

AN ABSTRACT OF THE DISSERTATION OF

Barbara A. Han for the degree of Doctor of Philosophy in Zoology presented on September 26, 2008.

Title: The Effects of an Emerging Pathogen on Amphibian Host Behaviors and Interactions.

Abstract approved:

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Contemporary environmental change encompasses massive biodiversity loss and increasing numbers of emerging diseases worldwide. As part of a global biodiversity crisis, amphibians are disappearing at unprecedented rates. *Batrachochytrium dendrobatidis* is an emerging infectious pathogen prominently associated with many declines. Chapter 1 reviews the past decade of research on this system and highlights areas where knowledge is notably lacking.

Host behavior remains a crucial determinant of host-pathogen dynamics yet studies addressing the effects of *Batrachochytrium* on amphibian behaviors are virtually nonexistent. Remaining chapters examine behavioral responses of host species to *Batrachochytrium*. Chapter 2 examines how ancient behaviors that have persisted in amphibians for millions of years change with exposure to *Batrachochytrium*. I examined thermoregulatory behavior in tadpoles of four species (*Pseudacris regilla*, *Rana aurora*, *Bufo boreas*, *Rana cascadae*), and aggregation

behavior in two species that school as tadpoles (*B. boreas*, *R. cascadae*). Results suggest that some amphibians will continue seeking optimal temperatures and continue aggregating regardless of infection risk. I discuss the importance of behavioral plasticity and evolutionary inertia in interpreting host behavioral responses to infection.

Chapter 3 examines *Batrachochytrium* dynamics when multiple host species interact. I manipulated infection status in tadpoles of three naturally co-occurring hosts (*P. regilla*, *B. boreas*, *R. cascadae*) in various combinations and measured growth, survival and infection severity. There were strong interactions between species combinations and infection leading to pathogen-mediated mutualism and competition. Results also suggest that both species richness and species identity may be important factors moderating a dilution effect in this system.

Coexisting, interacting hosts must also contend with predators in a community. Chapter 4 explores *Batrachochytrium*-induced changes in antipredator behaviors in four species (*P. regilla*, *R. aurora*, *B. boreas*, *R. cascadae*). I also examined whether antipredator behaviors increased survivorship in the presence of lethal predators in *R. cascadae*, *R. aurora*. Exposure to *Batrachochytrium* changed activity rate and refuge use in *Bufo*, but not in the other species. Nonselective predation of *Batrachochytrium*-exposed prey by susceptible predators adds an unexplored dimension of complexity to this system.

Chapter 5 summarizes the ecological implications of studies presented in this dissertation.

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THE EFFECTS OF AN EMERGING PATHOGEN ON  
AMPHIBIAN HOST BEHAVIORS AND INTERACTIONS.

by

Barbara A. Han

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

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Barbara A. Han, Author

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## CONTRIBUTION OF AUTHORS

Chapter 2: Dr. Andrew Blaustein helped design the experiments, analyze data, and interpret results and Paul Bradley helped carry out experiments and collect data.

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THE EFFECTS OF AN EMERGING PATHOGEN ON  
HOST BEHAVIORS AND INTERACTIONS

CHAPTER 1.

A GLOBALLY EMERGING INFECTIOUS PATHOGEN OF  
AMPHIBIANS: REVIEW AND INTRODUCTION

Biodiversity loss ranks among the most pressing environmental issues of our time. Many scientists argue that we are experiencing the sixth mass extinction event in the history of our planet (Wake and Vredenburg 2008). Changes in global climate, the introduction of exotic, invasive species, and habitat destruction and degradation are major contributors to declining biodiversity (Chapin III et al. 2002). It is difficult to forecast the effects of species loss. One of the most unpredictable and unsettling consequences of declining biodiversity is its relationship with the emergence of infectious diseases which may be stimulated by altered environmental conditions (Daszak et al. 2000; Harvell et al. 2002; Patz et al. 2004). The rising number of emerging disease events around the world (Jones et al. 2008) has helped breach an anthropocentric mindset to spotlight the consequences of declining biodiversity for both human and wildlife populations (Millennium Assessment 2005).

Is infectious disease a cause or a consequence of biodiversity loss? A recent analysis shows that while species extinctions caused directly by infectious agents are historically rare, epizootics (wildlife disease epidemics) remain a threat to shrinking host populations or populations that are infected by pathogens that persist in reservoirs (de Castro and Bolker 2005).

### Amphibian declines and a novel pathogen

Amphibians have survived every mass extinction event since their emergence in the Late Devonian (approximately 360 million years ago, reviewed in Wake and

Vredenburg 2008). But in the past two decades, worldwide amphibian declines and extinctions have attracted international attention because they are occurring at unprecedented rates (Stuart et al. 2004). Amphibians may be especially prone to population declines and extinctions for a number of reasons. Amphibians have porous skin and most species have complex life-cycles that span both water and land during development. Their unique life history makes them vulnerable to anthropogenic changes in aquatic and terrestrial habitats, and increases their risk of exposure to waterborne, airborne, and terrestrial contaminants (reviewed in Blaustein et al. 2003). These challenges combine and interact with others including invasive species, climate change, ultraviolet-B radiation, a range of environmental contaminants (pesticides, nitrification), and infectious pathogens to precipitate population declines and extinctions (reviewed in Blaustein and Kiesecker 2002).

In the past decade, the interaction between abiotic factors and infectious agents has been examined in depth. For example, ultraviolet-B radiation increases mortality in amphibian embryos infected with a pathogenic water mold, *Saprolegnia ferax*, that is commonly found in permanent water bodies as a result of stocking fishes for recreational fishing (Kiesecker and Blaustein 1995). The eutrophication of aquatic habitats associated with agriculture, sewage, and livestock is linked to the infection of amphibian larvae by a parasitic trematode (*Ribeiroia ondatrae*) that causes multiple limbs and limb deformities in postmetamorphic amphibians (Johnson et al. 2007). Opportunistic bacterial infection by *Pseudomonas* can be lethal if amphibians are stressed by factors such as decreasing pH in aquatic environments (Brodtkin et al.

2003). In addition to these, researchers continue to discover novel pathogens capable of causing high mortality in local populations (Davis 2007). Though these infectious agents are not known to have caused extinctions, the past decade has witnessed mass mortality and population extinctions of several amphibian species associated with a newly emerging fungal pathogen, *Batrachochytrium dendrobatidis* (BD).

*Batrachochytrium* was discovered in the United States when researchers examined various species of moribund and recently deceased poison-dart frogs (*Dendrobates* spp.) at the National Zoological Park in Washington, DC in 1996-1997 (Pessier et al. 1999). Independently and concurrently, scientists were investigating sharp declines in several amphibian populations in the rainforests of Queensland, Australia and Central America between 1989-1999 (reviewed in Berger et al. 1999b), which were controversially hypothesized to be the result of epidemic disease (Alford and Richards 1997; Laurance et al. 1996; Laurance et al. 1997). This novel pathogen was isolated in pure culture in the late 1990s and described as a fungus pathogenic to amphibians (Berger et al. 1998; Longcore et al. 1999) and the etiologic agent of the disease amphibian chytridiomycosis.

The discovery of BD set off a decade of international, interdisciplinary research propelled by the urgency of ongoing catastrophic declines of amphibian populations in Australia, North America and Central America (Berger et al. 1998; Lips et al. 2006; Rachowicz et al. 2006; Ryan et al. 2008). Our current understanding of this host-pathogen system comes from related but distinct areas of research on the

biology of the BD pathogen, amphibians as hosts and studies examining the environmental context and geographic distribution of infection in natural populations.

### The amphibian chytrid fungus

*Batrachochytrium dendrobatidis* was placed in a new genus *Batrachochytrium* (Longcore et al. 1999) in the phylum Chytridiomycota. Members of this phylum are important saprobes of chitin, cellulose and keratin, and can be found in diverse environments (e.g. rainforest, temperate forest, desert, tundra; reviewed in Berger et al. 1999b). BD is the only species in the phylum that is pathogenic to vertebrates (Berger et al. 1998; Longcore et al. 1999). Though BD is newly described, genetics and life history characteristics suggest that it is evolutionarily adapted to parasitizing the keratinizing epidermal tissue of amphibians (Berger et al. 2005a).

The BD lifecycle begins when an infectious flagellated zoospore enters keratinized epidermal cells of an amphibian host and grows into a thallus (Berger et al. 2005a). Since keratin is necessary for BD growth and reproduction, infectious zoospores only infect postembryonic stages of amphibian hosts (larvae, juveniles, and adults). The lack of keratin in amphibian embryos protects them from infection. Within hosts, thalli typically cleave to form several clonal zoospores (a zoosporangium). Immature zoosporangia occur in deeper layers of the epidermis (the stratum corneum and stratum granulosum), while mature or empty zoosporangia occur in the most superficial cell layers. The developmental rate of BD tracks closely with

developmental stages of the epidermis. As superficial cell layers are shed and replaced with underlying cell layers, the embedded zoosporangia grow discharge papillae oriented towards the external surface of host tissue from which mature zoospores are released directly into the environment. In laboratory conditions, motile zoospores actively swim an average of 2 cm before encysting (Piotrowski et al. 2004), and are chemotactic towards various nutrient cues which presumably allow them to locate new hosts in the water (Moss et al. 2008). Zoospores are also attracted to BD colonies (Berger et al. 2005c), and are more successful at colonizing host tissue in groups rather than singly or in low numbers (Longcore et al. 1999). Thus BD infections usually consists of zoosporangia clustered in patches on the epidermis of infected hosts (Longcore et al. 1999; Weldon and Du Preez 2006), suggesting that high pathogen loads caused by reinfection of the same host (superinfection) may be common. These observations corroborate experimental evidence that still or slowly moving water may influence BD infection severity by leading to an accumulation of infectious zoospores in aquatic habitats (Tunstall 2007). Though flagellated and motile, BD resembles other members of the order Chytridiales that are most effectively transported by flowing water or host movement (Berger et al. 1999b; Kriger and Hero 2007a; Sparrow 1968).

The life history of BD is largely affected by temperature (Woodhams 2008). Laboratory studies show that optimal temperatures for BD growth are between 17-25°C (Piotrowski et al. 2004). BD does not appear to be sensitive to ultraviolet light (270-320 nm), but is easily killed by several disinfectants including commonly used

sodium chloride, sodium hypochlorite, and iodine (Johnson et al. 2003a; Webb et al. 2007). BD growth is also influenced by pH, with maximum growth rates occurring at near neutral pH (6.5-7) and growth slowing at very basic (pH 8-9) and acidic (pH 3-4) levels (Johnson and Speare 2005; Piotrowski et al. 2004).

BD is especially sensitive to desiccation and requires a moist environment for survival and transmission (Johnson et al. 2003a). In sterile water BD zoospores can remain viable for up to 2 months (Johnson and Speare 2003). Though BD is seemingly dependent on water for transmission, it has also been shown to successfully colonize bird feathers and snake skin (Johnson and Speare 2005). BD can also survive in sterile, moist river sand for up to 3 months, even with mild drying (between 1-3 hours, Johnson and Speare 2005). New evidence of an encysted form of BD suggests one possible mode of resistance to environmental fluctuation (Di Rosa et al. 2007). An initial study that utilized multilocus sequencing of BD isolates from different continents and host species showed a highly conserved genome, suggesting that all strains of BD are clones (Morehouse et al. 2003). However, a recent study shows that genetic recombination, most likely through sexual reproduction through a resistant spore stage, is occurring in BD found in geographically isolated host populations of the Sierra Nevada of California (Morgan et al. 2007). New evidence of genetic variability is perhaps not surprising, as the wide variation in pathogenicity and virulence observed between BD isolates suggests that key genetic differences underlie this variation (Berger et al. 2005b).

### Amphibian hosts

BD parasitizes amphibians, but has no host specificity within amphibians (Morgan et al. 2007) infecting frogs and salamanders from taxonomically diverse across multiple life history stages (e.g., Blaustein et al. 2005; Davidson et al. 2003; Garcia et al. 2006; Padgett-Flohr and Longcore 2007). BD causes a wide range of symptoms in infected hosts (Berger et al. 1998; Pessier et al. 1999). Infected adults may be lethargic, inappetent, and maintain an abnormal resting posture. There can be marked roughening of the epidermis due to discharge tubules protruding outwards from cells containing zoosporangia. Sloughing of the outermost epidermal cell layers is also common. Histology frequently reveals hyperplasia and hyperkeratinized epidermal cells (Berger et al. 1998), and in the stratum corneum these cell layers are shed all at once rather than continuously over time (Berger et al. 2005c). Shed skin may contain empty zoosporangia that have accumulated bacteria and other microorganisms after zoospores have been discharged into aquatic environments. BD infection is rarely observed on the dorsal epithelium of hosts (Berger et al. 2005c). Infection is typically concentrated in the ventral region, especially in hind limbs and the hypervascularized inguinal region known as the pelvic patch (or ‘drink patch’) in postmetamorphic hosts. Severe infection can lead to abnormal resting posture, loss of the righting reflex, seizures, and mortality (Berger et al. 1998)

Infection in tadpoles occurs exclusively in the keratinizing tissue of larval mouthparts (toothrows and jawsheaths) where BD causes visible depigmentation as a

result of damage to the mitotic zone of jawsheath tissues fated to be keratinized into tooththrows (Altig 2007). Infection in tadpoles is generally not lethal, even in species that experience lethal infections at postmetamorphic stages (i.e. *Rana muscosa* tadpoles, Rachowicz and Vredenburg 2004). There is at least one notable exception to this general pattern: the western toad (*Bufo boreas*) can experience acute mortality from BD infection at larval stages (Blaustein et al. 2005). Apart from limited empirical examples of BD-induced mortality in *Bufo boreas* and deformed mouthparts observed in some species in the field (e.g., (Knapp and Morgan 2006; Smith and Weldon 2007), little is known about how infection may influence tadpoles. One study showed that BD decreased mass at metamorphosis and larval period length in larvae of two amphibian species (Parris and Cornelius 2004).

