993; Rollins-Smith 1998). In Chapter 3, I investigate the relationship of post-metamorphic age and the prevalence and intensity of chytridiomycosis in two host species, the Pacific treefrog and the red-legged frog.

**Environmental temperature and susceptibility to chytridiomycosis**

It can be difficult to determine whether heterogeneity in infection prevalence or disease susceptibility is due to intrinsic species- or population-level traits of the host, or environmental conditions. Temperature is considered one of the most important environmental factors driving chytridiomycosis (Drew et al. 2006; Bosch et al. 2007; Kriger et al. 2007; Muths et al. 2008; Rodder et al. 2008; Daskin et al. 2011; Forrest & Schlaepfer 2011). As an aquatic microbe, Bd physiology is strongly influenced by the temperature of the surrounding environment (Piotrowski et al. 2004; Woodhams et al. 2008; Voyles et al. 2012). The optimal temperature for growth of Bd in culture is between 17° C and 23° C; above 25° C growth of Bd is halted and Bd mortality occurs near 30° C (Longcore et al. 1999; Piotrowski et al. 2004). However, environmental tolerances can vary between Bd strains (Johnson & Speare 2005; Raffel et al. 2013; Stevenson et al. 2013). Life history strategies of Bd can also be altered by environmental temperature. For example, colder temperatures cause Bd zoosporangia to develop and mature more slowly, but produce more and longer-lived zoospores overall (Hyatt et al. 2007; Woodhams et al. 2008; Voyles et al. 2012). Environmental conditions can also affect the amphibian host; as an ectotherm, amphibian physiology and immunology are both regulated in part by environmental temperature (Maniero & Carey 1997; Carey et al. 2009).

As models predict ongoing changes to the global climate, an increasing amount of attention is being paid to the synergy between climate change and chytridiomycosis (Pounds et al. 1999; Pounds et al. 2006; Rohr et al. 2008; Anchukaitis & Evans 2010; Blaustein et al. 2010). Many of these studies, however, concentrate on the predicted changes in annual or seasonal mean or extreme temperatures (Paaijmans et al. 2009; Paaijmans et al. 2010), despite many models predicting increases in temperature variability on shorter timescales (Easterling et al. 2000; Meehl & Tebaldi 2004; Schar et al. 2004; Rummukainen 2012). Few studies have investigated how the predicted increases in temperature variability can affect disease dynamics, despite the likelihood that such variability will differentially affect hosts and pathogens (Paaijmans et al. 2010; Ben-Horin et al. 2012; Rohr et al. 2013; Luis et al. 2014).

The relationship between temperature variability and an increase in disease susceptibility in amphibians was first postulated by Raffel et al. (2006) as the lag hypothesis. Given the thermal inertia of the host (Angilletta 2009) and the time required to produce necessary, or remove unnecessary, immune cells from the host (DeSantis & Strauss 1997; Allender & Fry 2008; Janeway 2008), Raffel et al. (2006) predicted that temperature-dependent immune parameters would lag behind environmental temperature shifts. Given the size differential between a host and pathogen (Lafferty & Kuris 2002) and the rate at which the metabolism of each organism can respond to changes in environmental temperature (Gillooly et al. 2001; Brown et al. 2004; Raffel et al. 2013; Rohr et al. 2013), the hypothesized lag would temporarily place amphibians in an
immune-compromised state following a shift in temperature. The *climate variability hypothesis*, as proposed by Rohr and Raffel (2010), builds off of the lag hypothesis to explain chytridiomycosis-associated amphibian population declines in the field. Investigating Bd-associated amphibian population declines in harlequin toads (*Atelopus spp.*) across Central and South America, the authors found declines were correlated with temperature variability. Further evidence that temperature variability may influence the outcome of Bd infection has come from laboratory studies. Cuban treefrogs (*Osteopilus septentrionalis*) displayed elevated susceptibility to Bd infection when exposed to random daily temperature fluctuations, or when exposed to a temperature decrease after acclimation to a warmer temperature (Raffel et al. 2013). Similar results were seen in rough-skinned newts (*Notophthalmus viridescens*) with high Bd infection intensities, which had an elevated risk of mortality following a temperature shift (Raffel et al. 2015).

To determine the breadth of association between temperature variation and chytridiomycosis susceptibility, I experimentally tested the *climate variability hypothesis* in larvae of two species of frogs found in the Pacific Northwest. In Chapter 2, I examine how a shift in temperature of the aquatic environment can affect host susceptibility to Bd infection and survival after exposure to Bd in both red legged frogs and western toads.

This dissertation investigates key biotic drivers (the ontogeny of susceptibility and population-level variation in susceptibility) and a key abiotic driver (temperature effects on susceptibility) of chytridiomycosis to help elucidate the patterns in amphibian susceptibility to this disease. Furthermore, it provides timely information to help conservation managers determine how best to direct conservation actions and funds to protect imperiled amphibians.
Literature Cited


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CHAPTER 2 – TEMPERATURE SHIFTS INFLUENCE THE FUNGAL DISEASE CHYTRIDIOMYCOSIS IN TADPOLES

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Abstract

Climate change represents one of the greatest challenges to biodiversity and conservation because it can compromise the integrity and functions of ecosystems. Many studies have investigated relationships between climate and disease by measuring host-parasite responses to changes in mean temperature. However, climate models also predict changes in the frequency and magnitude of temperature fluctuations. Because amphibian hosts invest more in immunity at warmer than cooler temperatures and parasites should acclimate to temperature shifts faster than hosts (creating lags in optimal host immunity), researchers have hypothesized that a temperature shift from cold to warm may result in increased amphibian sensitivity to pathogens, whereas a shift from warm to cold might result in decreased sensitivity. Support for components of this climate variability-based hypothesis has been provided by prior studies of the emerging pathogenic fungus *Batrachochytrium dendrobatidis* (Bd) that causes the disease chytridiomycosis in amphibians. However amphibians have a complex life cycle and the support thus far has been limited to the postmetamorphic stage only. To examine this hypothesis, we experimentally tested whether short-term temperature shifts before Bd exposure alter susceptibility to Bd in the larval stage of two amphibian species—western toads (*Anaxyrus boreas*) and red legged frogs (*Rana aurora*) in comparison to larvae that experienced a constant temperature. Both host species responded to temperature similarly. Both harbored elevated Bd infection intensities under the constant cold (15°C) temperature in comparison to the constant warm (20°C) temperature. Additionally, Bd abundance increased in both host species when shifted to 20°C compared to a constant
20° C, but Bd abundance in hosts decreased when shifted to 15° C compared to a constant 15° C, resulting in a strong shift-by-exposure temperature interaction. These results support the “lag effect” hypothesis, which predicts that short-term shifts from cold to warm and from warm to cold might increase and decrease amphibian susceptibility to pathogens, respectively. This study highlights the potential for species and stage differences in the temperature-dependence of chytridiomycosis.

**Introduction**

Climate change represents one of the greatest challenges to biodiversity and conservation because it might compromise ecosystem functions worldwide. Changes in climate have affected plant-animal interactions, predator-prey interactions and disease dynamics (Lafferty 2009; Rohr et al. 2011; Sheldon et al. 2011; Garcia et al. 2014). Many species, including pathogens and vectors have shifted their ranges to different latitudes and elevations (Hickling et al. 2005; Ostfeld & Brunner 2015). Climate-induced range shifts and species movements potentially affect host-pathogen interactions in complex ways (Rosenthal 2009; Altizer et al. 2013; Campbell et al. 2015).

Changes to annual or seasonal mean temperatures often are used to predict climate-change-induced effects on disease risk (Paaijmans et al. 2009; Paaijmans et al. 2010). However, many climate change models also predict increases in the frequency and magnitude of extreme weather events and increases in temperature variability at monthly to weekly timescales (Easterling et al. 2000; Meehl & Tebaldi 2004; Schar et al. 2004; Rummukainen 2012). Temperature variation at these shorter-term scales can affect ecological interactions (Stenseth et al. 2002; Diez et al. 2012; Paaijmans et al. 2013;
Vasseur et al. 2014). Yet few studies have investigated how increases in temperature variability affect disease dynamics despite the likelihood that such variability may differentially affect hosts and pathogens (Paaijmans et al. 2010; Ben-Horin et al. 2012; Raffel et al. 2013; Bannerman & Roitberg 2014; Luis et al. 2014; Raffel et al. 2015). Ectotherms, such as amphibians, are particularly sensitive to climate change (Blaustein et al. 2010; Lawler et al. 2010; Li et al. 2013) and are experiencing disease-associated population declines and extinctions worldwide (Stuart et al. 2004; McCallum 2007; Rohr et al. 2008; Wake 2012), making them an ideal group to investigate the relationship between temperature shifts and disease risk.

Chytridiomycosis is an emerging infectious disease of amphibians caused by the aquatic chytrid fungal pathogen *Batrachochytrium dendrobatidis* (Bd). It is widespread globally (Liu et al. 2013; Olson et al. 2013) and is associated with worldwide amphibian population declines (Stuart et al. 2004; Skerratt et al. 2007). The negative effects of Bd infection are more pronounced in post-metamorphic stages, often leading to death (Blaustein et al. 2005; Garner et al. 2009; Gervasi et al. 2013a). In larvae, Bd infection can cause host mortality in some species (Blaustein et al. 2005; Garner et al. 2009); however, as keratin is localized in the mouthparts (Marantelli et al. 2004; McMahon & Rohr 2015) the infection is generally limited, often resulting in sublethal effects (Han et al. 2008; Venesky et al. 2010; Buck et al. 2012; Gervasi et al. 2013a). Sublethal effects on tadpole feeding can carry over post-metamorphosis and affect frog survival (Smith 1987; Berven 1990). Additionally tadpoles of many species are important ecological members of aquatic communities and alterations to tadpole feeding have the potential to
cascade through the aquatic ecosystem (Alford 1989; Brönmark et al. 1991; Lamberti et al. 1992; Kupferberg 1997b).

Bd is non-linearly sensitive to temperature with an optimal growth range in culture between 17° and 23° (Piotrowski et al. 2004; Rohr & Raffel 2010; Raffel et al. 2013) and a temperature-dependent generation time of 4 to 10 days (Woodhams et al. 2008). The upper thermal limit for Bd growth in culture is between 25°C and 28°C, with Bd mortality occurring above 30°C (Longcore et al. 1999; Piotrowski et al. 2004). Bd has been shown to be reliably cleared from multiple amphibian species by extended exposure to 30°C (McMahon et al. 2014). Its lower thermal limit is below 4°C (Piotrowski et al. 2004). Additionally, life history strategies of the pathogen can be altered by environmental temperature, where colder temperatures can cause Bd zoosporangia to develop and mature more slowly (Voyles et al. 2012), but produce more and longer-lived zoospores overall (Hyatt et al. 2007; Woodhams et al. 2008).

Temperature is considered one of the most important environmental factors driving chytridiomycosis; climate change has been used to explain several major Bd outbreaks and amphibian population declines, because physiologies of both the host and pathogen are strongly influenced by environmental temperature (reviewed in Li et al. 2013; Rohr et al. 2013). Yet, the host and pathogen are not expected to share a uniform response to a given temperature (Brown et al. 2004; Paull et al. 2012; Rohr et al. 2013), and thermal responses measured in constant-temperature artificial environments might not reflect organism responses in more realistic variable-temperature environments. Providing evidence of the lack of a uniform response between Bd and amphibians to temperature shifts, Rohr and Raffel (2010) found a strong correlation between elevated
month-to-month temperature variability and Bd-associated amphibian population declines of *Atelopus* spp. across Central and South America. Further support of the relationship between chytridiomycosis and temperature variation has been provided by laboratory studies. In one study, Cuban treefrogs (*Osteopilus septentrionalis*) displayed reduced resistance to Bd infection when exposed to random daily temperature fluctuations or when exposed to a temperature decrease after acclimation to a warmer temperature (Raffel et al. 2013). Similar results were obtained in newts (*Notophthalmus viridescens*) exposed to Bd except both decreases and increases in temperature were associated with elevated Bd abundance relative to abundances at constant temperatures (Raffel et al. 2015).

The potential for temperature variability to increase disease severity in amphibians was first postulated by Raffel and colleagues (2006) and has subsequently been referred to as the *climate variability hypothesis* (Rohr & Raffel 2010). This hypothesis posits that parasites acclimate to the new temperature more rapidly than their hosts, leading to lags in host acclimation following a temperature shift that could make hosts more susceptible to infection (Raffel et al. 2013). This hypothesis assumes that (1) pathogens acclimate to the new temperature faster than the host because of their relatively smaller size and higher metabolic rate (Gillooly et al. 2001; Raffel et al. 2013), and (2) both host and parasite acclimation responses lead to increased performance at the new temperature, in accordance with the “beneficial acclimation hypothesis” of thermal biology (Angilletta 2009) However, Raffel et al. (2006) also pointed out potential complexities in acclimation of the ectotherm immune system that might lead to alternative predictions.
According to Raffel et al.’s (2006) “lag effect” hypothesis, changes in levels of temperature-dependent immune parameters might simply lag behind environmental temperature shifts (Fig. 1) because it takes time to produce necessary, or remove unnecessary, immune cells from the host. For example, amphibians are expected to require more immune cells at warmer temperatures to fight off faster-growing pathogens (Maniero & Carey 1997), and lags in production of new immune cells could lead to sub-optimal immunity following a temperature increase (Raffel et al. 2006). Conversely, the amphibian immune system is expected to be down regulated following a temperature decrease (Macela & Romanovsky 1970), with the removal of mature white blood cells determined by the rate of their respective half-lives (DeSantis & Strauss 1997; Janeway 2008). A lag in this process might lead to a brief period of elevated immune responsiveness relative to an already cold-acclimated host. Thus, the “lag effect” hypothesis predicts the opposite effect from the climate variability hypothesis following a temperature decrease, at least on a short timescale. These mechanistic hypotheses are not mutually exclusive, and it is unclear which effects might be more important for a given host-parasite combination.