The mechanisms of disease and mortality in this system are still unclear. Since BD infects keratinized epidermal tissues, an obvious hypothesis is that infection disrupts osmoregulation in hosts (Berger et al. 1998). A recent study examining pathogenesis of BD found that several electrolytes (sodium, potassium, chloride) were significantly depleted from infected hosts (Voyles et al. 2007). Authors hypothesize that intracellular infection by BD may severely reduce the active transport of these electrolytes across epidermal cells, which are essential in cell membrane function and the conduction of action potentials for smooth and cardiac muscle contractions (Voyles et al. 2007). One study observed acute mortality in larval hosts after 48 hours of exposure (Blaustein et al. 2005). Since larval mouthparts are not involved in osmoregulatory processes, and since death occurred at such a fast rate, authors suggest

the possibility that a lethal toxin may be involved. These hypotheses are not mutually exclusive, and it is possible that both factors may be contributing to patterns of infection and mortality.

Though mass mortality events proximately caused by chytridiomycosis have received international attention, the majority of amphibian species found with BD infections do not seem to develop chytridiomycosis and many are suspected to be asymptomatic carriers (Beard and O'Neill 2005; Davidson et al. 2003; Garner et al. 2006; Mazzoni et al. 2003; Simoncelli et al. 2005; Weldon 2004; Woodhams and Alford 2005; see [spatialepidemiology.net](http://spatialepidemiology.net) for a complete list of species). For example, postmetamorphic *Rana catesbeiana* and *Bufo marinus* can carry heavy pathogen loads but show no symptoms of disease (Berger et al. 2000; Daszak et al. 2004; Hanselmann et al. 2004). The invasion of *R. catesbeiana* in North America, and the invasion of *Bufo marinus* across Australia may exacerbate the declines of some amphibian populations by introducing a novel pathogen along with a nonnative, superior competitor. Aside from *Bufo boreas* tadpoles mentioned above, to our knowledge no other species display morbidity or mortality at larval stages from BD infection. Tadpoles are hypothesized to be a reservoir for BD persistence between epizootics of postmetamorphic stages (e.g. Lamirande and Nichols 2002; Rachowicz and Vredenburg 2004). Our understanding of why some species develop lethal chytridiomycosis while others remain uninfected, lightly infected, or heavily infected yet asymptomatic will benefit from studies on mechanisms of pathogenesis and resistance.

To explore this variation in susceptibility several studies have considered the interspecific variation in innate immunity provided by antimicrobial peptides. Antimicrobial peptides have evolved independently in several taxa and exemplify one of the most ancient forms of defense against infectious agents in multicellular organisms (Zasloff 2002). In amphibians, antimicrobial peptides are released from specialized glands in the epidermis can inhibit the growth of BD *in vitro* (Rollins-Smith 2003; Rollins-Smith 2002). A recent study of four anuran host species found that the *in vitro* effectiveness of their inhibitory peptides correlates negatively with BD infection severity observed in field surveys of wild populations (Woodhams et al. 2007a).

A related possibility is that natural assemblages of microbes found on amphibian skin provide an additional barrier to infection by BD and other infectious agents (Belden and Harris 2007; Brucker et al. 2008). A recent study found a significant difference in antimicrobial peptide assemblages on the skin of *Rana muscosa* hosts between populations with historically different BD infection severity in the Sierra Nevada of California (Harris et al. 2006). Researchers postulate that microbial communities composed of bacteria that can successfully colonize the complex, antimicrobial environment of amphibian skin may provide an additional line of innate defense against BD infection (Harris et al. 2006). These studies are a first step towards addressing variations in immunity that may underlie infection patterns in nature, and it is clear that much future work is needed to refine these initial studies (Fisher 2007; Garner 2007; Kurtz and Scharsack 2007; Woodhams et al. 2007b).

Furthermore, research examining adaptive immunity in amphibian hosts is almost nonexistent, and there are no studies examining any immune responses to BD for larval amphibians.

#### Environmental context of BD infection

The oldest museum record of any amphibian with retrospectively detectable BD infection dates to the 1930s (Weldon 2004), but chytridiomycosis epizootics observed in recent years suggest that environmental cofactors are involved in the recent emergence of BD. Early studies observed host mortality events associated with chytridiomycosis during cool seasons (Berger et al. 1998; Bradley et al. 2002). Various laboratory studies show that juvenile and adult amphibians exposed periodically to high temperatures (37°C) cleared infection (Woodhams et al. 2003), and infected tadpoles survived longer following metamorphosis when raised at temperatures above 25°C (Lamirande and Nichols 2002). A field study on adult *Rana yavapaiensis* in Arizona corroborates experimental work by showing that frogs sampled from thermal springs (22.8-35.8°C) were not infected with BD while conspecifics and heterospecifics found close by (some less than 30 meters away) harbored BD infection (Schlaepfer et al. 2007). These studies beg the question of whether amphibian hosts can mediate infection behaviorally by seeking thermal environments that will clear infection.

Though the effects of temperature on BD infection have been corroborated by several independent studies, the links between BD infection severity and long-term climate change remain controversial (Alford et al. 2007; Di Rosa et al. 2007; Lips et al. 2008; Pounds et al. 2007; Pounds et al. 2006). Two studies examining long-term patterns of climate change have shown an increase in infection severity with shifts in climate towards the hypothesized thermal optimum of the BD pathogen (Bosch et al. 2007; Pounds et al. 2006). However there is still some dispute about how retrospective climatic data should be analyzed statistically to account for error and spatial pattern (Lips et al. 2008). Field studies of current climate conditions conducted in distant geographic regions show that BD prevalence in host populations fluctuate seasonally, with persistent infections occurring in regions where the average summer maximum temperature falls below 30°C (Drew et al. 2006) and generally becoming more severe when hosts are found in cooler temperatures (Berger et al. 2004; Bradley et al. 2002; Kriger and Hero 2006; Kriger and Hero 2007b; Woodhams and Alford 2005). Shifts in climate that expand suitable areas for BD (Seimon et al. 2007) might also be expected to make other regions less suitable to persistent infection (Muths et al. 2008; Ron 2005).

A few studies have shown that the presence of environmental contaminants may interact with BD infection to negatively affect host growth, development and survival.(Davidson et al. 2007; Parris and Baud 2004). Another possibility is that increasing concentrations of ecological contaminants may decrease BD infection prevalence. For example, it is possible that the accumulation of trace elemental

contaminants may protect some species from severe BD infections by creating an inhospitable environment for BD growth and reproduction (Peterson et al. 2007). Additional research has examined the role of agriculture-related habitat alterations (Mendelson III et al. 2004; Simoncelli et al. 2005) in exacerbating BD infection. It is clear that scientific study of the environmental context of BD infection is just beginning, and much future work is needed to understand what factors underlie the patterns of BD infection we are beginning to observe in natural populations.

#### Distribution of *Batrachochytrium*

The recent emergence of BD has prompted scientists to consider whether historical declines can be attributed to previous outbreaks of chytridiomycosis. Retrospective analyses of museum samples address this question while simultaneously examining the historical distribution of the BD pathogen in geographically distinct areas.

The earliest known record of BD infection is from an adult *Xenopus laevis* frog dated to 1938 from southern Africa (Weldon 2004). There is speculation that BD infection could have spread worldwide through the international export of this species which was widely used in human pregnancy tests and as a model organism for embryology, immunology, and molecular biology (reviewed in Weldon 2004). A large-scale retrospective analysis of preserved museum specimens of 25 countries reveals endemism of BD across most of North America with infection persisting at

low levels in several cosmopolitan species of eastern North America (Ouellet et al. 2005).

Retrospective surveys may provide evidence that BD infection was present in populations historically. However, since BD detection in retrospective surveys is opportunistic and dependent on varying collection and survey methods, inferences about the role of BD in host population trends require careful interpretation. Furthermore, if BD is emerging as a novel pathogen in a particular area, retrospective surveys would be less useful since museum specimens would not be expected to harbor a novel infection (Blaustein and Kiesecker 2002). Similarly, retrospective surveys can provide some evidence that historical population declines are not associated with an epizootic of BD if no samples contain infection (e.g., Lampo and Señaris 2006). To my knowledge, all retrospective analyses have been conducted on postmetamorphic stages, thus there are no available data about historical distribution of BD among amphibian larvae.

Several countries have undertaken diagnostic surveys to determine the presence of BD in native species, and in exotic species that could be asymptomatic carriers of BD infection (Beard and O'Neill 2005; Daszak et al. 2004; Fisher and Garner 2007; Garner et al. 2006; Mazzoni et al. 2003; Sánchez et al. in press). Some of these were conducted in response to observed mortality events (e.g., Bosch et al. 2001), while others were undertaken preemptively to determine whether BD could be a threat to native populations (e.g., McLeod et al. 2008; Rowley et al. 2007). Though BD has been associated with some population crashes and extirpations, its presence in

field or retrospective surveys is not a conclusive indication that BD is the causal factor behind current or historical population declines (Daszak et al. 2005; Martinez-Solano et al. 2003). Additionally, BD is widespread, but there are still areas where it has not been detected, sometimes despite extensive sampling (Garcia et al. 2007; Rowley et al. 2007). The global distribution of BD infections are being compiled into a single public database (available at [www.spatialepidemiology.net/bd](http://www.spatialepidemiology.net/bd)) that also lists which species have been found positive and negative by country with relevant citations to primary literature. The aim of this project is to provide an interface for researchers to examine BD infection dynamics through global sharing of BD occurrence data. As survey efforts continue, data from long-term studies of host species from geographically disparate areas will shed light on spatial and temporal infection patterns that may exist in natural populations.

This global, collaborative effort may eventually produce enough data to empirically test two hypotheses about the emergence of BD. The novel pathogen hypothesis states that BD is emerging because of recent spread to naïve populations, possibly due to recently evolved increases in virulence (Rachowicz et al. 2005). A second hypothesis states that BD existed endemically in amphibian populations and has recently emerged as a result of environmental change impacting hosts, BD, or both (Rachowicz et al. 2005). While some scientists believe that BD infections are spreading in a wave-like manner (Berger et al. 1998; Laurance et al. 1996; Laurance et al. 1997; Lips 1999), others argue that many characteristics of BD, including its mode of transmission and its sensitivity to desiccation and temperature, support that BD was

endemic in amphibian populations until recently (McCallum 2005), or has returned to an endemic state after historical epizootic events (Retallick et al. 2004). These two hypotheses are not mutually exclusive, and there is evidence in support of both. For example, population genetics of BD shows little evidence that fungal genotypes correlate with locality. Moreover, field work has shown that invasive species such as bullfrogs and cane toads can carry infection to naïve populations (Berger et al. 2000; Daszak et al. 2004; Sánchez et al. in press), providing a plausible mechanism by which BD can spread to naïve populations and support for the novel pathogen hypothesis. However, evidence of genetic recombination (Morgan et al. 2007) together with laboratory studies showing differences in virulence between strains (Berger et al. 2005b; Retallick 2007), lend support to the hypothesis that BD is endemic. In short, the evidence for both of these hypotheses is mounting but inconclusive (Morgan et al. 2007; Rachowicz et al. 2005).

### Diagnostic tools

Much of the research described thus far was made possible through advances in diagnostic methods. Initial studies relied on histology and light microscopy to identify structures resembling zoosporangia in epidermal tissue (see Berger et al. 1999c; Green and Sherman 2001) or to observe motile zoospores from skin scrapings of infected hosts (Longcore et al. 1999). Since histological sections are likely to miss sparse infections (Kriger 2006), this method has been largely replaced by quantitative

PCR tests that are highly specific to BD-specific DNA sequences. Quantitative PCR can determine the presence of BD in host tissue as well as give an approximate measure of infection severity by estimating the concentration of zoospores in sampled tissue (Boyle et al. 2004). Though quantitative PCR is the most sensitive and accurate diagnostic test currently available, it has not been universally adopted because of its high cost (Kriger et al. 2006). Several other diagnostic methods have been developed (reviewed in Hyatt 2007), but visual detection using light microscopy and histology, and quantitative PCR currently remain the most widely adopted diagnostic tools.