We tested the general prediction that an amphibian shifted to a new temperature before Bd exposure would respond to infection differently from a host already acclimated to the exposure temperature. We postulated that the direction of the effect would depend upon the direction of the temperature shift, in accordance with the “lag effect” hypothesis of Raffel et al. (2006). Given the differences in size between the host and the pathogen, we assumed Bd would physiologically respond to the temperature shift faster than the host, such that an idealized host immune response to Bd exposure would temporarily lag
behind the temperature shift. Thus, we predicted that a temperature shift from cold to warm would result in an *increase* in susceptibility to Bd exposure, whereas a temperature shift from warm to cold would result in a *decrease* in susceptibility to Bd exposure. To test these predictions, we quantified susceptibility to Bd by measuring host survival and infection abundance after exposure to the pathogen.

**Materials and Methods**

To ensure that the animals used in our experiment were not previously infected with Bd, amphibians were collected as eggs from natural oviposition sites. Red-legged frog eggs were collected from a permanent pond located near Florence, Oregon, USA (Lincoln County, elevation 12 m; latitude/longitude: 44.088/-124.123) in the Oregon Coast Range on 11 February 2012. Western toad eggs were collected in Little Three Creeks Lake (Deschutes County, elevation 2,000 m; latitude/longitude: 44.009/-121.643) in the Cascade Mountain Range on 9 July 2011. Immediately after collection, eggs were transported to a laboratory at Oregon State University where they were maintained at 14°C and under a 12-12 photoperiod in 40-liter aquaria filled with dechlorinated water. Upon hatching, tadpoles were maintained at a density of approximately 200 individuals per aquarium and fed *ad libitum* a mixture of Tetramin fish food and ground alfalfa pellets (1:3 ratio by volume). Water was changed every seven days. The 40-day trials for each species were not run concurrently, but identical protocols were used for both species and both trials consisted of individuals of identical larval stage (Gosner stage 26).

**Acclimation Period**
Independent trials for each host species began with a 20-day acclimation period with 80 (Gosner stage 26) tadpoles randomly selected from and individually placed into 80 plastic 500 mL containers where they were housed for the duration of the acclimation period and experiment. Each container was filled with 14° C dechlorinated water and covered with a lid to help maintain water temperature and limit evaporation. Each container had 2-mm diameter holes drilled between the water line and the lid to allow air circulation into the container. Pairs of containers were then placed within 40 individual temperature-controlled chambers (to ensure independent replication of the temperature treatments) that were set at 15° C to avoid cold-shocking the tadpoles. Each temperature-controlled chamber was independently controlled via its own thermostat and the interior measured approximately 37 cm deep x 21 cm wide x 13 cm in height. Half of the 40 temperature-controlled chambers were then randomly selected to begin the acclimation period at 20° C (warm treatment) and the other half were kept at 15° C (cold treatment). The placement of temperature chambers within the laboratory was randomized, as was the placement of 500 mL containers within each temperature chamber. Furthermore, these temperatures are commonly observed in ponds where eggs are laid (personal observations).

**Temperature Shifts**

On day 20 of the experiment, half of the temperature chambers in each of the two initial temperature treatments (15° C and 20° C) were randomly selected to undergo a temperature shift, either from 20° to 15° C or from 15° C to 20° C. The other half of the temperature chambers underwent no shift in temperature. Thus, each of the temperature chambers was subjected to one of four temperature treatments: a constant 15° C (cold)
throughout the experiment, a constant 20° C (warm) throughout the experiment, a temperature shift from 15° C to 20° C (cold to warm), or a temperature shift from 20° C to 15°C (warm to cold).

Western toad tadpoles experienced more mortality than expected during the acclimation period, resulting in fewer individuals overall and an unbalanced number of individuals per treatment (Table 1). Thus on day 20, the remaining western toad tadpoles were evenly divided within temperature treatments but unevenly divided between Bd-exposure treatments to best allow for temperature treatment effects on Bd-exposed tadpoles. As this occurred on the day assigned tadpoles were scheduled to experience a temperature shift, all western toads either experienced a constant temperature throughout the experiment or experienced one temperature shift.

**Bd exposure**

On day 24, one of the two 500-mL containers within each temperature-controlled chamber was randomly selected to undergo a Bd-exposure treatment and the other was selected as a control. Tadpoles in the Bd-exposure treatment were exposed to a single inoculate of Bd strain JEL 274, which was grown in pure culture on 1% tryptone agar in 10-cm diameter Petri dishes. The Petri dishes were inoculated with liquid culture 10 days before the start of the experiment and incubated at 15° C. To harvest the zoospores, 10 plates were flushed with 15 mL of 15° C dechlorinated water and remained undisturbed for 10 minutes. The plates were scraped with a rubber spatula to release the zoospores and sporangia adhering to the agar. The inoculum from each plate was then pooled in a beaker and the number of moving zoospores was determined using a hemocytometer. After quantifying the zoospore concentration, the inoculum was diluted to 10,000
zoospores/mL. Individuals in the Bd-exposed treatments were exposed to 10 mL of inoculum transferred into the 500 mL container housing a tadpole. Control individuals were exposed to 10 mL of sham inoculum lacking the Bd culture, (made from 1% tryptone sterile agar plates following the same methods) similarly transferred into the 500 mL container housing each tadpole. Thus, the individual tadpole underwent their exposure treatment on day 24, four days after the water temperature shift for chambers in the two temperature shift treatments.

During the 40-d trial, survival and metamorphic status were checked daily. Water for each 500-mL container within the temperature chambers was changed every 12 days and consisted of dechlorinated water of the same temperature (15° C and 20° C). Animals that died were preserved in 95% ethanol after measuring mass and snout-vent length (SVL) measurements. Individuals that survived until the end of the trial (i.e., day 40) were euthanized in a 2% solution of MS-222, and then measured and preserved in 95% ethanol. Individuals that reached metamorphosis (Gosner stage 42: emergence of forelimbs) were euthanized, measured, and preserved as previously described.

**Determining infection status**

We used quantitative polymerase chain reaction (qPCR) to determine infection status and quantify Bd-infection intensity of all individuals in the Bd-exposure treatments. Additionally, we investigated Bd-infection status in eight randomly selected control individuals per species. To sample the individuals for Bd, we extracted whole mouthparts of the larvae using sterile dissection scissors. We conducted qPCR using an ABI PRISM 7500 sequencer (Applied Biosystems) according to the methods of Boyle et al. (2004) except that we used 60 µL of Prepman Ultra (Applied Biosystems, Carlsbad,
California), instead of the 40 µL in the DNA extraction. All samples were run in triplicate and averaged.

Statistical Analyses

Each temperature-controlled chamber was an experimental unit (whole plot) and the pairs of containers within each chamber acted as subplots. The whole plots were subjected to one of four temperature regimes consisting of a Bd-exposure temperature combined with a temperature shift status (constant cold, constant warm, shifted to cold, and shifted to warm). Further, subplots were subjected to one of two exposure treatments (Bd exposed and Bd unexposed).

Survival was compared between temperature treatments for each host species individually with Cox proportional hazards models (Cox 1972) using TIBCO Spotfire S+ version 8.1. Kaplan-Meier (or product-limit) survival curves were generated to visualize tadpole survival during the 16-day period after application of the exposure treatment. For each host species, the model consisted of the main effects of the temperature treatment, temperature shift status (constant versus shifted), and an interaction between the two variables. Because of losses of 22/80 toad tadpoles prior to the application of the exposure treatment (Table 1), we lacked the power to statistically compare survival between the Bd exposure treatments but were able to compare the survival among the temperature treatments for Bd-exposed toad tadpoles. Similarly, due to unexpected losses of some toads prior to Bd-exposure we lacked the power to statistically compare the Bd-associated survival between the two host species.

*Batrachochytrium dendrobatidis* infection abundance (Bd genomic equivalents) among temperature treatments and between host species was analyzed using R version...
3.11. We used a zero-inflated negative-binomial generalized linear model (function ‘zeroinf’ in package ‘pscl’) as described by Raffel et al. (2010), which includes a zero-inflation component that models infection status as a binomial process (binomial distribution with a logit link) and a count component that models infection intensity as a negative binomial process (negative binomial distribution with a log link). Our full model investigated the effects of all of the explanatory variables including, host species, exposure temperature, temperature shift status, and all two- and three-way interactions on Bd (*Batrachochytrium dendrobatidis*) abundance. Interpretation of this analysis required further reduced models to investigate the effect exposure temperature and temperature shift status for each species (species model) and the effect of temperature shift status for each Bd-exposure temperature and host species combination (Bd-exposure temperature model).

**Results**

*Infection Abundance*

We detected a significant host species by temperature shift status interaction ($\chi^2_{1} = 3.83, p = 0.050$; Table 2) and a significant Bd-exposure temperature by temperature shift interaction ($\chi^2_{1} = 7.50, p = 0.006$; Table 2). We investigated these interactions with reduced models to investigate effects on Bd abundance at the levels of species and exposure temperature.

Red legged frog tadpoles had significantly higher Bd abundance when they were exposed to infection at 15° C in comparison to 20° C ($\chi^2_{1} = 3.88, p = 0.049$; Fig. 3). The main effect of temperature shift was only marginally significant in the reduced species
model analysis ($\chi^2_1 = 3.50, p = 0.061$), but there was a significant effect of temperature shift for tadpoles exposed at 20°C in the reduced Bd-exposure model ($\chi^2_1 = 5.7, p = 0.017$), with tadpoles shifted from 15°C to 20°C having higher Bd abundance than tadpoles experiencing constant 20°C (Fig. 3). In contrast, there was no evidence that a temperature shift influenced Bd infection when tadpoles were exposed at 15°C ($\chi^2_1 = 0.6, p = 0.4$; Fig. 3). There was no statistically significant interaction between exposure temperature and temperature shift status for red legged frogs ($\chi^2_1 = 2.4, p = 0.13$).

We detected a significant interactive effect of exposure temperature and temperature shift on Bd abundance in Western toad tadpoles ($\chi^2_1 = 5.2, p = 0.023$). This was driven by elevated Bd abundance in tadpoles under the constant 15°C temperature when compared to tadpoles that experienced a temperature shift from 20°C to 15°C, but no evidence of an effect of shifting temperature from 15°C to 20°C prior to Bd exposure (Fig. 3). There were no statistically significant main effects of exposure temperature ($\chi^2_1 = 0.50, p = 0.5$) or temperature shift ($\chi^2_1 < 0.01, p = 0.9$) on Bd abundance in Western toads. Further, when investigating the exposure temperatures individually in the reduced Bd-exposure model, there was no evidence that a temperature shift influenced Bd infection after exposure at 15°C ($\chi^2_1 = 3.4, p = 0.066$) or 20°C ($\chi^2_1 = 2.5, p = 0.113$).

The general patterns for both species were similar (Fig. 3). Both species generally experienced an increase in Bd abundance when shifted to 20°C compared to a constant 20°C, but they generally experienced a decrease in Bd when shifted to 15°C compared to a constant 15°C.

All the red legged frog tadpoles survived until the end of the experiment, but many Western toad tadpoles died or metamorphosed on earlier dates. We therefore
assessed the possibility that the timing of Bd sampling or the proximity of a tadpole to metamorphosis might drive observed patterns of Bd abundance in Western toads. The model for Bd abundance on Western toads was not significantly improved by adding either a variable coding whether tadpoles were near metamorphosis when sampled ($X^2_1 = 4.00, p = 0.150$) or a covariate indicating the sampling date ($X^2_1 = 3.33, p = 0.068$). Furthermore, neither variable qualitatively changed the contribution of exposure temperature or temperature shift status to the model. Therefore, we omitted both covariates from the final model for Western toads.

**Survival**

Faster rates of mortality and an overall greater proportion of mortality of western toad tadpoles were observed in the constant temperature treatments (Fig. 2), however survival differences between exposure temperatures ($p = 0.27$) and temperature shift status ($p = 0.78$) were not statistically significant in this host species. Western toad survival was associated with Bd infection status, as 80% (20/25) of the Bd-exposed individuals that survived until the end of the experiment tested negative for Bd infection whereas only 40% (6/15) of Bd-exposed individuals that did not survive the experiment tested negative for Bd. Mortality was observed in only one individual of red legged frog tadpoles after application of the exposure treatment (Table 1).

**Discussion**

Our results suggest that Bd infection dynamics in tadpoles can be affected by a shift in water temperature before host exposure to the pathogen, and that the direction of temperature shift determines the outcome of Bd exposure. Similar patterns were observed
for the two host species when comparing tadpoles exposed to constant versus shifted temperatures. A shift from the warm temperature to the colder temperature was associated with a decrease in Bd abundance whereas a shift from the cold temperature to the warmer temperature increased Bd abundance. Importantly, we detected the effects of temperature shifts despite the host having a four-day head start on acclimating to the Bd exposure temperature relative to the pathogen. This suggests that we are likely underestimating the strength of these effects and that their magnitudes might have been larger if the host and pathogen experienced the shifts concurrently.

As we had predicted, western toad tadpoles experienced a lower Bd infection abundance after a shift from the warm to cold temperatures. However we failed to detect higher Bd abundances in western toad tadpoles after a shift from a cold to warm temperature as we had also predicted. A similar pattern was observed with the red legged frog tadpoles. Red legged frog tadpoles experienced an increase Bd infection abundance after a shift from the cold to warm temperature, as we had predicted. However we did not find evidence to support our hypothesis that red legged frog tadpoles would harbor higher Bd abundance after a shift from a warm to cold temperature as we had predicted. Thus for western toads, the predicted effects of a shifted temperature on Bd susceptibility were confirmed for a shift in one direction (warm-to-cold), and the predicted effects were confirmed for a shift in the opposite direction (cold-to-warm) in red legged frogs. Likewise, we found no evidence that temperature shifts affect Bd susceptibility in the opposite directions than we had predicted for either host species.