For BD detection in larval hosts, some researchers have suggested examinations of mouthpart deformities and discolorations that can develop as a result of BD infection (Knapp and Morgan 2006; Rachowicz and Vredenburg 2004). However, since there are several environmental factors that can also cause similar discoloration and deformation of tadpole mouthparts, this method leads to a high number of misdiagnoses and its reliability is controversial (Altig 2007; Padgett-Flohr and Goble 2007; Smith and Weldon 2007).

#### Current and future work

The development of reliable diagnostic tools and the establishment of a small number of long-term study sites have enabled some researchers to move forward from simple observational studies of BD presence and absence to begin examining the ecology and dynamics of infection and host populations (Briggs et al. 2005;

Woodhams and Alford 2005). Two such studies combine data from field and laboratory work with mathematical models to draw hypotheses about host population dynamics (Briggs et al. 2005) and BD transmission dynamics (Rachowicz and Briggs 2007). Another study compiled published BD occurrence data to inform ecological niche models generating testable predictions about where BD is most likely to persist, and which regions are most at-risk for severe infections (Ron 2005). These studies are among the first to explore infection dynamics on a larger scale and to question whether there are underlying patterns enabling us to predict where severe disease may occur.

After a productive decade of study there are still many important questions that remain unanswered and unexplored. For example, BD is known for lethal infections and mass mortality, but the majority of examined species seem to carry sublethal infections with few adverse effects. Why are there such drastic differences in infection among species? What are the community consequences of losing species to BD infection? And how might low-level, sublethal infections affect host populations and their communities?

Host behavior is one of the most interesting and understudied responses to infection. Infection can change the behavior of individuals within a single host species; it can alter numerous interactions between multiple host species composing a guild, and may influence the larger community by affecting predator-prey dynamics. To begin examining some of these broad questions for the first time, I examined various behavioral responses of native amphibian species that are susceptible to BD

infection and have been found infected in the field (Carey 2003; Pearl 2007). The Pacific treefrog (*Pseudacris regilla*) and the red-legged frog (*Rana aurora*) are two anuran species that frequently occur together in low-elevation, ephemeral aquatic habitats. The Cascades frog (*Rana cascadae*) and the western toad (*Bufo boreas*) are high elevation species that are native to temporary and permanent water bodies of the Oregon Cascades.

My dissertation work focuses on the larval stage where it is arguable that all behaviors are aimed at enhancing larval growth and development rates which are directly correlated to fitness (Morin 1983). For example, aggregation behaviors in larvae of some amphibians contribute to successful foraging, thermoregulation, and antipredator defense. Thermoregulatory behaviors allow tadpoles to seek temperatures that are optimum for development, which is crucial for amphibians developing in ephemeral habitats. Chapter 2 examines how exposure to BD influences aggregation and thermoregulatory behaviors, and explores whether amphibians can mediate a temperature-sensitive infection by temporarily seeking high temperatures to induce behavioral fever.

In Chapter 3, I present an experiment that moves from examining single species to explore how the interactions among multiple host species can influence infection dynamics in mixed species settings. I manipulated species combinations and BD infection in the laboratory to mimic naturally occurring species compositions in the Oregon Cascades.

In Chapter 4 I consider how BD may alter community interactions by focusing on antipredator behaviors of tadpoles in response to chemical cues from common amphibian predators. I also conducted feeding trials to determine whether any changes in antipredator behavior affected survivorship of hosts faced with lethal predators.

Chapter 5 summarizes the major conclusions drawn from each of these studies, and their ecological implications.

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CHAPTER 2.

ANCIENT BEHAVIORS OF LARVAL AMPHIBIANS  
IN RESPONSE TO AN EMERGING FUNGAL PATHOGEN,  
*BATRACHOCYTRIUM DENDROBATIDIS*

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## Abstract

Behaviors have evolved in response to various selection pressures over evolutionary time. However, not all behaviors are adaptive. Some presumably “ancient” behaviors, persistent for millions of years, may be detrimental in the face of novel selection pressures in modern times. These pressures include a multitude of emerging infectious diseases which may be stimulated by environmental changes. We examined how a globally emerging amphibian pathogen, *Batrachochytrium dendrobatidis* (BD), affected two key evolutionarily persistent behaviors displayed by amphibian larvae: aggregation and thermoregulation. Larval aggregation behavior is often essential for foraging, thermoregulation and antipredator defense, but varies among species. Thermoregulatory behavior speeds larval development in ephemeral habitats. Specifically, we examined whether aggregation and thermoregulatory behaviors changed when exposed to the BD pathogen in two species (*Bufo boreas* and *Rana cascadae*) whose larvae aggregate in nature. In laboratory choice tests, larvae of neither species avoided infected conspecifics. Pathogen-exposed *B. boreas* larvae aggregated, while unexposed *R. cascadae* larvae associated more frequently with BD-exposed conspecifics. There was no evidence of behavioral fever or altered thermoregulation in larvae of four species we examined (*Pseudacris regilla*, *Rana aurora*, *B. boreas*, *R. cascadae*). The absence of behavioral fever may suggest an inability of the larvae of some host species to mediate infection risk by this pathogen. Thermoregulatory behaviors may exhibit a high degree of evolutionary inertia in

amphibian hosts because they are linked with host physiology and developmental rates, while altered aggregation behaviors could potentially elevate pathogen transmission rates, leading to increased infection risk in social amphibian species.

## **Introduction**

Pathogens (both micro- and macro-parasites) present an important biotic selection pressure and often affect the behavior of their hosts (Altizer et al. 2003a; Freeland 1976; Moore 2002; Parris and Beaudoin 2004). In light of potentially strong selective forces imposed by some infectious diseases, it is not surprising that many animals behave in ways that may reduce disease risk. For example, some amphibians, crustaceans, and fishes avoid associating with diseased conspecifics (e.g. Behringer et al. 2006; Dugatkin et al. 1994; Kiesecker 1999); some primate species quarantine newcomers to ensure they are free from latent infection before engaging in normal social interactions (Altizer et al. 2003b; Freeland 1976); and various parasitized ectotherms seek temperatures above their thermal optima, generating behavioral fevers that control infection (e.g. Ouedraogo 2003; Roy et al. 2006). These and other behavioral defenses may mediate the severity of infectious diseases in natural populations.

Some behaviors (e.g., aggregation, thermoregulation) are expressed across modern taxa that diverged millions of years ago and may therefore be considered ancient

(Huey 1982; Parrish and Edelstein-Keshet 1999). However, not all behaviors are adaptive. When confronted with novel selection pressures such as emerging infectious pathogens, some historically adaptive behaviors, such as aggregation behavior, may be detrimental in novel situations (Blaustein and Bancroft 2007). These ‘evolutionary traps’ are commonly observed in wildlife populations experiencing habitat changes (Battin 2004; Schlaepfer et al. 2002). Previously adaptive behaviors continue to be expressed, resulting in maladaptive reactions to new conditions or environments (Koko and Sutherland 2001; Robertson and Hutto 2006; Schlaepfer et al. 2005). Thus, with regard to pathogens, behaviors may help mediate disease risk, or they may be maladaptive, increasing disease risk.

Behaviors can also be directly altered by a pathogen. Infectious pathogens may induce changes in host behavior leading to increased pathogen transmission (reviewed in Moore 2002). One example of this phenomenon is illustrated by ground-dwelling arctiid caterpillars that climb to the tops of branches when infected by an entomopathogenic fungus, enhancing pathogen dispersal by wind and rainfall (Yamakazi et al. 2004). Additionally, some parasites with complex life cycles (requiring more than one host species to reproduce) cause their intermediate hosts to lose certain antipredator behaviors, predisposing them to predation by definitive hosts wherein parasites reproduce (reviewed in Moore 2002). These and other examples of infection-induced behavioral changes increase the chances of successful pathogen dissemination and transmission, and confirm the importance of examining the role of behavior in disease ecology.

As infectious diseases emerge with increasing frequency and severity on a global scale, they present novel selection pressures to wildlife populations (Altizer et al. 2003a), and in some cases, may affect population viability (reviewed by de Castro and Bolker 2005). An emerging infectious disease, amphibian chytridiomycosis, has been associated with many amphibian population declines on a global scale (Daszak 2003), and is the putative agent for the decline of numerous frog populations (Lips et al. 2006; Pounds et al. 2006; Rachowicz et al. 2006; Schloegel et al. 2006). The fungal pathogen causing this disease, *Batrachochytrium dendrobatidis* (BD), has a waterborne infectious stage. Flagellated zoospores emerge from intracellular zoosporangia encysted within keratinizing epidermal tissue of amphibian hosts (Altig 2007; Longcore et al. 1999), which may affect larval, juvenile, and adult stages in a species-specific manner (Blaustein et al. 2005; Garcia et al. 2006; Rachowicz et al. 2006). Laboratory studies show that BD zoospores swim an average of 2 cm before encysting (Piotrowski et al. 2004), and zoospores were more successful at colonizing host tissue when in groups rather than singly or in low numbers (Longcore et al. 1999). Additionally, infection is commonly observed as patches of clustered zoosporangia in the epidermis, indicative of emergent zoospores re-encysting nearby the original point of infection, often on the same host (Longcore et al. 1999; Weldon and Du Preez 2006). These observations corroborate experimental evidence that still or slowly moving water may influence BD infection severity by leading to an accumulation of infectious zoospores in larval host habitats (Tunstall 2007). Though flagellated, BD resembles other members of order Chytridiales that are most

effectively transported by flowing water or moving hosts (Berger et al. 1999a; Kriger and Hero 2006; Sparrow 1968).

Thus, close proximity and direct contact between aggregating amphibians may be one important mode of BD transmission in the field. Dense aggregations formed by some larval amphibians may increase pathogen loads for each individual within the group. This may be particularly important for species like western toads (*Bufo boreas*) that can experience acute mortality as tadpoles from BD exposure (Blaustein et al. 2005). Despite potential negative consequences, aggregating tadpoles likely experienced benefits promoting social behavior, including protection from predation (via predator swamping and collective vigilance), and enhanced filter feeding through group-agitation of organic matter into the water column (Beiswenger 1977; Hoff et al. 1999; Watt et al. 1997). Aggregation behavior also promotes thermoregulation. Temperatures within aggregating groups of tadpoles are higher than peripheral temperatures (Brattstrom 1962; Brattstrom 1963; Hoff et al. 1999). Though not all amphibian species aggregate, general heat-seeking behaviors are shared by all amphibian larvae of temperate zones, as warm temperatures expedite larval development and facilitate metamorphosis before ephemeral habitats dry or freeze (Dupré and Petranka 1985). The BD pathogen shows a thermal optimum ranging between 10-25°C, with growth ceasing at 28°C (Piotrowski et al. 2004). (Woodhams et al. and colleagues) (2003) showed that housing adult frogs (*Litoria chloris*) in water baths at 37°C cleared experimental infection in less than 16 hours. *Rana yavapaiensis* adults from thermal springs in Arizona (>30°C) tested negative for BD compared to

conspecifics found in nearby habitats (ca. 550 m at temperatures between 20-24°C, (Schlaepfer et al. 2007). Collectively, these studies raise the interesting possibility that some hosts may clear BD infection through behavioral fever in the wild.

Thermoregulation of individuals and groups may mediate the risk of infection in amphibian larvae (Lefcort and Blaustein 1995). In this study, we sought to determine if hosts behaviorally respond to BD exposure by examining aggregation and thermoregulatory behaviors in larvae of multiple amphibian host species. Specifically, we tested whether social, schooling tadpoles of *B. boreas* and *Rana cascadae* (Cascades frog), 1) avoid BD-exposed conspecifics, and 2) alter aggregation behaviors as a result of exposure status. Because amphibian larvae often exhibit opportunistic cannibalism by preying on sick or injured tadpoles (Crump 1983), we also measured activity rates to discern whether changes in aggregation behavior by healthy conspecifics were an indirect result of morbidity in BD-exposed animals. To examine thermoregulatory behaviors we used larvae of four species: *Pseudacris regilla* and *Rana aurora* breed in the winter in lower elevation sites, and their larvae are found in cooler temperatures during development compared with *B. boreas* and *R. cascadae* larvae which are found at high elevation sites that develop in spring in warmer temperatures (Bancroft et al. 2008; O'Hara 1981). In these four species we tested 1) whether tadpoles induced behavioral fever as a response to BD exposure, and 2) if thermal preferences were altered as a result of exposure status. Thermoregulation and aggregation behaviors are closely tied to tadpole physiology and development and may present evolutionary constraints on potential adaptation to novel conditions

(Huey et al. 2003; Ricklefs and Wilkelski 2002). Thus, alterations of either of these evolutionarily persistent behaviors through BD infection may help explain some of the wide variation in susceptibility between host species. More generally, host behavioral responses to a globally emerging pathogen may reveal the adaptive potential of some host species to behaviorally respond to a novel biotic selection pressure.