Similarly, western toads experienced a reduced risk of mortality in both shifted temperature treatments compared to the constant temperature treatments. This pattern of
reduced risk of mortality associated with a shift in temperature prior to exposure to Bd was predicted for the shift to the cold temperature treatment because of predicted lag effects (Fig. 1). However, we predicted, but did not detect evidence of an increased risk of mortality associated with a shift to a warm temperature under the assumptions of the reverse lag effect.

We hypothesized that hosts exposed to a shifted temperature would respond to infection differently than hosts exposed to a constant temperature, and under the framework of the “lag hypothesis,” the direction of the temperature shift would differentially affect infection severity. We predicted that a temperature shift from cold to warm would leave hosts in a temporarily immune compromised state and result in an elevated Bd abundance after exposure when compared to hosts exposed to a constant warm temperature. Conversely, we predicted that a temperature shift from warm to cold would provide hosts with a temporarily elevated immune responsiveness and result in a decrease in Bd abundance after exposure when compared to hosts exposed to a constant cold temperature. Our results were consistent with predictions of the “lag effect” hypothesis in larval amphibians, and generally consistent with previous studies showing that a shift in temperature influences Bd infection in postmetamorphic amphibians (Raffel et al. 2013; Raffel et al. 2015). In particular, our finding of decreased resistance to infection following a temperature increase (relative to warm-acclimated tadpoles) mirrored a laboratory study of post-metamorphic red-spotted newts (Notophthalmus viridescens), where juvenile newts exhibited decreased Bd resistance following a shift from 15° C to 25° C (Raffel et al. 2015). These findings of fluctuating temperature effects on Bd infection across taxonomic groups and life-stages suggest that effects of
temperature shifts and chytridiomycosis susceptibility might be widespread within amphibians. However, our finding of increased resistance to Bd infection following a temperature decrease (relative to cold-acclimated tadpoles) was opposite the pattern observed in red-spotted newts and Cuban treefrogs (Raffel et al. 2013; Raffel et al. 2015), suggesting that there are important among-taxa differences in the underlying mechanisms driving the effects of temperature fluctuation on Bd infection.

We observed differences in Bd abundance on the frogs at the two constant temperature treatments. Higher Bd abundances were observed for both host species under the constant cold temperature treatment compared to the constant warm temperature treatment, and the constant cold temperature treatment was associated with the greatest proportion and rate of mortality for Bd-exposed western toads. These results are consistent with previous experiments that showed increased Bd abundance (Raffel et al. 2015) and Bd-induced mortality (Kilpatrick et al. 2010; Murphy et al. 2011; Raffel et al. 2015) associated with lower temperatures. This is despite Bd growing best in culture at about 23° C, which is much closer to the warm than cold temperatures in these experiments (Piotrowski et al. 2004; Woodhams et al. 2008). This might be because the larval immune response to Bd infection increases with increasing temperatures at a faster rate than the infectivity or growth rate of Bd (Raffel et al. 2013), or alternatively because of the differences between the growth rate of Bd in culture compared to the growth rate on host tissue (Venesky et al. 2013). Our results provide further evidence to suggest patterns of Bd growth in culture can differ from patterns of Bd growth on a host and that it is important to assess the host-parasite interaction when predicting effects of climate and climate change on disease risk.
In conclusion, our results provide additional evidence for climate variability affecting Bd infection in amphibians but suggest important among-taxa differences in the directionality of these effects. Our finding of increased host resistance to infection following a temperature decrease is consistent with the “lag effect” hypothesis of Raffel et al. (2006) but contradicts the climate variability hypothesis, which has been proposed as an explanation for patterns of Bd-associated amphibian population declines (Rohr & Raffel 2010; Raffel et al. 2013; Raffel et al. 2015). Our study highlights the complexity that temperature plays in determining the outcome Bd-amphibian interactions and the role that a fluctuating temperature may play in altering these interactions. Furthermore, this study increases the diversity of amphibian species and stages that have been shown to exhibit thermal acclimation effects on disease, and the broad generality of this pattern suggests that fluctuating-temperature effects on amphibian infection are widespread. Accurately predicting the effects of global climate change on infectious diseases, such as chytridiomycosis will require further understanding of how infectious agents respond to heterogeneity in temperatures and temperature fluctuations.

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Literature Cited


**Table 2.1.** Summary survival information for the two amphibian host species (red legged frogs and western toads). For both host species, tadpoles were equally divided between warm (20° C) and cold (15° C) temperature treatments for acclimation period (the first 20 days). Individuals that survived until day 20 were equally divided into a constant temperature or shifted temperature treatment. Individuals that survived until day 24 were divided into one of two exposure treatments, Bd-exposed or Bd-unexposed control treatment for the remainder of the 40-day experiment.

| Acclimation temperature | Sample size at start of acclimation period | Sample size at end of acclimation period | Temperature treatment application | Losses between end of acclimation period and exposure | Sample size in exposure treatments (Bd+ | Bd-) | Number of survivors at end of experiment (Bd+ | Bd-) |
|-------------------------|-------------------------------------------|-----------------------------------------|----------------------------------|------------------------------------------|---------------------------------|---------------------------------------|
| **Red legged frog (*Rana aurora*)** | | | | | | |
| Hot (20° C) | 40 | 40 | Constant Hot (20° C) | 0 | 10 | 10 | 10 | 10 |
| | | | Shifted to Cold (20° C) | 0 | 10 | 10 | 10 | 9 |
| Cold (15° C) | 40 | 39 | Constant Cold (15° C) | 0 | 10 | 10 | 10 | 10 |
| | | | Shifted to Hot (15° C) | 0 | 10 | 9 | 10 | 9 |
| **Western toad (*Anaxyrus boreas*)** | | | | | | |
| Hot (20° C) | 40 | 36 | Constant Hot (20° C) | 0 | 10 | 7 | 2 | 4 |
| | | | Shifted to Cold (20° C) | 0 | 10 | 9 | 10 | 6 |
| Cold (15° C) | 40 | 28 | Constant Cold (15° C) | 2 | 10 | 2 | 4 | 0 |
| | | | Shifted to Hot (15° C) | 4 | 10 | 0 | 9 | NA |
Table 2.2. Full model investigating *Batrachochytrium dendrobatidis* (Bd) abundance. Model investigated effects of host species, exposure temperature, temperature shift status, and all two- and three-way interactions on Bd abundance in red legged frogs (*Rana aurora*) and western toads (*Anaxyrus boreas*).

<table>
<thead>
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<th>Predictor</th>
<th>$\chi^2_{1}$</th>
<th>p-value</th>
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<td>Exposure Temperature</td>
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</tr>
<tr>
<td>Temperature Shift</td>
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<td>Species * Temperature Shift</td>
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<td>Exposure Temperature * Temperature Shift</td>
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<td>0.006</td>
</tr>
<tr>
<td>Species * Exposure Temperature * Temperature Shift</td>
<td>0.871</td>
<td>0.351</td>
</tr>
</tbody>
</table>
Figure 2.1. Hypothesized lag effect showing the relationship between fluctuating temperatures (over days to weeks) and the optimal levels of a hypothetical temperature-dependent host immune parameter. The immune parameter follows and lags behind temperature changes – resulting in periods of a compromised immune status after a temperature increase, and resulting in an over-active (or unnecessarily costly) immune status after a temperature decrease. Modified from Raffel et al. (2006).
Figure 2.2. Kaplan-Meyer survival plots comparing survival under four temperature regimes for western toad (Anaxyrus boreas) tadpoles following exposure to Bd (Batrachochytrium dendrobatidis). The symbol (+) indicates when an individual tadpole was lost due to either metamorphosis or imminent metamorphosis resulting in euthanasia. Survival plots for red legged frogs are not displayed as only 2 of 80 individuals of this host species died during the experiment.
Figure 2.3. Mean *Batrachochytrium dendrobatidis* (Bd) infection abundance (± SE) levels. Infection levels measured at death or 16-days after Bd exposure, in both western toad tadpoles (*Anaxyrus boreas*) and red legged frog tadpoles (*Rana aurora*), and between the two temperatures at the time of Bd-exposure on day 24 (cold [15° C] versus warm [20° C]) and between shifted temperature status (constant versus shifted temperature). Bd infection abundance is quantified as the log (1 + Bd genomic equivalents) per excised larval mouthparts of all individuals exposed to the pathogen.
CHAPTER 3 – HOST AGE ALTERS SUSCEPTIBILITY TO AN EMERGING INFECTIOUS DISEASE OF AMPHIBIANS

Paul W. Bradley and Andrew R. Blaustein
Abstract
Parasites and pathogens are often aggregated in a minority of susceptible hosts within a population, with a majority of individuals harboring low infection intensities and only a few individuals experiencing high levels. Variation in local environmental conditions or differences in host vigor, sex, behavior, or age can all affect the risk of exposure to pathogens as well as susceptibility to infection. However, determining the relative importance of host traits to explain observed heterogeneity of infections is a challenge. Elucidating host-pathogen dynamics has become an urgent priority as the impacts of emerging infectious diseases on populations and species have become more profound, contributing to the worldwide loss of biodiversity. The emerging infectious fungal pathogen *Batrachochytrium dendrobatidis* (Bd), which causes the disease chytridiomycosis, is associated with many amphibian population declines worldwide. However, susceptibility and vulnerability to Bd varies with life stage, species, populations and ecological parameters. For many amphibian host species, post-metamorphic stages are generally more susceptible than the larval stage. Investigation of the effects of Bd infection at different ages within a life stage, however has received little attention. This study investigated the hypotheses that recently-post-metamorphic frogs are more sensitive to chytridiomycosis than older frogs and that sensitivity to Bd infection decreases with age. We examined the relationship between frog age and prevalence and intensity of Bd infection in two host species, the Pacific treefrog (*Pseudacris regilla*) and the red legged frog (*Rana aurora*). Age had a strong effect on susceptibility to infection, infection intensity, and survival – but not in the directions we
had predicted. In both host species, frogs of a recently-post-metamorphic age were not at a heightened risk of infection or disease. An increase in age was associated with frogs becoming more susceptible to Bd infection, harboring higher infection intensities and greater risk of mortality following exposure.

**Introduction**

Heterogeneity in the prevalence and intensity of parasitic infection in host populations is well documented in numerous disease systems (Poulin 1996; Coltman et al. 1999; Wilson et al. 2002; Hudson et al. 2008). Parasites and pathogens are generally aggregated in a minority of susceptible hosts within a population, with a majority of individuals harboring low infection intensities (Shaw & Dobson 1995). This distribution can result from differences in host risk of exposure or susceptibility to infection following exposure (Anderson & May 1978). Risk of exposure can be a function of environmental variation across the landscape or can occur via variation in host traits such as intrinsic susceptibility, vigor, sex, behavior, or age. However, assessing the relative strength of host traits determining the outcome of infection can be challenging. This is particularly true for assessing the role of host age in affecting infection outcome.

Age-related changes in infection prevalence can have several different causes (Wilson et al. 2002; Duerr et al. 2003). Infection prevalence can increase as a host ages due to a prolonged period of exposure to the pathogen or due to increased transmission from cumulative host-host contacts over time. Infection prevalence can decrease with host age due to differential survival between infected and uninfected individuals in the population, resulting in an elevated proportion of uninfected or resistant survivors.
Infection prevalence can decrease due to acquired immune responses or other age-related changes in host behavior or traits that decrease their risk of exposure or infection. Assessing how infection dynamics differ, as a function of host ages is important for predicting infection patterns that may help mitigate population declines or extirpations associated with wildlife diseases.

One wildlife disease that has been associated with numerous population declines worldwide is chytridiomycosis, which can infect numerous amphibian species. Amphibian species respond in different ways to Bd exposure (Daszak et al. 2004; Searle et al. 2011; Reeder et al. 2012; Gervasi et al. 2013a), although mortality is common in many host species (Blaustein et al. 2005; Gahl et al. 2011a; Searle et al. 2011; Gervasi et al. 2013a). However, mortality can be dependent upon a variety of factors For example, survival after exposure to Bd is often context dependent with further heterogeneity in host susceptibility or pathogen virulence resulting from both abiotic and biotic factors (Rodder et al. 2008; Searle et al. 2010; Blaustein et al. 2011; Forrest & Schlaepfer 2011; Ortiz-Santaliestra et al. 2011). Susceptibility and sensitivity to infection may differ across life-stages, but many amphibian species suffer only sublethal effects of exposure as larvae (Rachowicz & Vredenburg 2004; Searle et al. 2013) with heightened sensitivity to Bd infection often observed in the post-metamorphic stage. Many species of frogs appear to exhibit an elevated susceptibility to chytridiomycosis immediately following metamorphosis (Bradley et al. 2002; Lamirande & Nichols 2002; Rachowicz & Vredenburg 2004; Briggs et al. 2010), leading some to suggest that the stage when frogs are at most risk to the diseases is immediately after metamorphosis (Gahl et al. 2011a; Tobler & Schmidt 2011; Pask et al. 2013; Narayan et al. 2014). Nonetheless, there is a
paucity of experimental research investigating the effect of post-metamorphic age on the susceptibility to infection with Bd. This is surprising because it is assumed that individuals immediately following metamorphosis would be more susceptible to Bd than those that are older because of immunosuppression that occurs during the metamorphic transition.

In this study, we investigated how susceptibility to Bd infection, and the disease chytridiomycosis, varies across the first nine months post-metamorphosis, specifically testing the hypotheses that (1) frogs are more sensitive to chytridiomycosis immediately following metamorphosis, and (2) that susceptibility of frogs to chytridiomycosis decreases with age.

**Materials and Methods**

To test our hypotheses experimentally, we used two species of anuran amphibians: the Pacific treefrog (*Pseudacris regilla*) and the red legged frog (*Rana aurora*). These species were selected because both have been observed in the field with Bd infections (Pearl et al. 2007; Piovia-Scott et al. 2011; Reeder et al. 2012), and both species are susceptible to chytridiomycosis with death as a potential outcome after infection (Kleinhenz et al. 2012; Gervasi et al. 2013a). Furthermore, it is common for these two species to undergo metamorphosis at approximately the same time in the Willamette Valley of Oregon, where we collected eggs of these species, allowing both species to be investigated simultaneously. The study was performed using individuals collected as eggs, and reared under uniform conditions through metamorphosis and post-metamorphic ages.
The experiment was conducted over two years. The first year of the experiment consisted of five age trials run consecutively. Each trial occurred 60-days after the previous and each trial with Bd-naïve frogs that differed in post-metamorphic age by approximately 60-days (1-, 3-, 5-, 7-, 9-months post-metamorphosis). The second year of the experiment consisted of three age trials run concurrently, with each trial comprised of Bd-naïve frogs that differed in post-metamorphic age by approximately one week (1-, 2-, 3-weeks post-metamorphosis).

**Year 1 Trials – ages 1-, 3-, 5-, 7-, 9-months post-metamorphosis run consecutively**

To ensure that subjects had not been previously exposed to Bd, all individuals utilized in the experiment were collected as eggs. Pacific treefrog eggs were collected from >20 clutches found in a temporary pond near Corvallis, Oregon, USA (Benton County, elevation 93 m; latitude/longitude: 44.572/-123.301) and red-legged frog eggs were collected from >10 clutches located in a permanent pond located near Florence, Oregon, USA (Lincoln County, elevation 12 m; latitude/longitude: 44.088/-124.123). For both species, eggs were collected on the same day (11 February 2012) and consisted of early-stage embryos (Gosner 1960). Immediately after collection, eggs were transported to Oregon State University where they were placed in 40-L aquaria filled with dechlorinated water. Upon hatching larvae were kept at a density of approximately 200 individuals per aquarium and fed a mixture (1:3 ratio by volume) of Tetramin fish food and ground alfalfa pellets *ad libitum*. Aquaria were kept at 14° C, under a natural photoperiod, and water was changed every seven days.

On 27 May 2012, at Gosner (1960) stage 25, 250 larval tadpoles of each species were transported to outdoor mesocosms at the Lewis Brown Horticulture Farm near
Corvallis, Oregon, USA (Benton County, Oregon; elevation 71 m). Mesocosms were 0.9 m in diameter and filled to a depth of 0.4 m with approximately 322 L of well water. To establish a natural microbial aquatic community, mesocosms were inoculated with 1 L of pond water collected from nearby wetlands, 100 oak (*Quercus* spp.) leaves, 50 g of alfalfa pellets, and allowed to sit for 30 days before the addition of larvae. Individuals of each species were equally divided among five mesocosms with a density of 50 individuals per mesocosm. Larvae were checked weekly until they reached Gosner (1960) stage 41 (at least one forelimb visible), and then were checked daily. For both species, metamorphic climax occurred between 9 July and 15 August 2012 and upon metamorphosis frogs were moved from the aquatic mesocosms to terrestrial mesocosms (0.9 m wide x 0.7 m across x 0.3 m deep). Terrestrial mesocosms were located adjacent to the aquatic mesocosms, lined with wet sphagnum moss. Newly metamorphosed frogs were fed 1-week-old crickets *ad libitum*. A mesh screen covered both the aquatic and terrestrial mesocosms to keep predators out and to keep frogs from escaping. After completing metamorphosis, frogs were transported back to Oregon State University and kept in 40-L glass terraria. All individuals were kept at a constant 14° C ambient temperature and 12:12d (light:dark) photoperiod. Individuals in the first year were allowed to acclimate to the laboratory environment for 7 days before the start of the first trial.

At the start of each trial, 36 individuals of each species were randomly selected from the pool of available Bd-naïve individuals. Frog mass was collected from each individual in the trial. For each species, average mass was calculated and each frog was placed into an individual 600-mL (12.5 cm x 9 cm round) glass beaker, where it was
housed for the duration of the trial. Each beaker had a mesh screen secured to the top to provide air circulation into the beaker but prevent the escape of frogs. The 36 individuals in each trial were randomly assigned to and equally divided among one of three exposure treatments: (a) a fixed dose of Bd zoospores (n=12), (b) a mass-specific dose of Bd zoospores (n=12), or (c) an unexposed control treatment (n=12). The fixed dose of Bd zoospores consisted of 10 mL of inoculum containing 10,000 zoospores/mL for a total of 100,000 zoospores. The mass-specific dose consisted of 10 mL of inoculum containing a concentration of zoospores calculated at 5,000 zoospores multiplied by the average mass (in grams) of all 36 individuals in that trial (Tables 3.1 and 3.2). Ten days prior to the start of each trial, 1% tryptone agar Petri dishes for use in the upcoming trial were inoculated with liquid Bd culture (Bd strain JEL 274) and incubated at 22° C. This inoculation occurred concurrently with the passage of the culture into a new beaker of 1% tryptone broth. Thus, for each trial, the culture used to inoculate the Petri dishes had been re-passaged approximately 60 days prior. To harvest the zoospores at the start of each trial, Petri dishes were flushed with 15 mL of 22° C dechlorinated water and remained undisturbed for 5 minutes. The dishes were scraped with a rubber spatula to release the zoospores and sporangia adhering to the agar. The inoculum from each dish was then pooled in a beaker and the number of moving zoospores was determined using a hemocytometer. After quantifying the zoospore concentration, the inoculum was diluted to either 10,000 zoospores/mL for the fixed dose or to the calculated mass-specific dose (Table 3.1). Individuals in the Bd-exposed treatments were exposed to 10 mL of inoculum poured directly on their dorsal surface while they were housed in beakers. This volume of inoculum was sufficient to cover the bottom of the beaker with a thin film.
Control individuals were exposed to 10 mL of sham inoculum lacking the Bd culture, (made from 1% tryptone sterile agar plates following the same methods) poured onto their dorsal surface. The first trial began on 11 August 2012 with the initiation of subsequent trials every 60 days.

Over each 15-day trial, position in the beaker (side or bottom), survival, and water level within the beaker were monitored daily. To maintain a thin film of water covering the entire bottom of a beaker, 5 mL of 14°C dechlorinated water was added daily to each beaker if necessary; no beaker was allowed to dry out before adding more water. Individuals were fed crickets (*Acheta domestica*) on the fifth, ninth, and 14th day and the water was changed in the beaker on the ninth day. The frogs utilized in the first year were fed 6 crickets; individuals in the one- and three-month post-metamorphosis trials were fed one-week old crickets, and individuals in the five- seven- and nine-month post-metamorphosis trials were fed two-week old crickets. Animals that died during the trial were preserved in 95% ethanol. Individuals that survived until the end of the trial (i.e. day 14) were euthanized in a 2% solution of MS-222, and then preserved in 95% ethanol. Individuals were removed from the available pool to be used in a trial. To limit the role that decreasing animal density might potentially play over the length of the nine-month study, frog density was limited to approximately 30 individuals per holding terrarium at all times.

We used quantitative polymerase chain reaction (qPCR) to quantify Bd-infection intensity of all individuals in the Bd-exposure treatments. Additionally, we investigated Bd-infection status in two unexposed individuals per species per trial as well as all unexposed individuals that died during the trials. To sample the individuals for Bd, we
used a sterile, fine-tipped, dry swab (Medical Wire and Equipment, Corsham, Wiltshire, England) and swabbed the right ventral surface of individual frogs 10 times including the feet, legs, and drink patch. Individual swabs were placed into sterile screw-capped vials. Bd-DNA was extracted by adding 60 µL of Prepman Ultra (Applied Biosystems, Carlsbad, California), heating the vial for 10 min at 100°C, cooling the vials for 2 min, obtaining the supernatant, then diluting it to a 10% solution. We conducted qPCR using an ABI PRISM 7500 sequencer (Applied Biosystems) according to the methods of Boyle et al. (2004). All samples were run in triplicate and averaged. If a sample tested positive for Bd-DNA in only one replicate we reanalyzed the sample. If a second analysis was required, we re-swabbed the individual on their left side and analyzed the sample from the second swabbing. An individual was considered Bd-positive if more than one sample (run once) or more than two samples (run twice) were positive.

*Year 2 Trials – ages 1-, 2-, 3-weeks post-metamorphosis run concurrently*

Methods were the same as in Year 1 except for the following. Eggs were collected on 15 February 2013 (red legged frog) and 8 March 2013 (Pacific treefrog) and tadpoles were transported to the outdoor mesocosms on 20 May 2013. Metamorphic climax occurred between 3 and 18 July 2013. After moving the post-metamorphic frogs from the mesocosms to the laboratory, trials began after individuals acclimated for two days. Due to the unpredictable timing of metamorphosis, sample sizes differed in the first trial investigating Pacific treefrogs nearest to metamorphosis (Table 3.1). All three age trials in Year 2 were run concurrently with all individuals among all age classes undergoing the same exposure procedure on the same day (20 July 2013). Thus, controlling for a change in density within holding terraria between the ages was not required. Due to the relatively
small body size of recently-post metamorphic frogs, the frogs utilized in Year 2 were fed four 1-week-old crickets.

**Statistical Analyses**

We performed statistical analysis using TIBCO Spotfire S+ version 8.1 for Windows. We used logistic regression to test the prediction that infection prevalence in Bd-exposed individuals was negatively associated with age. The most parsimonious model explaining the odds of an individual becoming and maintaining an infection had the following explanatory variables: Bd exposure treatment, species, age, survival, and a Bd exposure treatment by species interaction.

The infection intensity values obtained by qPCR were not normally distributed and it was necessary to log transform (log-mean genome equivalents per individual + 1) these data prior to investigating this response variable. To test the prediction that infection intensity of infected individuals was negatively associated with age, we used a generalized linear model. The model was selected after performing a backwards stepwise comparison process starting with the following explanatory variables: species, Bd-exposure treatment, survival, mass, age, post-exposure day of death, and proportion of observations with individual on the side of the beaker. After investigating the variance inflation factor and performing a simple linear regression, frog mass was determined to be highly correlated with age and was removed from the model. The most parsimonious model had the following explanatory variables: species, Bd-exposure treatment, survival, and age.

We used a Cox proportional hazards model to investigate survival differences between the three exposure treatments and two host species, as well as to test our
prediction that risk and rates of mortality after Bd exposure would increase as hosts aged.

We performed Kaplan-Meier analyses to generate survival curves to visualize the
differences in survival among exposure treatments and ages.

Results

Infection after Bd exposure was not consistent across host ages (Table 3.3).
Infection prevalence increased with age ($\chi^2 = 4.89$, $df=1$, $p = 0.027$) and an individual
was 5.8% more likely to become infected with each 60-day increase in age. However at
any given age, the likelihood of obtaining an infection after exposure did not differ
between species ($\chi^2 = 0.212$, $df=1$, $p = 0.6$) or between the fixed dose and mass-specific
dose Bd-exposure treatments ($\chi^2 = 0.246$, $df=1$, $p = 0.6$). None of the individuals in the
control treatment were infected. However all individuals in the Bd-exposed treatments
that died were infected and mortality before the end of a trial was associated with an
increase in the likelihood of infection ($\chi^2 = 13.3$, $df=1$, $p < 0.001$) when compared to
survivors of the same age.

Host age was positively associated with infection intensity ($t_{336} = 6.23$, $p < 0.001$)
when controlling for host species, Bd-exposure treatment, and survival outcome (Fig.
3.1). Each additional 60-day increase in age was associated with an increase in median
infection intensity by 36.6% (95% CI of 24%-48%). Host species was a significant
predictor of infection intensity ($t_{336} = 4.89$, $p < 0.001$). Red legged frogs displayed 44.9%
lower median intensities (95% CI of 12%-77%) than Pacific treefrogs when holding the
other predictors constant. However, the particular Bd-exposure treatment utilized (fixed
dose or mass-specific dose) was not associated with infection intensity ($t_{336} = 1.92$, $p =$
Survival outcome strongly affected infection intensity at death ($t_{336} = 12.1$, $p < 0.001$). Individuals that survived the 15-day trial had lower median infection intensities by a factor of 19.2 (95% CI of 18.7-19.6).

More mortality occurred in the Bd-exposure treatments with death occurring in 67/382 (17.5%) individuals exposed to Bd, compared with 5/190 (2.6%) unexposed control individuals (Figs 3.2 and 3.3). The negative effect on survival after exposure to the pathogen was not consistent across ages or between host species. The risk of mortality after exposure to Bd differed between species ($LR = 181$, $df = 4$, $p < 0.001$) with 50/67 (74.6%) of the observed Bd-related mortality occurring in red legged frogs resulting in an increase in risk of mortality by a factor of 5.8 (95% CI of 3.3-10.1) compared to Pacific treefrogs of the same age (Figs 3.2 and 3.3). For either species and for any given age, the risk of mortality after exposure to Bd did not differ ($LR = 181$, $df = 4$, $p = 0.57$) between the two Bd exposure treatments. The risk of mortality after Bd-exposure increased with age for both amphibian species (Figs 3.2 and 3.3). Mortality in either of the Bd-exposed treatments was not observed until the third trial (3-weeks post-metamorphosis) for red legged frogs, or until the fifth trial (3-months post-metamorphosis) for Pacific treefrogs.