## **Methods**

*Animal collection and rearing.* Red-legged frog (*R. aurora*) and Pacific treefrog (*P. regilla*) eggs were collected from the Willamette Valley of Oregon (January-February, elevation: 71.6 m; Benton County). *Rana cascadae* and *B. boreas* eggs were collected from the Three Creeks recreational area (March-April, elevation: 2000 m; Deschutes County). All animals were reared in 38 liter tanks at temperatures ranging between 11-14°C on a 14:12 hr photoperiod. Post-hatching, all tadpoles were fed a 2:1 mixture of ground fish flakes and alfalfa pellets *ad libitum* and transferred to lower densities. At developmental stage 25-26 (Gosner 1960), tadpoles were inoculated with either BD or a control treatment (see below). Complete water changes were conducted weekly. For brevity, we refer to animals inoculated with the BD pathogen as “BD<sup>+</sup>” and animals exposed to the control agar-wash without BD as “BD<sup>-</sup>”. Importantly, tissue from animals used in these experiments were not retrospectively sampled to quantify pathogen concentration, thus BD<sup>+</sup> and BD<sup>-</sup> designations refer only to exposure status.

To confirm that our inoculation methods are sufficient to produce infection, we quantified BD pathogen loads in additional, non-experimental (new) larvae of these species. We inoculated 6 tadpoles held together in opaque plastic containers filled with 1 liter dechlorinated water. We quantified BD loads for each tadpole using real-time PCR. DNA was extracted from tissue of excised oral discs using a DNAeasy 96 well kit (Qiagen, Valencia, California) and quantified using a spectrophotometer (Nanodrop Technologies, Wilmington, Delaware). The real-time PCR was based upon established methodology (Boyle et al. 2004) using an ABI 7300 Real-time PCR system (Applied Biosystems, Foster City, California). Twenty five  $\mu\text{L}$  reactions contained: 5  $\mu\text{L}$  of 20ng/L template DNA and 20  $\mu\text{L}$  of master mix (containing 900nM forward and reverse primers, 125nM MGB probe, and Taqman Master Mix). We obtained BD genome equivalent standards from D. Boyle (as used in Boyle et al. 2004) and included triplicates of each standard serially diluted on each plate ( $10^{-1}$ ,  $10^0$ ,  $10^1$ ,  $10^2$ ) and a duplicate of the high standard ( $10^3$ ). Unknown samples were run in triplicate and values that differed by a coefficient of variation greater than 0.2 were rerun for greater accuracy. Values obtained from the quantitative PCR reaction are the mean BD zoospore genome equivalents per nanogram of excised mouthpart tissue. This measure accounts for differences between species based on size alone (i.e., more mouthpart tissue containing more zoospores).

#### Aggregation experiment

Inoculation regime. Ninety tadpoles of each species were held in 39 L aquaria. For  $BD^+$  treatments, aquaria were each inoculated with four culture dishes of isolate JEL274 grown in pure culture for 10 days on 1% Tryptone-agar culture dishes. Five mL water was added to each culture dish, which was scraped 5 times with a disposable pipette to dislodge BD colonies. Liquid containing zoospores and sporangia was added directly to aquaria. This process was repeated for control culture dishes (1% Tryptone agar without BD), and inoculate was added directly to aquaria holding  $BD^-$  tadpoles. Experiments were conducted 14 days after inoculation. Animals were inoculated and held in multiple aquaria to avoid pseudoreplication (testing inoculated animals from a single tank).

Test chambers and data collection. Opaque commercial plastic containers (capacity: 39.7L, dimensions: 0.16 x 0.86 x 0.42 m) were filled with 7.6 L dechlorinated water. Chambers were partitioned on either end by mesh screening, creating 0.10 m x 0.16 m enclosures to hold groups of 5 tadpoles of two treatments ( $BD^+$  or  $BD^-$ ) from two species (*R. cascadae* and *B. boreas*). A line drawn on the bottom of each chamber divided the remaining area in half (0.42 x 0.30 m). Chambers were rotated 180° between trials to remove possible effects of chamber orientation. The treatment sides were also randomized for each chamber in all trials. A focal tadpole (either  $BD^+$  or  $BD^-$ ) was placed in the center of each container. For brevity, we refer to the side of each test chamber nearest the  $BD^+$  group of conspecifics as the “ $BD^+$  side”, and the side of each test chamber nearest the un-exposed group of conspecifics as the “ $BD^-$

side”. All animals were allowed to acclimate to test chambers for 20 minutes. As a proxy for aggregation, we recorded the side of the chamber occupied by the focal tadpole every 10 minutes for 240 minutes ( $N_{BD^+}=16$ ,  $N_{BD^-}=16$ , for each species). Observations were made over a 240 minute period to capture an accurate mean for aggregation activity, as tadpole movements and behaviors can show high variability. All focal and stimulus tadpoles were used only once during the experiment. Test trials conducted before the experiment and observations made throughout the experiment showed that focal and stimulus animals were not repelled by the mesh used to create partitions, as they were observed touching, clinging, or very close to the mesh filters at both ends of test chambers.

Using a binomial test, we tested the null hypothesis that stimulus tadpoles would not spend a significant proportion of time on either side of the test chamber. Therefore, we compared the number of focal animals that spent the majority of time on the side of the chamber containing  $BD^+$  stimulus animals to a null proportion of 0.5.

Activity rate. Tadpoles were inoculated as described above, resulting in a  $BD^-$  and a  $BD^+$  treatment. Single tadpoles were randomly chosen and placed in clear plastic chambers containing 1 L dechlorinated water. A grid was placed underneath each chamber so that gridlines were visible through the bottom of each chamber (grids= $25\text{ mm}^2$ ). Tadpoles were left to acclimate to new conditions for 1.5 hours. We quantified tadpole activity by counting the number of lines crossed by each tadpole in 30 seconds continuously over a 2 hour period. All observations were made from behind black

curtains with small viewing windows to ensure that tadpoles would not respond to the presence of observers. We counted a tadpole as having crossed a gridline if the tadpole swam horizontally across a gridline. Vertical movement through the water column was not quantified. We replicated each treatment 15 times for each species. Treatments were randomly assigned and observers were blind to treatments during data collection.

#### Thermoregulation experiment

Inoculation regime. We tested four species: *R. cascadae*, *B. boreas*, *P. regilla*, and *R. aurora*. At stage 25 (Gosner 1960), *R. cascadae* and *B. boreas* were inoculated with BD isolate JEL 274 (*B. boreas* isolate) and *R. aurora* and *P. regilla* tadpoles were inoculated with BD isolate JEL 215 (*ranid* isolate). Infectious zoospores were harvested from 1% tryptone-agar culture dishes by adding 20 mL filtered water to each dish and pouring the inoculum from each plate directly into holding aquaria after a 20 minute period to allow for zoospore discharge. We used two culture dishes per tank of 20 tadpoles for each species. Animals were inoculated and held in multiple aquaria to avoid pseudoreplication (testing inoculated animals from a single tank). Animals were exposed to respective treatments for 40 days at the start of trials. We replicated each treatment 16 times for each species.

Test chambers and data collection. Thermal gradients were 1.2 m x 0.1 m lanes filled to a depth of 4.5 cm with 4 L dechlorinated water. Six digital temperature loggers

(Thermacron i-Buttons, Dallas Semiconductor) placed along the length of each lane (25 cm apart) recorded temperatures every 10 minutes. A hot plate underneath one end of each lane heated the water and dry ice contained in a separated well (7 x 7 x 6 cm) at the opposite end of each lane cooled the water. Each lane was aerated at two locations (30 cm from either end) to minimize vertical and horizontal temperature stratification. Thermal gradients were allowed to establish for a minimum of 30 minutes (Figure 1).

A single tadpole was added to the center of each of eight lanes and allowed to acclimate for 15 minutes. We recorded the location of each tadpole every 10 min for 180 min. To approximate the water temperature at the tadpole as closely as possible, the distance between each of the 6 temperature loggers was further divided into 3 cm increments. The nearest temperature at each tadpole was extrapolated using the following formula:

$$T_{tadpole} = T_x + g \left( \frac{T_y - T_x}{g_{y,x}} \right)$$

where  $T_x$  is the temperature of the logger nearer the cold end of the thermal gradient,  $T_y$  is the temperature of the logger nearer the warm end,  $g$  is the grid mark where the tadpole was located, and  $g_{y,x}$  is the total number of grid marks between the two loggers. Some ectotherms produce behavioral fever by periodically occupying high temperature environments over time, which may cause less damage from heat stress than continuous occupation of feverish temperatures (Kluger et al. 1998). Thus, as an additional measure of behavioral fever, we also counted the number of times tadpoles

were found in temperatures  $\geq 26^{\circ}\text{C}$ , the temperature above which BD growth is presumably inhibited in laboratory cultures (Piotrowski et al. 2004). We also quantified activity rates of tadpoles within thermal gradients by counting the number of grid marks (spaced approximately 3 cm apart) crossed by the tadpole during a 30-second period every 10 minutes over 180 minutes. Test chambers for all experiments were sterilized with 10% bleach solution after every trial.

## **Results**

### Inoculation

Tadpoles of all four species tested positive for BD infection, confirming that our inoculation methods are sufficient to produce infection. All control (BD<sup>-</sup>) tadpoles tested from the four species were negative for BD. There were species specific variations in BD infection severity. The mean log BD zoospore genome equivalents per nanogram of excised mouthpart tissue are as follows: *B. boreas*, 0.53 (N=8); *P. regilla*, 0.11 (N=6); *R. cascadae*, 0.01 (N=8); *R. aurora*, 0.35 (N=5). Since it is not possible to match BD infection severity in tadpoles *a priori* without sacrificing them, our hypotheses and results examine the effects of BD exposure (not infection).

### Aggregation behavior

Neither  $BD^+$  nor  $BD^-$  focal tadpoles of *R. cascadae* and *B. boreas* avoided  $BD^+$  conspecifics (Figure 2). However, we observed other changes in aggregation behavior as a result of BD-exposure. The majority of  $BD^+$  *B. boreas* focal tadpoles were found more frequently on the  $BD^+$  side of test chambers ( $p < 0.01$ , binomial test).  $BD^-$  *B. boreas* focal tadpoles did not depart from random in their allocation of time between the two sides of the chamber ( $p = 0.19$ ).  $BD^+$  *R. cascadae* focal tadpoles were associated randomly with both sides of test chambers ( $p = 0.18$ ), but a majority of  $BD^-$  *R. cascadae* focal tadpoles were observed more frequently on the  $BD^+$  side of test chambers ( $p < 0.03$ ; Figure 2). Activity rates between  $BD^+$  and  $BD^-$  tadpoles did not differ in *R. cascadae* ( $p = 0.9$ ;  $t_{15} = 1.32$ ), but  $BD^+$  *B. boreas* were more active than  $BD^-$  tadpoles ( $p < 0.02$ ;  $t_{15} = -2.29$ ; Figure 3).

#### Thermoregulatory behavior

There were no differences in mean temperatures selected by  $BD^+$  tadpoles compared to  $BD^-$  tadpoles for any of the four tested species: *B. boreas* ( $p = 0.37$ ), *R. cascadae* ( $p = 0.23$ ), *P. regilla* ( $p = 0.2$ ), and *R. aurora* ( $p = 0.89$ ; Mann-Whitney-Wilcoxon (MWW) test; Figure 4 and Table 1). *Pseudacris regilla* and *R. aurora* generally chose colder temperatures compared to *R. cascadae* and *B. boreas* (Table 1 and Figure 4). There was no significant difference in the mean number of observations at temperatures  $\geq 26^\circ\text{C}$  for any species in either treatment ( $BD^+$  or  $BD^-$  tadpoles; Table 1, Figure 5). Generally, activity rates quantified within thermal gradients corroborated activity rate results observed for *R. cascadae* and *B. boreas*. There was no difference

in activity rates within thermal gradients for *R. cascadae* between BD treatments. BD<sup>+</sup> *B. boreas* and *R. aurora* tadpoles were more active compared with BD<sup>-</sup> conspecifics, although these differences were not statistically significant (Table 1). There was no difference in activity rates between treatments in *P. regilla*.

There were no differences in body or tail length between treatments in any of the four species tested (MWW tests, Table 1).

## **Discussion**

The behavioral repertoire of an animal has been molded over evolutionary time in response to numerous abiotic and biotic selection pressures. Yet extant behaviors that have persisted for millions of years may be maladaptive in a continuously changing environment that includes an onslaught of emerging infectious diseases (Morens et al. 2004). Our study examined two evolutionarily persistent ancestral behaviors in amphibian hosts in relation to a newly emerging infectious agent. Thermoregulatory behaviors, in particular, the ability to produce behavioral fever, may be one way that ectothermic hosts mediate or clear infection (Kluger et al. 1998; Lefcort and Blaustein 1995; Schlaepfer et al. 2007; Woodhams et al. 2003). Aggregation behaviors in many host species may increase disease risk (Kermack and McKendrick 1927; Rowley and Alford 2007).