**Discussion**

Host age can play an important role in determining both the risk of pathogen exposure and the outcome of pathogen exposure in many disease systems (Anderson & May 1991; Hudson & Dobson 1995; Hudson et al. 2002). Infected hosts are often not uniformly distributed across ages within wild populations (Gregory et al. 1992; Duerr et
al. 2003; Jolles et al. 2008; Raffel et al. 2009; Johnson et al. 2011) as even small differences in host competence can result in a skewed distribution of parasites or pathogens (Anderson & May 1978). Investigating the relationship between host age and disease in natural populations can be challenging. This is because it can be difficult to tease apart the relative effect of aging alone, against the background of other host traits that change over time, such as the strength of an acquired immune response which can be affected by previous pathogen exposure, or age-associated changes in host behavior that may alter the risk of pathogen exposure.

In our study, frog age had a strong effect on susceptibility to Bd infection, Bd infection intensity, and survival after exposure to the pathogen, but not in the directions that we had predicted. In both amphibian species, the youngest (newly metamorphosed) frogs were not at a heightened risk of infection or mortality when compared with older frogs. Rather, an increase in age was associated with an increase in frog susceptibility to infection, elevated infection intensities, and greater rates of mortality after exposure to the pathogen. This relationship between frog age and Bd infection prevalence did not differ between the two host species, nor did it differ between the two Bd-exposure treatments. Mortality during the 15-day trials was a strong predictor of positive infection status, and every individual that died during the trials tested positive for infection. Despite spending more time in the presence of the pathogen, survival of the entire 15-day trial was on average associated with a median infection intensity of only 5.21% of the infection intensity observed in individuals that died during the trial. Additionally, host species identity predicted infection intensities, with frogs harboring lower intensities despite on average exhibiting a larger mass.
As expected, exposure to the pathogen resulted in increased mortality. However, risk of mortality was contingent upon age. As seen with infection susceptibility and infection prevalence, the specific Bd-exposure treatment did not result in differences in survival. Moreover, the risk of mortality after exposure differed between the two host species. Red legged frogs were more likely to die during the 15-day trial, despite harboring lower infection intensities. This suggests that differences in tolerance to Bd infection may exist between these two host species or alternatively that red legged frogs may be better able to resist Bd infection than Pacific treefrogs but that this resistance could be costly and might indirectly lead to frog mortality.

Prior to the start of the experiment we predicted that over this range of ages, body size would be highly and positively correlated with frog age. As such, if host susceptibility to Bd infection was dose dependent, then an inoculum made up of a fixed dose of Bd zoospores regardless of frog age would decrease in relative strength as the individual frogs increased in both size and age. Comparing an unexposed control treatment to only a fixed-dose inoculation, would not allow us to properly test our prediction that older and larger frogs would be less sensitive to Bd exposure, as increases in age would be confounded with relatively smaller inoculation doses as frogs aged and grew. We thus included an additional Bd exposure treatment, based on a mass-specific dose. The amount of Bd necessary for this mass-specific dose was calculated from the average mass of the individuals at the time of exposure, and increased in Bd zoospore numbers and concentration as the frogs grew. The two Bd exposure treatments differed in Bd zoospore concentrations yet did not result in different infection intensities or risk of mortality. This held true despite the fixed dose treatment maintaining a total number of
100,000 zoospores regardless of frog mass, and the mass-specific dose treatment ranging between 23,000 to 71,000 zoospores for Pacific treefrogs and between 42,000 to 112,000 zoospores for red legged frogs (Table 3.3).

One could hypothesize that our observations were to be expected if a larger frog body size allowed for a larger infection to take hold for a given inoculation intensity (Kuris et al. 1980), with the increased risk of mortality associated with increased size rather than with age. However if sensitivity to Bd were a function of frog surface area and exposure dose, then we also would expect that infection intensity and risk of mortality would increase with Bd inoculation dose when controlling for frog size. However we did not observe differences in infection intensity or in mortality between the two Bd-exposure treatments, suggesting that the observed differences in sensitivity to Bd were due to an age-effect rather than a mass-effect. Furthermore, this also implies that there is no dose effect within the range of zoospore concentrations in this experiment for these two species of frogs – differences in the concentration of Bd zoospores exposed to a frog do not affect the outcome of exposure.

Others have also observed low infection prevalence rates as well as the survival of infected recently-post-metamorphic Pacific treefrogs in both the laboratory and the field. In a laboratory study, Garcia et al. (2006) showed that exposure to Bd did not increase mortality for recently-post-metamorphic Pacific treefrogs, whereas it did increase mortality in Anaxyrus boreas (western toads) and Rana cascadae (Cascades frog). Similarly, Searle et al. (2013) showed that when larval Pacific treefrogs were exposed to Bd in outdoor mesocosms some individuals cleared the infection during metamorphosis. In field surveys, Piovia-Scott et al. (2011) observed Bd infection prevalence to be lower
in recently-post-metamorphic Pacific treefrogs than in adults and suggested that Bd growth rate might be limited in the Pacific treefrog at the younger ages. Our results are consistent with these studies and provide further evidence to suggest that the Pacific treefrog might be a tolerant carrier of Bd (Padgett-Flohr & Hopkins 2009; Reeder et al. 2012). However, the ability of the Pacific treefrog to tolerate high levels of infection may depend on frog age.

Changes in sensitivity to Bd exposure as frogs aged could have been due to the post-metamorphic development of the frog immune system, which is not fully matured until well after metamorphosis (Flajnik et al. 1987; Rollins-Smith 1998). Mounting an immune response to pathogen exposure can be energetically costly (Sheldon & Verhulst 1996; Ricklefs & Wikelski 2002), and it is possible that the observed increases in sensitivity could have been caused by the changing costs of an immune response to Bd relative to other energetic needs of development or maturity (Kara 2009; Demas et al. 2012). On the other hand, the observed mortality in older frogs could have been the result of the self-inflicted damage potentially caused by the stronger immune reaction to Bd infection, but only after the frog immune system has matured sufficiently (Rollins-Smith & Woodhams 2011).

Investigators using both field and laboratory studies have observed heterogeneity in susceptibility to chytridiomycosis at different ages or stages. Mass mortality events of recently-post-metamorphic frogs have been reported while sympatric larvae of the same species survive nearby (Berger et al. 1998; Bosch et al. 2001; Bradley et al. 2002), implying heterogeneity in susceptibility across life-stages and elevated risk after metamorphosis. Similarly in the laboratory, Bd infected larvae often die soon after
metamorphosis (Rachowicz & Vredenburg 2004; Rachowicz et al. 2006; Garner et al. 2009). Likewise, field studies comparing frogs across post-metamorphic ages have also found elevated Bd infection prevalence and intensities in frogs of younger ages (Briggs et al. 2010; Russell et al. 2010; Walker et al. 2010).

Few experimental studies have investigated the role of post-metamorphic age on the susceptibility to chytridiomycosis and the results have not been consistent. Lamirande and Nichols (2002) observed survival in Bd-exposed sub-adult and adult Blue-and-yellow poison dart frogs, but mortality in recently-post-metamorphic frogs. Ortiz-Santaliestra et al. (2013) showed that American toads (*Anaxyrus americanus*) exposed to Bd 20-d after metamorphosis survived better than those exposed immediately after metamorphosis, however leopard frogs (*Lithobates pipiens*) exhibited no differences in survival between the two ages. A third study (Langhammer et al. 2014) found high mortality in Bd-exposed lab-reared (and Bd-naïve) young coqui frogs (*Eleutherodactylus coqui*) and greater survival in field-caught adult coqui frogs of unknown ages and unknown Bd-exposure history.

In conclusion, our results demonstrate that age can affect amphibian susceptibility to Bd infection. However our prediction that younger frogs would be more susceptible to Bd than older frogs was not supported. For both red legged frogs and Pacific treefrogs, the recently post-metamorphic age was the least susceptible to chytridiomycosis, with sensitivity to Bd increasing as frogs aged. This suggests that the timing or seasonality of Bd exposure may influence amphibian population dynamics for susceptible species. This also suggests that individuals of a susceptible species can act as reservoirs for Bd as recently-post-metamorphic frogs, but may become susceptible hosts as they age. Thus,
understanding the effect of post-metamorphic age on Bd infection is important for predicting the impacts of chytridiomycosis and managing imperiled amphibian populations.

**Acknowledgments**

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Daszak, P., A. Strieby, A. A. Cunningham, J. Longcore, C. Brown, and D. Porter. 2004. Experimental evidence that the bullfrog (*Rana catesbeiana*) is a potential carrier of


Table 3.1. Summary information for Pacific treefrogs (*Pseudacris regilla*) in the experiment investigating the effect of aging on susceptibility to chytridiomycosis. Information includes the approximate post-metamorphic age in each of the eight trials, the sample size for frogs in each exposure treatment and average mass for frogs, the average mass for frogs in each trial, and the concentration of *Batrachochytrium dendrobatidis* (Bd) zoospores in the mass-specific dose treatment for that trial.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Approximate Time Since Metamorphosis</th>
<th>Exposure Treatment</th>
<th>Sample Size</th>
<th>Average Mass in grams for Exposure Treatment (SE)</th>
<th>Average Mass in grams for Trial (SE)</th>
<th>Mass-Specific Bd-Exposure Concentration (zoospores/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1 week</td>
<td>Mass-specific</td>
<td>11</td>
<td>0.448 (0.024)</td>
<td>0.482 (0.029)</td>
<td>2,348</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Control</td>
<td>10</td>
<td>0.479 (0.039)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>2 weeks</td>
<td>Mass-specific</td>
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<td>0.483 (0.022)</td>
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<td>0.528 (0.022)</td>
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<tr>
<td>3</td>
<td>3 weeks</td>
<td>Mass-specific</td>
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<td>0.504 (0.039)</td>
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<td>4</td>
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<td>5 months</td>
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<td>7</td>
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<td>8</td>
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<td>1.382 (0.068)</td>
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Table 3.2. Summary information for red legged frogs (*Rana aurora*) in the experiment investigating the effect of aging on susceptibility to chytriomycosis. Information includes the sample size for frogs in each exposure treatment and average mass for frogs, the average mass for frogs in each trial, and the concentration of *Batrachochytrium dendrobatidis* (Bd) zoospores in the mass-specific dose treatment for that trial.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Approximate Time Since Metamorphosis</th>
<th>Exposure Treatment</th>
<th>Sample Size</th>
<th>Average Mass in grams for Exposure Treatment (SE)</th>
<th>Average Mass in grams for Trial (SE)</th>
<th>Mass-Specific Bd-Exposure Concentration (zoospores/mL)</th>
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<tr>
<td>1</td>
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<tr>
<td>8</td>
<td>9 months</td>
<td>Fixed</td>
<td>12</td>
<td>2.119 (0.122)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mass-specific</td>
<td>12</td>
<td>2.215 (0.173)</td>
<td>2.245 (0.092)</td>
<td>11,219</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Control</td>
<td>12</td>
<td>2.398 (0.177)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 3.3. The proportion infected of Pacific treefrogs (*Pseudacris regilla*) and red legged frogs (*Rana aurora*) after exposure to *Batrachochytrium dendrobatidis* (Bd). Included are the proportion infected in the fixed dose treatment (10,000 zoospores per mL) or the mass-specific dose treatment, and the difference in concentration of Bd zoospores per mL between the two Bd exposure treatments for each trial. Trials one through three were performed on frogs that differed in post-metamorphic age by approximately one week (1-, 2-, 3-weeks post-metamorphosis). Trials four through eight were performed on frogs that differed in post-metamorphic age by approximately 60-days (1-, 3-, 5-, 7-, 9-months post-metamorphosis).

<table>
<thead>
<tr>
<th>Trial</th>
<th>Proportion Infected After Fixed Dose Treatment</th>
<th>Proportion Infected After Mass-Specific Dose Treatment</th>
<th>Difference between the Mass-Specific and Fixed Dose Exposure Concentrations (zoospores/mL)</th>
<th>Proportion Infected After Fixed Dose Treatment</th>
<th>Proportion Infected After Mass-Specific Dose Treatment</th>
<th>Difference between the Mass-Specific and Fixed Dose Exposure Concentrations (zoospores/mL)</th>
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<tr>
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<td>0.9167</td>
<td>-5,740</td>
</tr>
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<td>2</td>
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<td>1.000</td>
<td>-7,422</td>
<td>1.000</td>
<td>0.6667</td>
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<tr>
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<tr>
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<td>1.000</td>
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<tr>
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<td>1.000</td>
<td>1.000</td>
<td>1,092</td>
</tr>
<tr>
<td>7</td>
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<td>1.000</td>
<td>-2,637</td>
<td>0.8333</td>
<td>0.7500</td>
<td>1,180</td>
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<tr>
<td>8</td>
<td>1.000</td>
<td>1.000</td>
<td>-2,867</td>
<td>1.000</td>
<td>0.9167</td>
<td>1,219</td>
</tr>
</tbody>
</table>
Figure 3.1. *Batrachochytrium dendrobatidis* (Bd) infection intensity over eight trials. Infection intensities (on the log scale) measured on the day of death or euthanasia for the Pacific treefrog (*Pseudacris regilla*) and the red legged frog (*Rana aurora*). Trials one through three were performed on frogs that differed in post-metamorphic age by approximately one week (1-, 2-, 3-weeks post-metamorphosis). Trials four through eight were performed on frogs that differed in post-metamorphic age by approximately 60-days (1-, 3-, 5-, 7-, 9-months post-metamorphosis).
Figure 3.2. Kaplan-Meyer survival plots comparing survival among the eight age trials for Pacific treefrogs (*Pseudacris regilla*). Survival in the unexposed control treatment (a), after exposure to *Batrachochytrium dendrobatidis* (Bd) in the fixed dose exposure treatment (b), and after exposure to Bd in the mass-specific exposure treatment. Trials one through three were performed on frogs that differed in post-metamorphic age by approximately one week (1-, 2-, 3-weeks post-metamorphosis). Trials four through eight were performed on frogs differed in post-metamorphic age by approximately 60-days (1-, 3-, 5-, 7-, 9-months post-metamorphosis).
Figure 3.3. Kaplan-Meyer survival plots comparing survival among the eight age trials for red legged frogs (*Rana aurora*). Survival in the unexposed control treatment (a), after exposure to *Batrachochytrium dendrobatidis* (Bd) in the fixed dose exposure treatment (b), and after exposure to Bd in the mass-specific exposure treatment. Trials one through three were performed on frogs that differed in post-metamorphic age by approximately one week (1-, 2-, 3-weeks post-metamorphosis). Trials four through eight were performed on frogs differed in post-metamorphic age by approximately 60-days (1-, 3-, 5-, 7-, 9-months post-metamorphosis).
CHAPTER 4 – DIFFERENCES IN SENSITIVITY TO THE FUNGAL PATHOGEN \textit{BATRACHOCHYTRIUM DENDROBATIDIS} AMONG AMPHIBIAN POPULATIONS

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Abstract
Contributing to the worldwide biodiversity crisis are infectious diseases, which can lead to extirpations and extinctions of hosts. For example, the infectious fungal pathogen *Batrachochytrium dendrobatidis* (Bd) is associated with worldwide amphibian population declines and extinctions. Sensitivity to Bd varies with species, season, and life stage. However, there is little information on whether sensitivity to Bd differs among populations, which is essential for understanding Bd-infection dynamics and for formulating conservation strategies. We experimentally investigated intraspecific differences in host sensitivity to Bd across 10 populations of wood frogs (*Lithobates sylvaticus*) raised from eggs to metamorphosis. We exposed the post-metamorphic wood frogs to Bd and monitored survival for 30 days under controlled laboratory conditions. Populations differed in overall survival and mortality rate. Infection load also differed among populations but was not correlated with population differences in risk of mortality. Such population-level variation in sensitivity to Bd may result in reservoir populations that may be a source for the transmission of Bd to other sensitive populations or species. Alternatively, remnant populations that are less sensitive to Bd could serve as sources for recolonization after epidemic events.

Introduction
Infectious disease is one of the principle threats to global biodiversity (Daszak et al. 2000; Fisher et al. 2012; McCallum 2012) and is increasing in both number and impact (Jones et al. 2008). Most pathogens infect multiple host species (Woolhouse et al. 2001), and one way a disease can emerge is through the transmission from a reservoir
host species to a sensitive species (Engering et al. 2013). Reservoir hosts can harbor and transmit the pathogen without succumbing to disease, potentially maintaining the long-term persistence of a disease across a landscape (Schmidt & Ostfeld 2001; Ostfeld & Keesing 2012) and driving sensitive populations to extinction (Keesing et al. 2010; McCallum 2012).

Differences in infection susceptibility to emerging infectious diseases have been studied extensively at the species level (Haydon et al. 2002; Power & Mitchell 2004; Hughes & Macdonald 2013), but little is known about variability in susceptibility at the population level. Our aim was to investigate differences in infection susceptibility and disease sensitivity to an emerging disease at the population level under controlled experimental conditions in populations of an amphibian host species. Because amphibians are undergoing worldwide population declines and disease is one major contributing factor in these declines (Stuart et al. 2004), amphibians are an ideal model system to examine population differences in disease susceptibility.

The infectious chytrid fungus, *Batrachochytrium dendrobatidis* (Bd), which causes the disease chytridiomycosis (Berger et al. 1998; Longcore et al. 1999), has been associated with numerous amphibian population extirpations and species extinctions (e.g. Stuart et al. 2004; Lips et al. 2006; Vredenburg et al. 2010). Several experimental studies have shown differences in how host species respond to Bd infection (Blaustein et al. 2005; Garcia et al. 2006; Gahl et al. 2011a; Searle et al. 2011; Van Rooij et al. 2012; Gervasi et al. 2013a), and field studies have revealed differences in chytridiomycosis sensitivity within a species across a landscape of environmental gradients (Kriger et al. 2007; Van Sluys & Hero 2009; Savage et al. 2011). At smaller spatial scales, field studies
suggest differences in how Bd is manifested at pond level (Briggs et al. 2005; Brem & Lips 2008; Briggs et al. 2010). However, there is little information on population-level differences in Bd sensitivity. Wood frogs (*Lithobates sylvaticus*) are an excellent species to examine population-level differences in Bd susceptibility. Wood frogs infected with Bd have been observed in the field (Chatfield et al. 2009; Davidson & Chambers 2011), and exposure to Bd can cause mortality in the laboratory (Searle et al. 2011).

Furthermore, this species exhibits strong site fidelity and has a limited home range (Bellis 1965; Vasconcelos & Calhoun 2004), which allows identification of genetically distinct populations (Relyea 2002; Squire & Newman 2002; Cothran et al. 2013; Hua et al. 2013). As synchronous breeders, wood frog eggs from different populations can be collected at approximately the same time across a landscape. This oviposition behavior allowed us to collect wood frog eggs of approximately the same age for our experiment from different populations. We raised these individuals to metamorphosis under common-garden conditions and exposed the recently metamorphic frogs to Bd in a controlled laboratory experiment to test the hypothesis that wood frog populations differ in their sensitivity to Bd infection.

**Materials and Methods**

*Husbandry*

We acquired Wood frogs as eggs to ensure individuals in this study were not previously exposed to Bd. Eggs were collected from 10 populations in northwestern Pennsylvania (U.S.A.) that were 4-80 km apart (Table 4.1). The pathogen is endemic in the region from which we collected eggs (Groner & Relyea 2010). We are unaware of
any published evidence of Bd-infected wood frogs in the region despite monitoring attempts (Glenney et al. 2010; Groner & Relyea 2010). However, it is unknown if any of the populations we collected had previously been exposed to Bd. We collected 10 egg masses from each population; eggs consisted of early-stage embryos (Gosner 1960). The eggs were collected from 4 to 11 April 2011. Immediately after collection, eggs were transported to the University of Pittsburgh, where they were placed in 100-L outdoor pools filled with 90 L of aged well water.

To ensure all eggs hatched at approximately the same time, eggs from populations collected on 11 April (locations: Bowl, Log, Road, and Reed) were held indoors at approximately 20° C in 14-L plastic containers containing 10 L of aged well water, while eggs from the other populations remained outdoors. Once hatched, free-swimming tadpoles were transferred to outdoor pools. Across all 10 populations, all eggs hatched within 48 hours between 24 and 25 April. During the period, when some populations were housed indoors and some populations outdoors (i.e. 11 to 24 April), the outdoor average daily minimum and maximum temperature was 4° and 13° C respectively.

After all 10 populations had free-swimming tadpoles, we moved them into 100-L outdoor mesocosms. Each mesocosm contained 90 L well water, 1 L pond water, 5 g ground alfalfa, and 100 g dried oak leaves (Quercus spp.). We let these mesocosms sit for 15 d to allow the community to develop. We randomly assigned three mesocosms to each population and stocked each mesocosm with 25 tadpoles. Each mesocosm was covered with 60% shade cloth to exclude predators and prevent the escape of the wood frogs as they metamorphosed.

Upon metamorphosis, individuals were transferred to 1-L containers where they
were kept until tail absorption (Gosner 45). Recently metamorphic frogs were fed crickets *ad libitum* before being shipped overnight to Oregon State University (OSU), Corvallis, Oregon (U.S.A.).

Upon arrival at OSU, frogs were transferred to 40-L glass terraria. Terraria were housed in a temperature-controlled room (14 °C) with a 12:12 hour photoperiod, and frogs were allowed to acclimate for 48 hrs. At the start of the experiment, we measured the mass and snout vent length (SVL) of each frog and placed single individuals into 14 x 1 cm Petri dishes with 10 mL of dechlorinated water, where they were housed for the duration of the experiment. Each Petri dish had a lid with three 4-mm holes to provide air circulation. Over the 30-day experiment, we changed the water in the Petri dishes every 7 days and individuals were fed 4, 1-week old crickets twice per week.

*Bd exposure*

Half of the individuals from each population were randomly selected to be either in the Bd-exposed or unexposed treatments. Individual frogs were exposed to Bd strain JEL 274, which was originally isolated from a western toad (*Anaxyrus boreas*) in Colorado (U.S.A.) (Annis et al. 2004). This strain was selected because it was putatively an equally novel strain for each of the ten populations as well as having been deemed one of the more virulent strains associated with major amphibian populations declines (Rosenblum et al. 2013). The pathogen was grown in pure culture on 1% tryptone agar in 10-cm diameter Petri dishes. The Petri dishes were inoculated with liquid culture 8 to 16 days prior to the start of the experiment and incubated at 22 °C. To harvest the zoospores, each plate was flushed with 15 mL of 22 °C dechlorinated water and remained undisturbed for 5 minutes. The plates were scraped with a rubber spatula and the inoculum from each
plate was then pooled in a beaker. The number of moving zoospores was determined using a hemocytometer, and then the solution was diluted to a concentration of $1.03 \times 10^4$ zoospores/mL.

Individuals in the Bd-exposed treatment were exposed to 15 mL of inoculum ($1.55 \times 10^5$ total zoospores) poured directly on their dorsal surface. When added to the 10 mL of water already in the Petri dishes, this additional volume of liquid brought the total volume to 25 mL, which covered the bottom of the Petri dish with a thin film and kept the individuals in constant contact with the water covering the bottom. Control individuals were exposed to 15 mL of inoculum solution lacking the Bd culture (made from 1% tryptone sterile agar plates following the same methods), which we added to the 10 mL of water already in the Petri dishes.

All individuals were observed daily for 30 days following the inoculations. Animals that died during the experiment were preserved in 95% ethanol. Individuals that survived until the end of the experiment (i.e., day 30) were euthanized in a 2% solution of MS-222 and then preserved in 95% ethanol.

We used quantitative polymerase chain reaction (qPCR) to determine Bd-infection prevalence and to quantify Bd-infection load. Following standard protocols (Searle et al. 2010; Gervasi et al. 2014), we randomly selected six Bd-exposed individuals per population. This subsampling examined those individuals that died prior to the end of the experiment to investigate differences in infection load at the time of death as opposed to potential differences in infection loads among individuals that survived the 30-day experiment. Additionally, we quantified Bd-infection status in three randomly sampled unexposed individuals from the control treatment from each population.
To sample individuals for Bd, we used a sterile, fine-tipped, dry swab (Medical Wire and Equipment, Corsham, Wiltshire, England) and swabbed the right ventral surface of individual frogs 10 times, including the feet, legs, and drink patch. We placed each swab into a sterile screw-capped vial. We extracted the DNA by adding 60 µL of Prepman Ultra (Applied Biosystems, Carlsbad, California), heating the vial for 10 minutes at 100 °C, cooling the vials for 2 minutes, and then extracting the supernatant. We diluted supernatant to a 10% solution and then performed the qPCR. We conducted qPCR using an ABI PRISM 7500 sequencer (Applied Biosystems) according to the methods of Boyle et al. (2004). All samples were run in triplicate and averaged. If a sample tested positive for Bd-DNA in only 1 or 2 replicates, we reanalyzed the sample. If a second analysis was required, we re-swabbed the individual on its left side and analyzed the sample from the second swabbing. An individual was considered Bd-positive if all 3 samples (run once) or 4 out of 6 samples (run twice) were positive.

**Statistical Analyses**

We performed statistical analyses in TIBCO Spotfire S+ version 8.1 for Windows. We used a Cox proportional hazards (CPH) model, which allows one to compare the survival of two or more groups (Cox 1972) and provides a hazard ratio (HR) to quantitatively compare the relative survival of groups.

We began with a CPH model to compare the effect of Bd exposure (Bd-exposed versus unexposed), population, and mass on wood frog survival. This analysis allowed us to determine the strength of the effect of exposure to Bd among populations and detect a Bd-exposure-by-population interaction because the survival of non-exposed individuals was high across all populations, whereas the survival of exposed individuals differed
among populations. As a result, we used a subsequent CPH model to examine the effect of population and mass for individuals that were exposed to Bd. This analysis allowed us to determine the strength of the effect of population identity given Bd-exposure and to explicitly test the hypothesis that populations of wood frog differed in their response to Bd exposure. For each of the two CPH analyses, models with all possible combinations of sets of explanatory variables were compared and the model with the largest likelihood ratio (LR) was selected as the most parsimonious (Parmar & Machin 1995). To compare survival among the ten populations when exposed to Bd, we performed a Bonferroni adjustment to maintain an $\alpha = 0.05$ (Gotelli 2012).

We also examined whether individuals exposed to Bd had population-level differences in mass at the start of the experiment. To test for differences in mass, we performed a 1-way analysis of variance (ANOVA) followed by a Tukey-Kramer procedure due to unequal sample size among populations. Additionally, to determine the strength of the relationship between the masses of the 10 populations and the HR of each population we used a simple linear regression (SLR).

A Fisher’s exact test was used to test for differences in infection prevalence. To test for difference in infection loads among populations, we began by log transforming the infection loads obtained by qPCR (log-mean genome equivalents per individual + 1), which was necessary to successfully normalize the data. We then analyzed the effects of population and mass on log-transformed infection loads with an ANCOVA. We then examined the relationship between mass and log-transformed infection loads and the relationship between frog mass and frog length (SVL) with SLR.
**Results**

Survival of unexposed wood frogs was 96%, whereas survival of Bd-exposed wood frogs was 27%. The selected CPH model that tested whether Bd-exposure treatment affected wood frog survival (Table 4.2) contained main effects of all the explanatory variables: Bd-exposure, population, and mass (LR = 303, df = 11). A model including the main effects of all explanatory variables as well as an interaction between Bd exposure and mass was considered but rejected because it explained results no better and was less parsimonious. Across all populations, Bd exposure increased the risk of mortality by a factor of 56.9 (95% CI 26.2, 123.8, df = 11, LR = 303, *p* < 0.001) relative to unexposed animals, yet among populations there was a 6-fold difference in hazard ratios, which ranged from 10.9 to 66.4 (Table 4.3). Additionally, frogs of a smaller mass had a 41% increase in the risk of mortality after exposure to Bd (CI 22.1%, 61.7%, df = 11, LR = 303, *p* < 0.001) for each 0.05 g decrease in mass from the mean mass (0.43 g SE 0.006) of individuals in the Bd-exposure treatment. However, when investigating each population individually, mass was a significant predictor of mortality in only three populations: Blackjack (df = 2, LR = 36.5, *p* < 0.001), Mallard (df = 2, LR = 21.4, *p* = 0.017), and Reed (df = 2, LR = 19, *p* = 0.044), increasing the risk of mortality by factors of 2.03 (95% CI 1.34, 3.08), 1.87 (95% CI 1.12, 3.13), and 1.57 (95% CI 1.01, 2.44) respectively.