Tadpole aggregations are likely formed and mediated on the basis of various cues (Hoff et al. 1999; O'Hara 1981; Wassersug 1973). Some of these cues may be altered as a result of infection and altered cues may lead to changes in individual host behaviors (Kavaliers and Colwell 1992; Kavaliers and Colwell 1995), and the behavioral interactions between hosts in groups, although the latter possibility has not been well-studied experimentally. Among the two more social species we examined, neither *B. boreas* nor *R. cascadae* avoided  $BD^+$  groups of conspecifics.  $BD^-$  *B. boreas* tadpoles associated randomly with conspecifics regardless of exposure status while  $BD^+$  tadpoles associated with other  $BD^+$  conspecifics.  $BD^+$  individuals were also more active than  $BD^-$  tadpoles.  $BD^+$  *B. boreas* tadpoles may aggregate with other  $BD^+$  individuals more frequently because they are responding to cues from conspecifics sharing similar, high activity rates. Sharing similar activity patterns with conspecifics may further decrease individual conspicuousness to predators. It may therefore be advantageous for “hyperactive”  $BD^-$  *B. boreas* to associate with conspecifics that share similar activity levels in the field.

Contrary to expectation, unexposed *R. cascadae* tadpoles associated more frequently with  $BD^+$  conspecifics, whereas  $BD^+$  tadpoles showed random associations with conspecifics regardless of exposure status. There was no difference in activity rate between  $BD^+$  and  $BD^-$  *R. cascadae*. The larvae of many anuran species, including those examined in this study, exhibit opportunistic cannibalism of conspecifics in the field (Crump 1983) and in the laboratory (*pers. obs.*). Therefore, one possible explanation for the attraction of  $BD^-$  tadpoles to  $BD^+$  conspecifics could be that  $BD^-$

tadpoles were seeking opportunities to consume moribund conspecifics. However, since activity rates of  $BD^+$  *R. cascadae* tadpoles did not differ from  $BD^-$  tadpoles, we could not attribute this trend to perceived opportunities for cannibalism from lowered activity rates. It remains to be tested whether altered chemical cues emitted from infected or moribund conspecifics attract uninfected conspecifics, or whether the BD pathogen itself may present cues that are attractive to potential hosts (Berger et al. 2005a). Also, our observations of host activity rates were made on individual tadpoles. Groups of tadpoles may exhibit different activity rates than solitary tadpoles, or single tadpoles in the presence of conspecifics (e.g. Griffiths and Foster 1998).

In the four species we tested, there was no evidence that thermoregulatory behaviors were altered in  $BD^+$  tadpoles compared to controls. Instead, all species occupied mean temperatures that paralleled their native habitats. *Pseudacris regilla* and *R. aurora* collected from similar cooler habitats occupied colder temperatures expected of winter breeding compared to *R. cascadae* and *B. boreas*, which inhabit warmer temperatures during larval development in summer months (Bancroft et al. 2008; Brattstrom 1963; Putnam and Bennett 1981). Furthermore, we did not detect any evidence of behavioral fever in these species. Our results suggest that during larval stages these anuran species do not engage in behavioral fever as a result of BD exposure, even though temperatures  $\geq 26^\circ\text{C}$  were consistently available in all thermal gradients.

We observed thermoregulatory and aggregation behaviors of individual hosts separately in a controlled laboratory environment. Temperatures selected by

amphibian larvae in laboratory settings may differ widely from those selected in natural settings (Brattstrom 1963). This may be due partly to conspecific aggregations contributing to thermoregulation (reviewed in Hoff et al. 1999). In at least two of the four species tested (*R. cascadae*, *B. boreas*), conspecific aggregations aid thermoregulation by raising water temperatures within larval aggregations. If schooling tadpoles can routinely reach sufficiently high temperatures in the field to regulate BD-infection levels, there may not exist a strong force to select for behavioral fever in response to BD infection. Field studies that track both body temperatures of aggregating individuals and infection status over time would provide valuable insight to this system.

From an evolutionary standpoint, our data also lead us to speculate about the potential interactions between altered aggregation and conserved thermoregulation behaviors in response to BD infection in the field. For example, contrary to the trend we observed, if some infected *B. boreas* in a given host population avoid infected conspecifics, these individuals may avoid superinfection (increased pathogen load through infected neighbors or through reinfection of self) thus standing a better chance of surviving infection by avoiding the accumulation of a lethal BD load. However, hyperactivity of these hosts may render them more conspicuous to predators, increasing their chances of being removed from the population through predation. Similarly, if some infected *B. boreas* tadpoles in the population exhibit tendencies towards behavioral fever unlike the majority of the infected population, these individuals may also become more conspicuous, increasing their chances of becoming

prey. Both scenarios would slow the evolution of adaptive behaviors in response to BD infection in the host population. Although speculative, these and similar conjectures highlight the importance of considering multiple behaviors in a natural context, as well as in context with one another. Behaviors are considered among the most labile characteristics of organisms and may form the basis of trait evolution (Wcislo 1989; West-Eberhard 1989). However, there are costs to behavioral plasticity that may render some behaviors relatively slow to change (Huey et al. 2003). As hosts encounter rising numbers of emerging diseases, it will be important to consider the relative roles of both behavioral plasticity and behavioral inertia in interpreting host responses to these novel selection pressures.

The lack of avoidance behavior in susceptible tadpoles suggests that the risk of successful BD transmission to susceptible tadpoles may increase, especially in social species. Moreover, if susceptible tadpoles cannot or do not avoid infected conspecifics (such as *R. cascadae*), they could experience increased infection risk through close proximity, direct contact, or cannibalizing infected conspecifics. Indeed, heightened risk of infection resulting from consuming infected conspecifics is one hypothesis that may explain the general infrequency of opportunistic cannibalism in nature (e.g. Pfennig et al. 1998) despite the developmental advantages gained through consuming conspecifics (Heinen and Abdella 2005).

The alteration of ecologically important host behaviors in response to nonlethal infection has not been examined in the amphibian-chytridiomycosis system. The transmission dynamics that may arise from interactions between susceptible and

infected hosts remains a challenging area for future study. In particular, the relationship between ancient host behaviors and transmission of non-lethal infections warrant further examination. It is likely that social behaviors such as aggregation and pathogen-mediated behavioral changes in social species are making important and understudied contributions to the distribution and prevalence of horizontally transmitted pathogens and their hosts in nature.

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Table 2.1. Mean body and tail lengths (mm) of tadpoles tested in thermal gradients, the mean number of lines crossed in a 30-s period as a measure of activity rate within thermal gradients, and the mean temperatures selected by each species in a 180-min period for two treatments (BD+ and BD-). Fever activity is the number of times tadpoles were observed in temperatures  $\geq 26^\circ\text{C}$  over 180-min. Reported statistics are from Mann-Whitney-Wilcoxon rank sum tests (MWW).

	<i>Pseudacris regilla</i>		<i>Rana aurora</i>		<i>Bufo boreas</i>		<i>Rana cascadae</i>	
	BD <sup>+</sup>	BD <sup>-</sup>	BD <sup>+</sup>	BD <sup>-</sup>	BD <sup>+</sup>	BD <sup>-</sup>	BD <sup>+</sup>	BD <sup>-</sup>
<b>Mean body (mm)</b>	5.07	5.67	12.03	11.47	7.64	7.57	11.69	11.38
SE	0.34	0.45	0.53	0.61	0.26	0.28	0.27	0.33
MWW test	Z=-0.83; p=0.41		Z=-1.07; p=0.28		Z=-0.44; p=0.66		Z=1.17; p=0.24	
<b>Mean tail (mm)</b>	8.69	9.37	17.89	16.67	10.81	10.86	19.93	19.56
SE	0.35	0.52	1.21	1.11	0.34	0.39	0.58	0.60
MWW test	Z=-1.54; p=0.12		Z=0.64; p=0.52		Z=0.15; p=0.88		Z=0.38; p=0.71	
<b>Activity rate</b>	2.83	2.91	4.84	3.68	7.60	4.55	2.23	2.89
SE	0.50	0.87	0.65	0.97	1.56	0.93	0.36	0.58
MWW test	Z=0.44; p=0.66		Z=1.72; p=0.09		Z=1.73; p=0.08		Z=-0.64; p=0.52	
<b>Mean selected °C</b>	20.10	21.00	19.30	19.40	25.40	24.50	25.30	25.70
SE	0.66	0.54	0.63	0.51	0.70	0.89	0.32	0.59
MWW test	Z=-1.28; p=0.2		W=338; p=0.89		W=362; p=0.37		W=294; p=0.23	
<b>Fever activity</b>	3.19	3.86	2.06	2.81	10.29	7.93	6.19	7.00
SE	0.86	1.15	0.65	0.97	1.04	1.59	1.03	1.18
MWW test	Z=-0.25; p=0.60		Z=-0.34; p=0.63		Z=0.55; p=0.29		Z=-0.22; p=0.59	

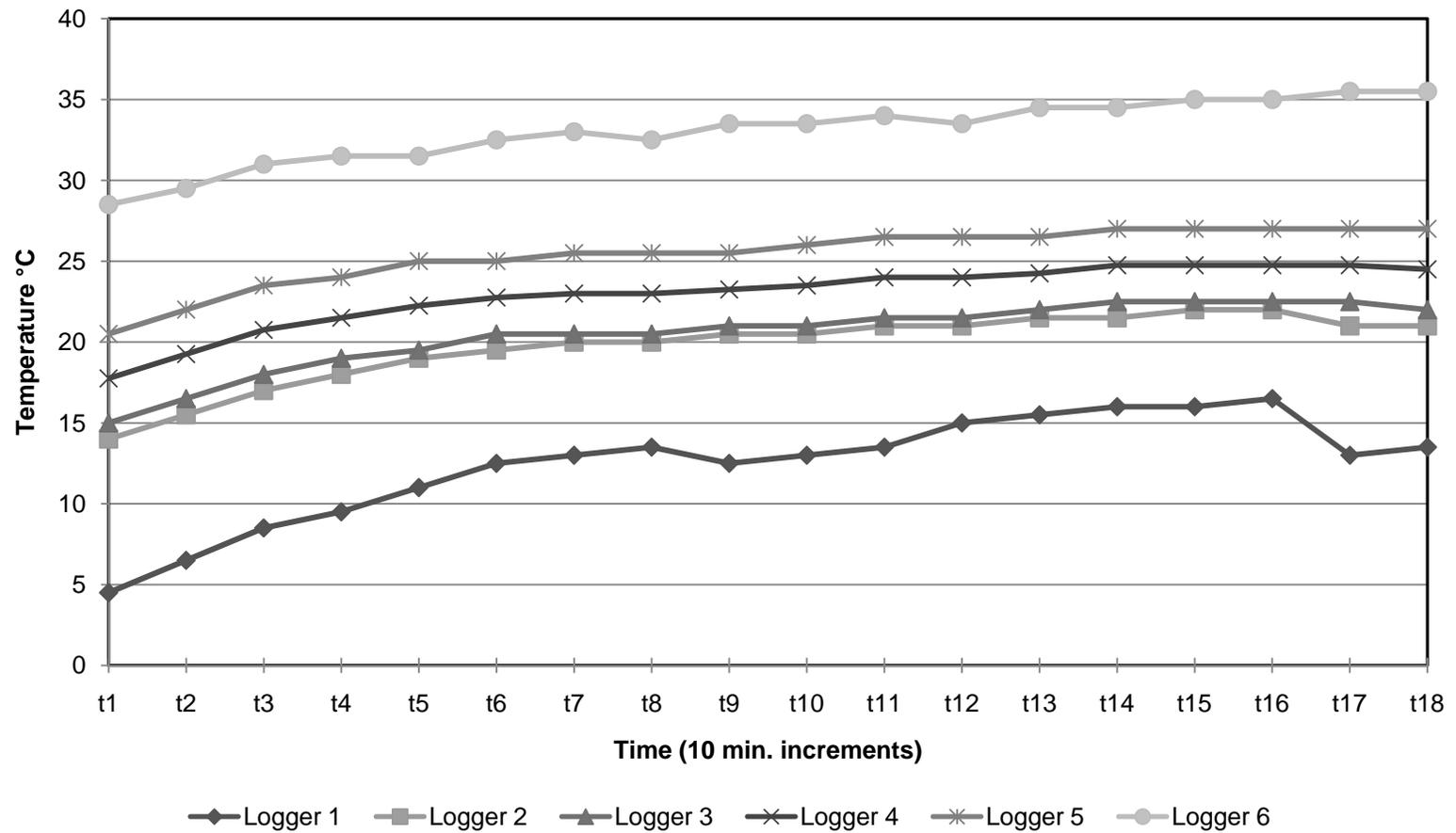


Figure 2.1. Temperature profile for one representative thermal gradient. Temperatures were collected 18 times using 6 temperature loggers each collecting data at 10 minute intervals.