For the CPH model that examined the effect of population and frog mass only in the presence of Bd, the largest likelihood ratio was obtained when population and mass were both included as explanatory variables (LR = 82.6, df = 10). Of the 45 pairwise comparisons among the 10 populations, 6 differed in survival after Bonferroni correction.
Two populations (Graveyard and Turkey Track) had high mortality and associated larger HR values; each differed significantly from several other populations with low mortality and smaller HR values (Fig. 4.1, Table 4.4). Survival of individuals from the Graveyard population differed from both the Log and Reed populations and had an increased mortality risk by a factor of 4.9 and 6.1 respectively. Survival of individuals from the Turkey Track population differed from the Square, Log, Relyea, and Reed populations; mortality risk increased by a factor of 4.2, 5.7, 6.5, and 7.4 respectively.

Frog mass differed (ANOVA, df = 9, \( p < 0.001 \)) among populations (Table 4.3). However, population differences in mean mass were not correlated with the population hazard ratios (SLR, df = 8, adjusted \( R^2 = 0.008, p = 0.330 \)), suggesting that mean mass of the population was not related to population-level differences in risk of mortality.

All of the 30 individuals subsampled from the control treatment tested negative for Bd infection. Further, 59 of 60 individuals subsampled from the Bd-exposed treatment tested positive for Bd infection. We found no differences in infection prevalence (Fisher’s exact test, \( p = 1 \)), but infection load differed (ANCOVA, \( F_{9,39} = 2.66, p = 0.016 \)) across populations (Fig. 4.2; Table 4.5) after accounting for mass and ranged from 0.5 to 450 Bd genomic equivalents. Additionally, infection load was positively correlated with frog mass (SLR, df = 57, adjusted \( R^2 = 0.099, p = 0.009 \)); an increase in the median infection load by a factor of 16.8 (95% CI .378, 733) was associated with each 0.1 g increase in body mass. Additionally, there was a mass-by-population interaction in infection load (ANCOVA, \( F_{9,39} = 2.48, p = 0.024 \)) and frog mass was positively correlated with frog length (SLR, df = 365, Adj. \( R^2 = 0.584, p < \))
frog mass increased 0.058 g for every millimeter increase in frog length (95% CI 0.052, 0.063).

**Discussion**

The negative effect of exposure to Bd was not consistent across the 10 populations (4.3). Two populations with high levels of mortality (Graveyard and Turkey Track) differed in survival from several other populations, whereas two other populations with high levels of survival (Reed and Relyea) composed nearly half of all Bd-exposed individuals that survived the 30-day experiment.

Heterogeneity of host responses to a pathogen can result in complex host-parasite dynamics (Dobson 2004; Metcalf et al. 2013; Streicker et al. 2013) at both the species and population levels. A population that is less sensitive to Bd infection may better survive exposure to the pathogen and act as reservoir population, which would allow the disease to persist (Haydon et al. 2002; Mitchell et al. 2008). A reservoir population made up of individuals with elevated infection tolerance may produce and shed more infectious propagules into the environment, or alternatively maintain normal health and behavior, leading to a longer period of shedding propagules or a higher host-host contact rate. Either of these possibilities may allow persistence of chytridiomycosis in the ecosystem, potentially increasing the risk to sympatric species less tolerant to Bd (Venesky et al. 2012) as well as other nearby wood frog populations.

Alternatively, populations made up of individuals that are less sensitive to Bd infection could remain as remnants in the face of epidemics and serve as a source for recolonization after epidemic events. Such individuals would be instrumental in species
persistence and potentially could be used in conservation efforts (Venesky et al. 2012; Scheele et al. 2014). Several layers of complexity may exist when considering such scenarios (Longo et al. 2014; Vander Wal et al. 2014). However, it would be important to determine the processes that contribute to heterogeneity for conservation efforts (Streicker et al. 2013).

Our results suggest a decoupling of infection load, frog mass, and population-level survival patterns in wood frogs. Infection load was positively correlated with mass (Fig. 4.2), yet mass was positively correlated with overall survival of frogs in the Bd-exposed treatment and differences in population mean mass were not correlated with population hazard ratios. Whereas we observed population-level differences in survival (Fig. 4.1), neither mass nor infection load accounted for these differences.

When data across all populations were pooled, we observed a greater proportion of mortality and a faster rate of mortality in smaller individuals within the Bd-exposed treatment. These results are similar to those reported by others (Carey et al. 2006; Searle et al. 2011; Tobler & Schmidt 2011). However, when we investigated the populations individually, we found this same relationship in only three of ten populations (Blackjack, Mallard, and Reed). Further, of the populations that differed in survival (Table 4.4), in only one of six contrasts (Turkey Track – Relyea) did the populations also differ in mass.

Infection load measured at death differed among the ten populations but did not explain population-level differences in survival. Of the six population comparisons that differed in survival (Table 4.4), only two also differed in pathogen load (Turkey Track – Relyea and Turkey Track – Square). Of those two comparisons, pathogen load and survival were positively associated for one comparison (Turkey Track – Relyea) and
negatively associated for the other (Turkey Track – Square). The remaining four comparisons had similar levels of infection load as measured at death despite differences in population survival, suggesting that these wood frog populations differed in their ability to tolerate infection loads of a given magnitude. With near 100% mortality in the Bd-exposed treatment, neither the Graveyard nor Turkey Track populations tolerated similar infection loads that resulted in lower levels and rates of mortality in the Log or Reed populations.

Despite uniform sample sizes across all ten populations when individual tadpoles were moved to the outdoor mesocosms, we initiated the laboratory experiment with unequal sample sizes based on the number of animals that successfully metamorphosed in each group (Table 4.1). There, however, were no clear trends between the losses prior to the start of the laboratory experiment and any explanatory or response variable investigated.

In a study of chytridiomycosis in post-metamorphic common midwife toads (*Alytes obstetricans*), Tobler and Schmidt (2011) investigated survival across three populations under controlled conditions. Using individuals collected as 1-year-old larvae that tested positive for Bd infection, they too found that populations differed in their response to Bd exposure. However in our study, all individuals were raised from eggs to metamorphosis under similar conditions and housed in the same laboratory under identical environmental conditions. Thus, the differences in mortality we observed can be explained by intrinsic biology (e.g., genetic differences) of individuals among the populations; neither abiotic and biotic environmental differences among ponds nor differences in host density are necessary to explain observed population-level differences
in survival in the presence of chytridiomycosis. To our knowledge, this is the first study to empirically show population-level differences in chytridiomycosis-related survival experimentally under identical environmental conditions with individuals previously unexposed to the pathogen.

Our study was designed to test the hypothesis that wood frog populations differ in their response to exposure to Bd. However, our study does not reveal the mechanism or mechanisms responsible for population-level differences in sensitivity to chytridiomycosis. Differences in anti-microbial peptides (Rollins-Smith & Conlon 2005; Woodhams et al. 2006), skin microbiota (Harris et al. 2009; Lam et al. 2009), MHC genotypes (May et al. 2011; Savage & Zamudio 2011), or behavior (Rowley & Alford 2007; Venesky et al. 2011; Hossack et al. 2013) may all influence sensitivity to Bd and may all vary across populations. Our experiment was performed on animals collected as eggs and raised under identical conditions; thus, our results strongly suggest one of the above-mentioned mechanisms or some other genetic component is affecting the sensitivity of individuals to chytridiomycosis in this species.

Our experiment demonstrates that there is heterogeneity in wood frog sensitivity to Bd infection at the population level. Furthermore, because this experiment was performed under controlled environmental conditions, the observed population-level differences in survival after exposure to the pathogen can be credited to intrinsic biological factors of the host populations rather than to environmental differences between the locations from where the populations were collected.

Whereas population-level heterogeneity in sensitivity may result in reservoir populations acting to maintain or amplify the pathogen across the landscape, there is,
however, another side to this coin. After an epidemic episode such heterogeneity could allow for the recovery (Newell et al. 2013) or emigration and recolonization of areas that previously were inhabited by members of a host metapopulation. Moreover, individuals of populations surviving such an episode could be used in conservation efforts and captive breeding programs with the intention of reintroduction or translocation of individuals to areas where the species had been extirpated. However, the sensitivity of a population to infection is complex at both the individual and population levels. Heterogeneity in sensitivity could be due to within-host differences in resistance or tolerance or within-population differences in host behavior, in addition to being context dependent. Once surviving individuals are released into the field, these causes could be acted upon by natural selection in complex ways that might negate the artificial selection performed in captivity. Similarly, a lack of sensitivity observed under controlled conditions might not equate to persistence in the dynamic natural environment.

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Literature Cited


Table 4.1. Information on the populations of wood frog used in the study of heterogeneity of sensitivity to chytridiomycosis.

<table>
<thead>
<tr>
<th>Population</th>
<th>Location</th>
<th>Date Collected</th>
<th>Sample Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blackjack</td>
<td>41 39.934 N, 80 30.762 W</td>
<td>4 April 2011</td>
<td>48</td>
</tr>
<tr>
<td>Bowl</td>
<td>41 55.625 N, 79 48.234 W</td>
<td>11 April 2011</td>
<td>38</td>
</tr>
<tr>
<td>Graveyard</td>
<td>41 41.062 N, 80 02.837 W</td>
<td>5 April 2011</td>
<td>40</td>
</tr>
<tr>
<td>Log</td>
<td>41 58.147 N, 79 35.922 W</td>
<td>11 April 2011</td>
<td>37</td>
</tr>
<tr>
<td>Mallard</td>
<td>41 41.518 N, 80 30.046 W</td>
<td>4 April 2011</td>
<td>26</td>
</tr>
<tr>
<td>Reed</td>
<td>41 58.801 N, 79 58.093 W</td>
<td>4 April 2011</td>
<td>52</td>
</tr>
<tr>
<td>Relyea</td>
<td>41 37.341 N, 80 27.261 W</td>
<td>6 April 2011</td>
<td>34</td>
</tr>
<tr>
<td>Road</td>
<td>41 53.078 N, 79 36.320 W</td>
<td>11 April 2011</td>
<td>24</td>
</tr>
<tr>
<td>Square</td>
<td>41 50.486 N, 80 14.402 W</td>
<td>7 April 2011</td>
<td>32</td>
</tr>
<tr>
<td>Turkey Track</td>
<td>41 37.823 N, 79 54.769 W</td>
<td>4 April 2011</td>
<td>36</td>
</tr>
</tbody>
</table>
### Table 4.2

Candidate Cox proportional hazards models of survival of frogs exposed to *Batrachochytrium dendrobatidis* (Bd) among the ten populations, in increasing order of likelihood ratio.

<table>
<thead>
<tr>
<th>Model</th>
<th>Likelihood Ratio (LR)</th>
<th>df</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>15.4</td>
<td>1</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>P</td>
<td>18.4</td>
<td>9</td>
<td>&lt; 0.031</td>
</tr>
<tr>
<td>P+M</td>
<td>30.9</td>
<td>10</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Bd</td>
<td>220</td>
<td>1</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Bd+M</td>
<td>252</td>
<td>2</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Bd*M</td>
<td>253</td>
<td>3</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Bd+P</td>
<td>279</td>
<td>10</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Bd*M+P</td>
<td>303</td>
<td>12</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Bd+M+P</td>
<td>303</td>
<td>11</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Model</th>
<th>Likelihood Ratio (LR)</th>
<th>df</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>29.6</td>
<td>1</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>P</td>
<td>59.7</td>
<td>9</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>P+M</td>
<td>82.6</td>
<td>10</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

Key: M, mass measured at the start of the experiment (mg); P, population.
Table 4.3. Summary information for 10 populations of wood frogs used in the study of heterogeneity of sensitivity to chytridiomycosis in decreasing order of hazard ratio when comparing survival between the Bd-exposed and unexposed control treatments.

<table>
<thead>
<tr>
<th>Population</th>
<th>Mean Body Size (SE)</th>
<th>Mean Body Size 95% CI</th>
<th>Median Survival Time</th>
<th>Hazard Ratio</th>
<th>Hazard Ratio 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Graveyard</td>
<td>0.450 g (0.010)</td>
<td>0.430, 0.470</td>
<td>4.5 days</td>
<td>66.4</td>
<td>8.21, 537</td>
</tr>
<tr>
<td>Bowl</td>
<td>0.348 g (0.012)</td>
<td>0.324, 0.371</td>
<td>4 days</td>
<td>59.4</td>
<td>7.46, 473</td>
</tr>
<tr>
<td>Turkey Track</td>
<td>0.446 g (0.010)</td>
<td>0.424, 0.467</td>
<td>4 days</td>
<td>59.1</td>
<td>7.39, 472</td>
</tr>
<tr>
<td>Mallard</td>
<td>0.429 g (0.014)</td>
<td>0.400, 0.459</td>
<td>5 days</td>
<td>31.2</td>
<td>3.54, 274</td>
</tr>
<tr>
<td>Blackjack</td>
<td>0.446 g (0.010)</td>
<td>0.423, 0.468</td>
<td>5 days</td>
<td>27.6</td>
<td>5.73, 133</td>
</tr>
<tr>
<td>Log</td>
<td>0.405 g (0.009)</td>
<td>0.387, 0.423</td>
<td>27 days</td>
<td>21.5</td>
<td>2.68, 173</td>
</tr>
<tr>
<td>Road</td>
<td>0.407 g (0.019)</td>
<td>0.366, 0.446</td>
<td>7 days</td>
<td>21.3</td>
<td>2.58, 176</td>
</tr>
<tr>
<td>Reed</td>
<td>0.427 g (0.011)</td>
<td>0.404, 0.450</td>
<td>NA</td>
<td>20.3</td>
<td>2.59, 160</td>
</tr>
<tr>
<td>Square</td>
<td>0.428 g (0.010)</td>
<td>0.408, 0.449</td>
<td>6 days</td>
<td>20.2</td>
<td>2.54, 160</td>
</tr>
<tr>
<td>Relyea</td>
<td>0.531 g (0.014)</td>
<td>0.502, 0.560</td>
<td>NA</td>
<td>10.9</td>
<td>1.33, 88</td>
</tr>
</tbody>
</table>

* Individuals in the Bd-exposed treatment for the Reed and Relyea populations never reached 50% mortality.
Table 4.4. Summary information for the 6 wood frog population contrasts that differed in survival after exposure to *Batrachochytrium dendrobatidis* (Bd), and after a Bonferroni-adjustment to maintain an $\alpha = 0.05$.

<table>
<thead>
<tr>
<th>Population by population comparison</th>
<th>Hazard Ratio</th>
<th>Hazard Ratio 95% CI</th>
<th>df</th>
<th>Likelihood Ratio (LR)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Graveyard : Log</td>
<td>4.9</td>
<td>2.16, 11.0</td>
<td>2</td>
<td>66.4</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Graveyard : Reed</td>
<td>6.1</td>
<td>2.71, 137</td>
<td>2</td>
<td>59.4</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Turkey Track : Square</td>
<td>4.2</td>
<td>1.87, 9.52</td>
<td>2</td>
<td>59.1</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Turkey Track : Log</td>
<td>5.7</td>
<td>2.49, 13.1</td>
<td>2</td>
<td>31.2</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Turkey Track : Relyea</td>
<td>6.5</td>
<td>2.41, 17.5</td>
<td>2</td>
<td>27.6</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Turkey Track : Reed</td>
<td>7.4</td>
<td>3.25, 16.9</td>
<td>2</td>
<td>21.5</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>
Table 4.5. Summary statistics for infection load over the ten populations of wood frog including sample size, median infection load, mean infection load, and 95% CI for mean infection load (log (1+ Bd genomic equivalents)) for each population.

<table>
<thead>
<tr>
<th>Population</th>
<th>Sample Size</th>
<th>Median Infection Load</th>
<th>Mean Infection Load (SE)</th>
<th>Mean Infection Load 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Graveyard</td>
<td>6</td>
<td>2.92</td>
<td>2.99 (0.20)</td>
<td>2.47, 3.50</td>
</tr>
<tr>
<td>Bowl</td>
<td>6</td>
<td>2.75</td>
<td>2.79 (0.31)</td>
<td>2.00, 3.59</td>
</tr>
<tr>
<td>Turkey Track</td>
<td>6</td>
<td>4.09</td>
<td>4.33 (0.38)</td>
<td>3.34, 5.31</td>
</tr>
<tr>
<td>Mallard</td>
<td>6</td>
<td>2.34</td>
<td>2.80 (0.43)</td>
<td>1.70, 3.90</td>
</tr>
<tr>
<td>Blackjack</td>
<td>6</td>
<td>3.07</td>
<td>3.08 (0.27)</td>
<td>2.38, 3.79</td>
</tr>
<tr>
<td>Log</td>
<td>6</td>
<td>3.64</td>
<td>3.45 (0.34)</td>
<td>2.59, 4.31</td>
</tr>
<tr>
<td>Road</td>
<td>5</td>
<td>2.73</td>
<td>2.77 (0.70)</td>
<td>0.82, 4.72</td>
</tr>
<tr>
<td>Reed</td>
<td>6</td>
<td>3.79</td>
<td>3.88 (0.41)</td>
<td>2.82, 4.94</td>
</tr>
<tr>
<td>Square</td>
<td>6</td>
<td>3.40</td>
<td>3.33 (0.30)</td>
<td>2.57, 4.09</td>
</tr>
<tr>
<td>Relyea</td>
<td>6</td>
<td>4.49</td>
<td>4.29 (0.66)</td>
<td>2.60, 5.97</td>
</tr>
</tbody>
</table>
Figure 4.1. Kaplan-Meier survival curves comparing the proportion survival of wood frogs in the Bd-exposed treatment. Survival over the 30-day experiment in the Graveyard, Log, and Reed populations (a) and in the Turkey Track, Square, Log, Relyea, and Reed populations (b).
Figure 4.2. The *Batrachochytrium dendrobatidis* (Bd) infection intensity in the 10 populations of wood frog. Boxes, median and interquartile range whiskers representing the 2 most extreme data points within 1.5 x IQR from the edge of the box. Summary statistics including 95% CI for the mean infection load for each population is in Table 4.5.
CHAPTER 5 – CONCLUSION

Paul W. Bradley
The “biodiversity crisis” represents an urgent and profound loss, the magnitude of which is unprecedented in the modern era. The current extinction rate is estimated to be up to 1000 times greater than the expected background rate (Pimm et al. 1995; De Vos et al. 2015) and nearly one-fifth of all vertebrate species are under the threat of extinction (IUCN 2012). Many scientists now agree that we are in the midst of Earth’s sixth major mass extinction event (Wake & Vredenburg 2008; Kolbert 2009; Barnosky et al. 2011; Ceballos et al. 2015).

In acknowledgement of the growing number of species extinctions, over 100 members of the United Nations signed the World Charter for Nature in 1982, which states, “Every form of life is unique, warranting respect regardless of its worth to man, and, to accord other organisms such recognition, man must be guided by a moral code of action” (Noss & Cooperrider 1994). All species have intrinsic value and the extinction of any one species is a loss. However, amphibians are important for a variety of instrumental and intrinsic reasons, and their losses are exceptionally troublesome.

Amphibian abundance and in turn the magnitude of their ecological impacts are often underappreciated. In many areas of the tropics amphibians are the most numerically abundant of all land vertebrates (Stebbins & Cohen 1995). Additionally, amphibians are important players in ecosystem energy flow. As larvae, they can exert strong ecological effects on algae and aquatic plants (Altig et al. 2007), and in their post-metamorphic life stage they often can be the chief vertebrate predator on invertebrates in many freshwater, arboreal, or fossorial environments (Stebbins & Cohen 1995). Further, as ectotherms they are an exceptionally efficient at transferring energy from aquatic plants, invertebrates, or aquatic environments to organisms elsewhere in the food web or elsewhere in the
terrestrial environment (Pough 1983; Pough et al. 2004). Thus, because of that which amphibians feed upon, and that which in turn feeds upon amphibians, the loss of amphibians has cascading effects throughout both aquatic and terrestrial food webs.

We can also assign value to extant amphibians because of the economic and aesthetic benefits they provide to humankind. Many cultures consume amphibians as part of their regular diet (Warkentin et al. 2009), and many other cultures care for them and consider them pets (Schlaepfer et al. 2005). Additionally, pharmaceuticals have been discovered or adapted from amphibian metabolites (Clarke 1997; VanCompernolle et al. 2005; Lu et al. 2008). For many the chorus of frogs calling and croaking holds aesthetic value (Wilson 1984). Moreover, many cultures consider amphibians to be signs of good luck due to their historical and cultural relationship with rains and reproduction (Collins et al. 2009).

Lastly, many consider amphibians to be “canaries in a coal mine” or bioindicators of environmental degradation (Vitt et al. 1990; Blaustein 1994; Morell 1999; Halliday 2000). There are several traits shared by many amphibians that may make them particularly sensitive to environmental changes. These include, an amphibious nature exposing them to changes in either the aquatic or terrestrial environments, skin that is permeable to many compounds and substances, using sunlight to regulate their body temperature, which could expose them to dangerous UV-B radiation, and lastly their tight relationship with water can increase their sensitivity to both extreme cold and drought (Stebbins & Cohen 1995).

Unfortunately, as part the global loss of biodiversity many amphibian populations worldwide are declining or disappearing altogether (Stuart et al. 2004). The causes of
these declines are complex and often context dependent (Kiesecker et al. 2001; Blaustein & Kiesecker 2002; Blaustein et al. 2010; Blaustein et al. 2011; Doddington et al. 2013), however one cause that has been linked to declines across both geographic ranges and taxonomic groups is infectious disease (Daszak et al. 1999; Blaustein et al. 2011; Hoverman et al. 2012; Price et al. 2014). The role that infectious disease can play in regulating wildlife populations has only recently been appreciated (Anderson & May 1978; Hudson et al. 2008) and further, the ability for pathogens to drive populations of their hosts to extinction is only beginning to be understood (Smith et al. 2006).

Amphibians are susceptible to many infectious diseases (Densmore & Green 2007), however one disease that has been associated with numerous population declines and species extinctions is chytridiomycosis (Skerratt et al. 2007; Vredenburg et al. 2010; Adams et al. 2013). This disease is caused by the fungal pathogen Batrachochytrium dendrobatidis (Bd) (Berger et al. 1998; Longcore et al. 1999), which can be found on every continent where amphibians are found and has been documented in over 500 host species (Olson et al. 2013).

In this thesis I investigated several biotic factors and an abiotic factor that can influence chytridiomycosis in several frog species. Enhanced knowledge on this topic is necessary if we are to limit further losses of amphibian biodiversity and the cascade effects caused by the losses of these ecologically important species. In Chapter 2, I tested the climate variability hypothesis and the “lag effect” following short-term temperature shifts in larvae of two species of amphibian that host Bd. I also investigated the degree to which temperature shifts can affect Bd infection prevalence and infection intensity, in addition to synergistically with Bd to affect survival patterns. Similar effects were
observed in both red legged frog (*Rana aurora*) and western toad (*Anaxyrus boreas*) tadpoles, when comparing tadpoles exposed to constant versus shifted temperatures. A shift from the warm temperature to the colder temperature was associated with a decrease in Bd abundance whereas a shift from the cold temperature to the warmer temperature increased Bd abundance. In Chapter 3, I explored the ontogeny of susceptibility to Bd infection in red legged frogs (*Rana aurora*) and Pacific treefrogs (*Pseudacris regilla*) over the first nine months post-metamorphosis. Previous research had suggested that frogs that had recently undergone metamorphosis where more susceptible to chytridiomycosis than at any other age or stage of development. However, I found that the youngest frogs were the least susceptible to chytridiomycosis and increasing age was associated with an increase in the likelihood of Bd infection, increase in infection intensity, and increase in risk of mortality following infection. In Chapter 4, using individuals collected as eggs and raised in a common garden environment, I examined differences in response to Bd infection among 10 distinct populations of wood frog (*Lithobates sylvaticus*). I detected differences in survival after Bd-exposure among the populations but did not observe differences in Bd infection intensity. This suggests that populations of wood frog share a similar level of resistance to Bd infection, but can differ in their levels of tolerance to Bd infection of a given intensity.

Bd-associated amphibian population declines are complex phenomena with numerous factors interacting to affect the severity and ultimate outcome of Bd exposure. One central theme is present in my research – at varied levels of biological organization, amphibians do not all respond similarly to Bd. Even under controlled laboratory conditions, amphibian host species did not react similarly to Bd exposure or other
treatments. No one model amphibian host species will suffice in exploring or predicting chytridiomycosis in natural systems. Likewise, as documented in Chapter 4, intraspecific populations of an amphibian host species did not respond similarly to Bd exposure. Furthermore, the results in Chapter 3 highlight differences in susceptibility to chytridiomycosis among individuals that differed in age by only a few weeks – despite those individual frogs consisting of members of the same population, cohort. In turn, management decisions should only cautiously utilize research performed on non-target amphibian host species, populations, developmental stages, or ages. Thoughtfulness is required when interpreting and extrapolating results from one study system to a focal system.

Lastly, the experiments outlined in this dissertation were performed in the laboratory environment under controlled conditions. And it is important to note that the laboratory setting is far from the natural environment that Bd or any of the amphibian host species inhabit. Thus some degree of caution is required when interpreting the results and predicting how faithfully they would be mirrored in a more realistic environment or in natural communities.

In summary, this dissertation provides further evidence for interspecific differences in host susceptibility to chytridiomycosis; host species did not respond identically to Bd exposure as I documented in both Chapter 2 and Chapter 3. Moreover, as described in Chapter 3, I observed that heterogeneity in susceptibility can be caused in part by interspecific differences in tolerance to Bd infections. In addition to interspecific differences, additional biotic drivers of this heterogeneity in susceptibility to chytridiomycosis were also documented. In Chapter 3, I elucidated the relationship of
increasing post-metamorphic host age on increasing susceptibility to chytridiomycosis and in Chapter 4 I described intraspecific population-level differences in susceptibility for individuals raised under identical environmental conditions. Lastly, in Chapter 2 I investigated temperature as a potential abiotic driver of chytridiomycosis dynamics and found that temperature shift prior to Bd exposure can alter host susceptibility to chytridiomycosis.

This dissertation describes and helps disentangle the biotic drivers and abiotic drivers of chytridiomycosis, one of the causes of worldwide biodiversity loss. Conservation of these imperiled amphibians will depend in part on managing emerging infectious diseases such as chytridiomycosis. Understanding the roles of species identity, population identity, host age, and temperature on the impact of chytridiomycosis on amphibian populations will help focus conservation efforts and help mitigate the losses due to this disease.
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