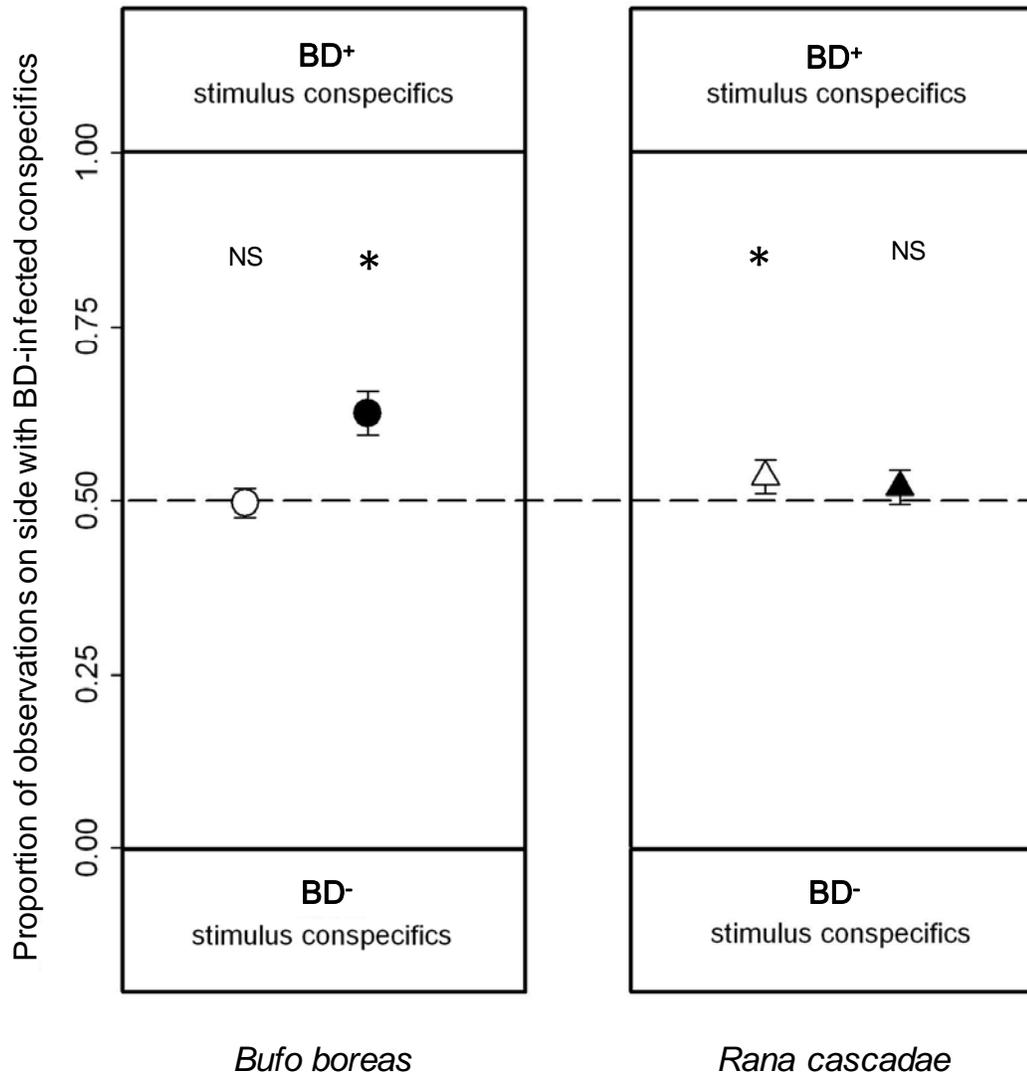


Figure 2.2. The proportion of focal tadpoles of *B. boreas* and *R. cascadae* that were observed associating with BD<sup>+</sup> conspecifics ( $N=15$ ). Shaded symbols represent BD<sup>+</sup> tadpoles, open symbols represent BD<sup>-</sup> tadpoles. Bars represent  $\pm 1$  standard error.

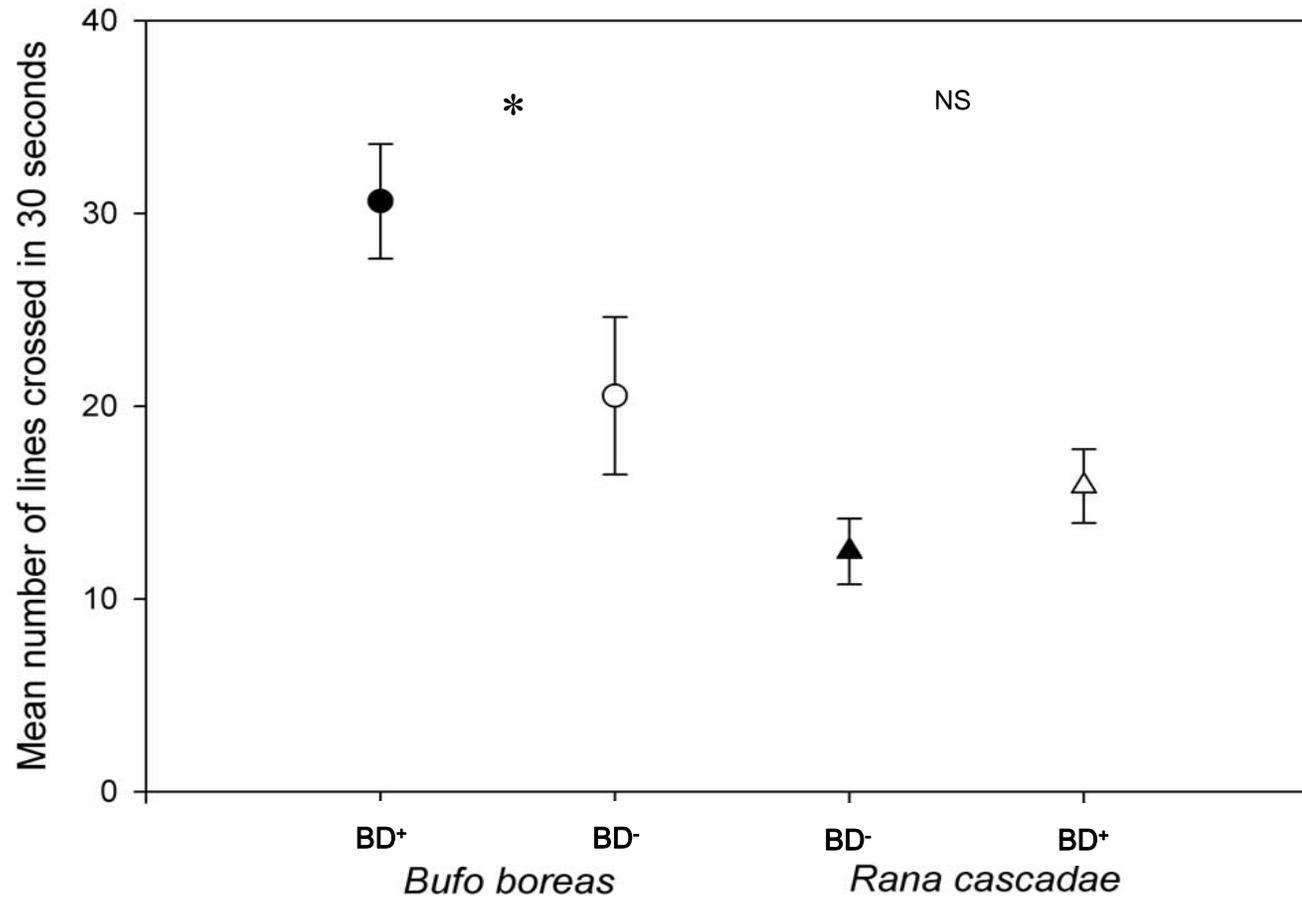


Figure 2.3. The activity rate of individual *B. boreas* ( $N=15$ ) and *R. cascadae* ( $N=15$ ) in two treatments. Shaded symbols represent BD<sup>+</sup> tadpoles, open symbols represent BD<sup>-</sup> tadpoles. Bars represent  $\pm 1$  standard error.

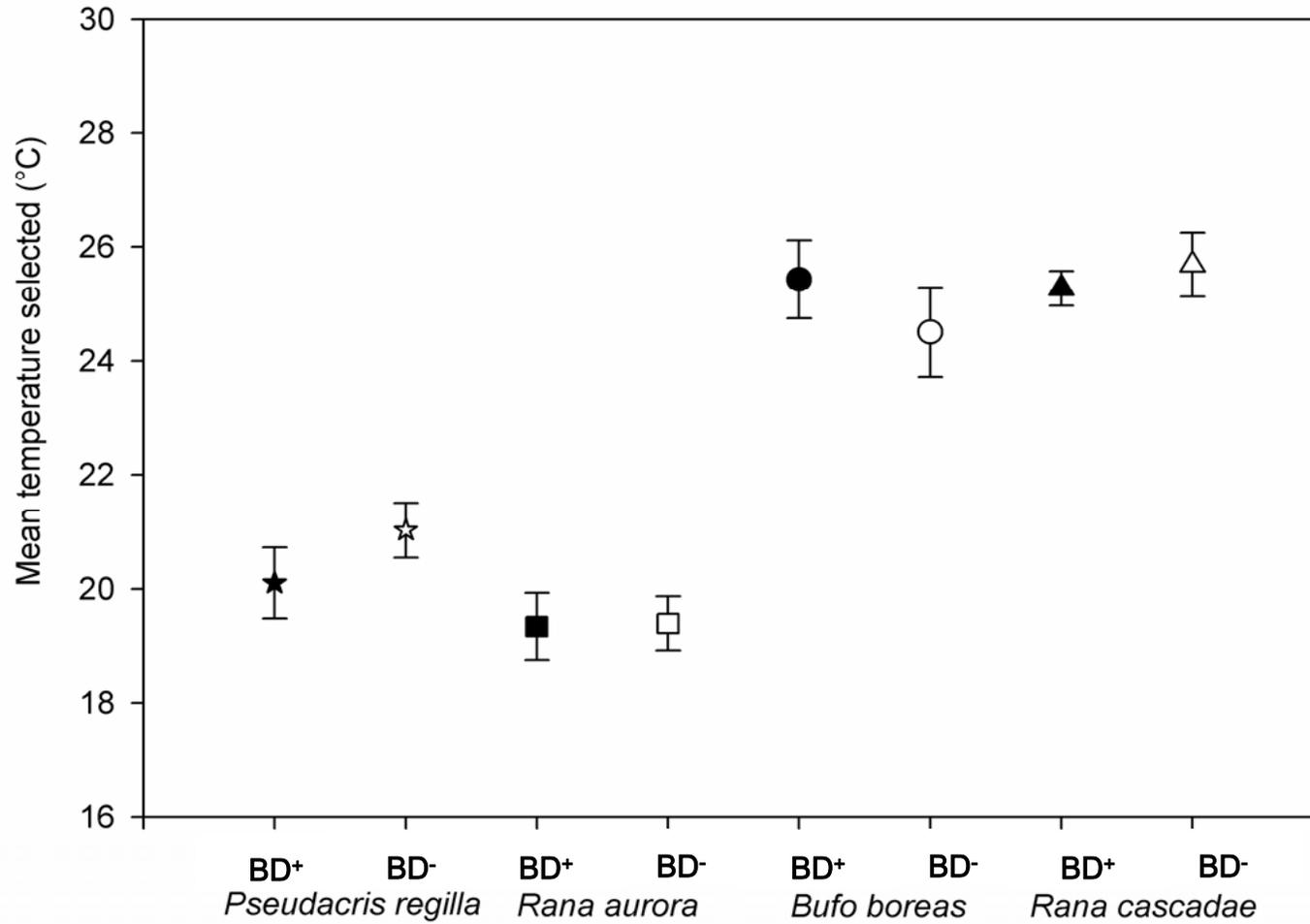


Figure 2.4. Mean temperatures selected by individual BD<sup>+</sup> and BD<sup>-</sup> tadpoles of four species ( $N=16$ ). Shaded symbols represent BD<sup>+</sup> tadpoles, open symbols represent BD<sup>-</sup> tadpoles. Bars represent  $\pm 1$  standard error.

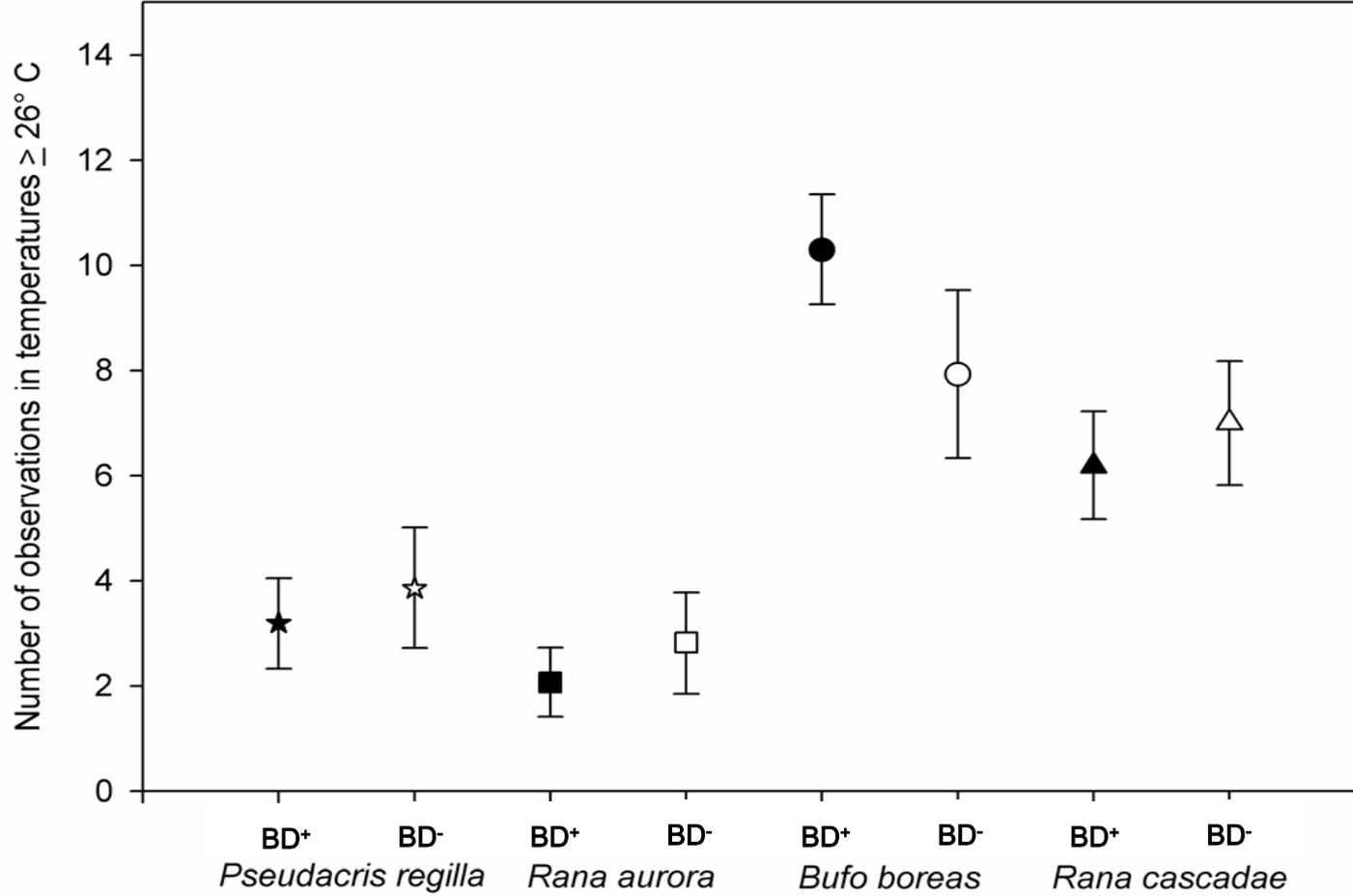


Figure 2.5. Mean number of observations of individual BD<sup>+</sup> and BD<sup>-</sup> tadpoles of four species in temperatures exceeding 26°C in four species as a proxy for behavioral fever ( $N=16$ ). Shaded symbols represent BD<sup>+</sup> tadpoles, open symbols represent BD<sup>-</sup> tadpoles. Bars represent  $\pm 1$  standard error.

CHAPTER 3.

PARASITE-MEDIATED COMPETITION, MUTUALISM,  
AND DIVERSITY EFFECTS IN AMPHIBIAN HOSTS.

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Andrew Storfer and Andrew R. Blaustein

**Abstract**

The current biodiversity crisis encompasses mass extinctions events exemplified by unprecedented, rapid declines of amphibian populations worldwide. Numerous factors are contributing to this crisis including infection by a recently emerged fungal pathogen, *Batrachochytrium dendrobatidis* that is prominently associated with many of these declines. Although data are accumulating on how *Batrachochytrium* affects single host species, in ecological communities, multiple co-occurring host species can interact with one another to influence infection dynamics. In a laboratory experiment, we examined the impacts of *Batrachochytrium* on larvae of three co-occurring amphibian host species (*Bufo boreas*, *Pseudacris regilla*, and *Rana cascadae*) combined in single and mixed-species combinations and inoculated with *Batrachochytrium* or maintained in a pathogen-free control treatment. Growth, survival and infection severity were compared to examine the influence of species composition on *Batrachochytrium*-infection dynamics. There were strong interactions between species combinations and *Batrachochytrium*-infection leading to parasite-mediated mutualism and competition between species. Specifically, *R. cascadae* and *P. regilla* showed increased growth and survivorship as a result of competitive release from high *B. boreas* mortality caused by infection and interspecific competition working synergistically in mixed-species combinations. Pathogen load decreased with increasing species richness, but pathogen load also varied between species combinations of equal richness and density suggesting that both species richness and

species identity may be important factors moderating a dilution effect in this system. Our results provide quantitative evidence that host species composition and diversity may influence *Batrachochytrium* infection dynamics due to differential susceptibility of host species within a community, and that *Batrachochytrium* may promote species exclusion or coexistence by indirectly affecting species interactions.

## **Introduction**

The current biodiversity crisis is exemplified by mass extinction events on a global scale. Five major extinction events have been recognized and some believe a sixth such event is underway (Wake and Vredenburg 2008). As part of this crisis, members of the class Amphibia are experiencing unprecedented population declines and extinctions occurring at rates exceeding those of birds and mammals (Pounds et al. 1997; Stuart et al. 2004; Wake and Vredenburg 2008). Numerous factors are contributing to this amphibian crisis (Blaustein and Kiesecker 2002), including various infectious agents (Green et al. 2002; Johnson et al. 2003b; Romansic et al. 2007).

Pathogens and parasites (considered synonymous herein) are increasingly appreciated for their role in influencing community dynamics and ecosystem function (Bonsall and Hassell 1997; Hudson et al. 2006), particularly through affecting ecological interactions among hosts. For example, parasitism can alter social interactions between conspecifics (Altizer et al. 2003b; Behringer et al. 2006); reverse

competitive interactions between heterospecific hosts (Borer et al. 2007; Kiesecker and Blaustein 1999; Park 1948); or alter predator-prey interactions (Joly and Messier 2004; Parris and Beaudoin 2004), ultimately influencing host population dynamics (Grosholz 1992), food web structure (Lafferty et al. 2008; Thompson et al. 2005), and ecosystem processes (Brown et al. 2001).

Research in disease ecology has moved beyond studies of single host-single parasite relationships to studies that incorporate multiple co-existing host species (e.g. Dobson 2004; Holt and Dobson 2006; Keesing et al. 2006), emphasizing a perspective critical for the advancement of community ecology. Recent theoretical studies have examined disease dynamics associated with multiple host species sharing a common infectious pathogen, generating several hypotheses about host species coexistence (Dobson 2004) and the relationships between host diversity and disease prevalence (Keesing et al. 2006). The diversity-disease hypothesis states that species-rich communities harbor less disease than species poor communities (Elton 1958). Results from recent studies suggest that increasing species diversity may dilute infectious disease in a community by either decreasing the abundance of the most competent host species (e.g., Lyme disease carried by white-footed mice, (Schmidt and Ostfeld 2001) or by decreasing the abundance of the host species that are required by specialist pathogens (e.g., species-specific foliar pathogens of plants, (Mitchell et al. 2002). In these systems, decreasing disease burden is likely the result of a density-dependent mechanism where the abundance of key species drives disease burden.

In addition to hypotheses about biodiversity and disease, recent work has shown that typical interactions between coexisting host species may shift in the presence of a common infectious pathogen. For example, a host species may gain advantage over a competitively superior species if it is less vulnerable to infection by a shared pathogen (parasite-mediated competition, Hudson and Greenman 1998). In this scenario, parasitism may actually benefit one host species by infecting its competition (parasite-mediated mutualism, Rudolf and Antonovics 2005). Such parasite-mediated interactions can influence community composition by facilitating species coexistence or promoting competitive exclusion indirectly through infection.

Though well-supported by mathematical models, empirical support for parasite-mediated interactions and the diversity-disease hypothesis remain scarce (Ostfeld et al. 2006). A recently emerged pathogen of amphibians affords a unique opportunity to empirically examine how an infectious pathogen may influence diversity and indirectly mediate interactions between multiple coexisting host species.

The infectious fungus *Batrachochytrium dendrobatidis* infects hosts when waterborne zoospores penetrate keratinizing tissue in post-embryonic amphibians (larvae, juveniles, and adults; Altig 2007; Berger et al. 2005a; Longcore et al. 1999). Pathologies range from malaise to more severe symptoms such as sloughing of epidermal tissue, lesions, loss of righting reflex, seizures, and mortality (Berger et al. 2005c), although some host species can carry severe yet asymptomatic infections (Blaustein et al. 2005; Daszak et al. 2004; Sánchez et al. in press). Thus, in addition to its association with mass die-offs in some areas (e.g. Lips et al. 2006; Rachowicz et al.

2006), BD may present a continuing, sublethal stressor for amphibians in many other regions worldwide (updates on the spatial distribution BD and host species at <http://www.spatalepidemiology.net>). The large variation in species responses to BD infection (e.g. Blaustein et al. 2005) has made it difficult to predict the effects of chytridiomycosis in amphibian communities. Although data are accumulating on how BD affects single host species, in ecological communities, multiple co-occurring host species can interact with one another to influence infection dynamics. There are virtually no studies that examine the effects of BD on community structure (but see Parris and Cornelius 2004).

In aquatic communities, several amphibian species may develop, coexist, and interact with one another (Alford 1999; Hoff et al. 1999; Wells 2007). Competition among larvae may be among the most important determinants of amphibian species assemblages (Morin 1983). Intra- and interspecific competition among amphibian larvae can influence growth and development, ultimately affecting metamorphosis and fitness (Dupré and Petranka 1985). Moreover, competitive stress influences infection susceptibility in amphibians and other host-pathogen systems (e.g., (Opplinger et al. 1998; Parris and Cornelius 2004). If BD infection influences competitive interactions between larval hosts, even sub-lethal infections may have important effects on the larger community.

Widespread intra- and interspecific competition at larval stages and the relatively straightforward experimental manipulation of commonly observed host species combinations make the amphibian-chytridiomycosis system ideal for

examining parasite-mediated competition, mutualism, and the effects of host species diversity on infection severity in mixed-species settings. We used three sympatric amphibian host species, *Bufo boreas* (the Western toad), *Rana cascadae* (the Cascades frog), and *Pseudacris regilla* (the Pacific tree frog), in single, two-, and three-species combinations to examine whether BD infection mediates interactions between host species. We also examined the diversity-disease hypothesis by quantifying infection severity in single and mixed-species combinations. Finally, by maintaining total abundance and manipulating species composition, we tested whether host species richness, independent of host abundance, contributes to infection severity in host species.

## **Methods**

*Tadpole rearing.* Amphibian eggs were collected near Todd Lake (44° 1' 48", -121° 41' 23.9994", elevation: 1873 m) from the Oregon Cascades Range, (Deschutes County, OR): *Rana cascadae* (*Rana*; 5 clutches), *Bufo boreas* (*Bufo*; 5 partial clutches), and *Pseudacris regilla* (*Pseudacris*; 25 partial clutches). Eggs were reared and hatched in the laboratory (at Oregon State University) in 9 L aquaria with dechlorinated and aerated water until hatching. Upon hatching, conspecific tadpoles from different clutches were combined at densities of 150 tadpoles per aquarium.

Tadpoles were moved into experimental groups and inoculated with BD or a control treatment at the free-swimming stage (stage 25-27, Gosner 1960).

*Inoculation and species combination treatments.* Tadpoles were assigned to one of two pathogen treatments (BD<sup>+</sup> or BD<sup>-</sup>), and one of seven species combinations. These include single species groups of *Pseudacris* (P), *Bufo* (B) and *Rana* (R) alone, two species combinations, (PB, BR, PR) and a three species combination (PBR). Each combination maintained a total density of 6 tadpoles per experimental unit while varying host species composition. This 7 x 2 design was replicated 8 times in a total of 112 experimental containers (35 x 17.5 x 8.5 mm) that each held six tadpoles in 2.5 L filtered, dechlorinated water. Fifty-six culture dishes of *Batrachochytrium dendrobatidis* JEL strain 274 on 1% Tryptone agar were flooded with 5 mL filtered water and gently scraped with a rubber policeman three times across the diameter of the plate to dislodge colonies. The liquid BD suspension from each culture dish was combined to yield a total zoospore concentration of approximately  $1.46 \times 10^8$  zoospores/mL. The same method was used to obtain control inoculum using sterile plates (1% Tryptone agar) without BD. Experimental units were inoculated once at the beginning of the 40 day experimental period with 3 mL of either BD inoculum (BD<sup>+</sup> treatment) or control agar wash (BD<sup>-</sup> treatment). Animals were fed *ad libitum* a 2:1 mixture of ground alfalfa pellets and fish flakes (Tetramin) throughout the 40 day period. Containers were checked daily for mortality. Tadpoles of these and other amphibian species opportunistically cannibalize dead or dying tadpoles (*pers. obs.*,

(Crump 1983). Thus, if a tadpole was missing it was assumed to have been consumed by another tadpole in the same container. Tadpole carcasses were removed during daily observations and preserved in 70% ethanol using sterile forceps. After 40 days all remaining tadpoles were euthanized and preserved in 70% ethanol for real-time PCR quantification of BD loads in host tissue. We recorded both mass and body length for each tadpole prior to PCR.

*Quantifying infection using real-time PCR.* Mouthparts were excised from all BD<sup>+</sup> and a subsample of BD<sup>-</sup> tadpoles (~ 30%) for quantitative real-time PCR analysis. DNA was extracted from tissue using a DNeasy 96 well kit (Qiagen, Valencia, California) and quantified using a spectrophotometer (Nanodrop Technologies, Wilmington, Delaware). Real-time PCR was based on established methodology (Boyle et al. 2004) using an ABI 7300 Real-time PCR system (Applied Biosystems, Foster City, California). Twenty five  $\mu\text{L}$  reactions contained: 5 $\mu\text{L}$  of 20ng/L template DNA and 20 $\mu\text{L}$  of master mix (containing 900nM forward and reverse primers, 125nM MGB probe, and Taqman Master Mix). We obtained BD genome equivalent standards from D. Boyle (as used in Boyle et al. 2004) and included triplicates of each standard serially diluted on each plate ( $10^{-1}$ ,  $10^0$ ,  $10^1$ ,  $10^2$ ) and a duplicate of the high standard ( $10^3$ ). Unknown samples were run in triplicate and values that differed by a coefficient of variation greater than 0.2 were rerun for greater accuracy. Values obtained from the real-time PCR reaction are mean BD zoospore genome equivalents per nanogram of excised mouthpart tissue. This measure accounts for differences in

BD infection between species based on size alone (i.e., more mouthpart tissue containing more zoospores).

Statistical analyses. We used two-way analyses of variance to examine BD load, mass, and length in tadpoles, including day of death as a covariate for each analysis. We also examined mortality by performing an ANOVA on the number of days survived and we performed a log linear test on the proportion mortality of species in each experimental unit. Log-linear tests were conducted on each species separately, including combination (4 groups) and pathogen (2 groups) as main effects, and a combination x pathogen interaction term. Since the experimental design was not orthogonal (i.e., one cannot test the impacts on *P. regilla* in the *R. cascadae* combination group), analyses were first run by examining the interaction of species treatments with or without BD. Following significant species differences, subsequent tests were run on each species separately to compare between species combinations and BD treatments. Where appropriate, subsequent pairwise comparisons were run to determine statistical significance between specific treatments.

## **Results**

### *BD infection severity*

Real-time quantitative PCR analyses confirmed that inoculation was sufficient to cause BD infection in all species and species combinations (see Fig. 1 and Table 1). BD infection severity differed between species ( $F_{2,82}=3.36$ ,  $p=0.04$ ), but not by day ( $F_{1,82}=2.23$ ,  $p=0.14$ ). *Bufo* had the highest BD loads, *Rana* had the lowest, and *Pseudacris* showed intermediate loads (Table 1, Fig. 1a). All tadpoles from control treatments tested negative for BD (N=324 animals from 96 test chambers), confirming that there was no cross-infection between  $BD^+$  and  $BD^-$  treatments.

BD infection severity also differed across various species combinations. In the species combinations containing *Bufo* (B, BR, PR, PBR) there was a main effect of species combination ( $F_{3,22}=0.02$ ,  $p=0.02$ ) and the day covariate was significant ( $F_{1,22}=10.133$ ,  $p=0.004$ ). Tukey HSD multiple comparisons showed tadpoles from *Bufo*-only treatments (B) had higher BD loads than *Bufo* from the three-species PBR combination ( $p=0.02$ ; Fig. 1c). There was no effect of species combination ( $F_{3,23}=0.53$ ,  $p=0.67$ ) or day ( $F_{1,23}=0$ ,  $p=0.98$ ) on infection severity in *Pseudacris* or *Rana* (combination:  $F_{3,26}=0.01$ ;  $p=0.99$ ; day:  $F_{1,26}=1.36$ ;  $p=0.26$ ).

### Mortality

A log-linear test on proportion mortality showed a main effect of species ( $\chi^2=119.45$ , DF = 2,  $p<0.001$ ) in a model that also included pathogen as a main effect (but not species combination) and all interaction terms. Secondary analyses showed a main effect of species combinations on *Bufo* mortality ( $\chi^2=13.93$ , DF = 3,  $p=0.003$ ).

Specifically, *Bufo* mortality was lowest in single species treatments (B) and highest when *Bufo* were combined with *Rana* (BR; Table 1). *Pseudacris* had highest survivorship when combined with *Bufo* (PB; ( $\chi^2=9.51$ , DF=3,  $p=0.02$ ); Table 1). There was a pathogen effect for *Rana* ( $\chi^2=4.47$ , DF=1,  $p=0.03$ ) but no combination effect. *Rana* had higher survivorship in BD<sup>+</sup> treatments, with the lowest mortality occurring in BD<sup>+</sup> combinations where all species occurred together (PBR, Table 1).

Analyses of the mean number of days survived showed a significant main effect of species ( $F_{2,180}=55.98$ ;  $p<0.001$ ). Secondary species-specific analyses revealed a main effect of species combination on the mean number of days survived by *Pseudacris* ( $F_{3,52}=3.76$ ;  $p=0.02$ ). *Pseudacris* that were combined with *Bufo* (PB treatments) showed the highest mean number of days survived (Table 1).

### Mass and Length

Mean mass and length summaries for each species and all species combinations can be found in Table 1. Mass analyses revealed a species effect ( $F_{2,145}=10.72$ ;  $p<0.01$ ) and a significant species x pathogen interaction term ( $F_{2,145}=3.44$ ;  $p=0.04$ ). The day covariate was also significant ( $F_{1,145}=4.20$ ;  $p=0.04$ ), reflecting that surviving tadpoles were also increasing in mass as the experiment progressed. Species-specific length analyses revealed similar trends, with significant effects of pathogen ( $F_{1,145}=5.60$ ;  $p=0.02$ ), species ( $F_{2,145}=11.7$ ;  $p<0.01$ ), a species x pathogen interaction ( $F_{2,145}=5.14$ ;  $p=0.01$ ), and a significant day covariate ( $F_{1,145}=3.78$ ;  $p=0.05$ ). *Rana* was the largest

(in both mass and length) of the three species tested. Mass of *Rana* tadpoles in  $BD^+$  treatments was greater than those in  $BD^-$  treatments (Table 1).

Mass and length analyses were also conducted for species combinations, revealing main effects of pathogen (Mass,  $F_{1,137}=4.86$ ;  $p=0.03$ ; Length,  $F_{1,137}=10.2$ ;  $p=0.002$ ) and species combination (Mass,  $F_{6,137}=2.56$ ;  $p=0.02$ ; Length,  $F_{6,137}=2.40$ ;  $p=0.03$ ), with day as a significant covariate (Mass,  $F_{1,137}=11.44$ ;  $p=0.001$ ; Length,  $F_{1,137}=7.98$ ;  $p=0.005$ ). The pathogen x species combination interaction term was not significant ( $F_{6,145}=2.1$ ;  $p=0.06$ ). Species that were present with *Bufo* (*Pseudacris* from PB, and *Rana* from BR) weighed more compared to *Pseudacris* and *Rana* from single species treatments (P and R).

*Rana* from  $BD^+$  treatments had higher mass compared to those in  $BD^-$  treatments ( $F_{1,51}=5.77$ ;  $p=0.02$ ; Figure 2). *Rana* also grew longer in  $BD^+$  treatments ( $F_{1,51}=13.5$ ;  $p=0.001$ ), particularly when combined with *Bufo* ( $F_{3,51}=2.85$ ;  $p=0.05$ ; Fig. 2).

There was a significant main effect of pathogen ( $F_{1,48}=4.50$ ;  $p=0.04$ ) where *Pseudacris* that were combined with *Bufo* grew longer in  $BD^+$  treatments (PB; Fig. 2).

High mortality, and subsequent decomposition and cannibalism prevented the collection of adequate mass and length data from *Bufo* when combined with *Pseudacris* and/or *Rana* in  $BD^+$  treatments (PB, BR, and PBR combinations). Data for *Bufo* in  $BD^-$  treatments are summarized in Table 1.

## Discussion

Our results provide quantitative evidence that BD may significantly influence community composition and diversity via differential susceptibility of host species, and through indirect, parasite-mediated interactions between host species in a community.

The BD pathogen affected larvae of the three host species with varying degrees of severity. *Bufo boreas*, *Pseudacris regilla*, and *Rana cascadae* tadpoles harbored severe, intermediate, and very light to zero BD infection respectively across all species combinations. In addition, severely infected *Bufo* died more quickly than those with lighter infections. These results corroborate an earlier study that showed mortality rates were greatest in BD-exposed *Bufo* larvae, compared to *Pseudacris* and *Rana* mortality in single species experiments (Blaustein et al. 2005).

The variation in infection severity between species is particularly important when considering the contribution of each species to infection dynamics in more realistic mixed-species settings. Our results show that infection severity was directly associated with species composition. Specifically, *Bufo* experienced more severe infections when they occurred alone in single-species treatments compared to when they were combined with *Pseudacris* or *Rana*. We saw a trend of decreasing infection severity with increasing species richness in species combinations that contained *Bufo*, providing evidence for a dilution effect in a phenomenological sense. To examine this further, we compared infection severity in two-species combinations (*Bufo* from the

PB and BR combinations) where relative abundance and total density remained constant and species composition was manipulated. Infection severity of *Bufo* did not differ between PB and BR species combinations (Fig 1c), and therefore the dilution effect could be driven solely by total *Bufo* density. *Pseudacris* from two-species combinations (PB and PR) showed increased infection severity in the presence of *Bufo* and decreased severity in the presence of *Rana* (Fig1d), suggesting that the identity of species across various host species assemblages may also play an important role in infection dynamics. The presence of certain species may lead to increased pathogen loads independent of density and richness. The design of our experiment does not allow us to determine whether the dilution effect was a density-dependent result of decreasing numbers of *Bufo*, increasing species richness, or some combination of both factors. Future studies on the relative roles of species richness and species abundance will be important to determine the generality of the dilution effect for this and other host-pathogen systems.

Although transmission rates were not explicitly measured, it is possible that the observed patterns of infection severity may be the result of variation in within- vs. between-species BD transmission. Specifically, our results suggest that within-species BD transmission may be much greater among *Bufo* than between-species transmission to either of the other two species. *Bufo* to *Rana* and *Pseudacris* to *Rana* transmission was seemingly nonexistent, and *Rana* consistently showed low to zero pathogen loads regardless of species combination. Moreover, mean BD loads were generally lower in *Bufo* that were combined with other species, whereas BD loads in *Rana* and

*Pseudacris* did not change when each of these species were combined with *Bufo*. These results suggest that BD-infected *Bufo* might not pose a substantial threat to coexisting *Pseudacris* or *Rana* tadpoles in terms of enhanced transmission, but the BD pathogen may be more likely to persist in systems where *Bufo* are present. The patterns of infection severity that we observed lead us to speculate that *Rana* + *Pseudacris* combinations may be experiencing ‘transmission reduction’ in the field, whereby one weakly competent or non-host species effectively reduces pathogen loads in coexisting host species that are more susceptible to infection (Keesing et al. 2006). *Rana* may essentially be acting as a non-host mutualist for *Pseudacris* in the presence of BD, facilitating increased growth (body length) in addition to decreasing pathogen loads in *Pseudacris* when they occur with *Rana*. Transmission reduction among hosts in mixed species assemblages may be one mechanism contributing to a dilution effect in this system. However, to our knowledge there are no empirical examples of transmission reduction in the literature (Keesing et al. 2006; Schauber and Ostfeld 2002).

Importantly, there is still a general lack of consensus on whether the BD pathogen exhibits frequency- or density-dependent transmission (Rachowicz and Briggs 2007). It is also possible that BD could exhibit both forms of transmission (Rachowicz and Briggs 2007; Ryder et al. 2007). Moreover, experiments explicitly measuring inter- vs. intraspecific transmission rates have not been conducted (but see (Rachowicz and Vredenburg 2004). Since virtually all existing theoretical predictions hinge on these parameters, we anticipate the improvement of our conclusions by future

studies examining the transmission dynamics of the BD pathogen in more detail. In particular, studies examining the functional form of interspecific transmission will shed light on the mechanisms underlying the dilution effect in multi-host systems (Rudolf and Antonovics 2005).

In addition to pathogen transmission, our results highlight the importance of considering interactions between co-occurring species when examining infection dynamics. When *Bufo* and *Pseudacris* were combined in  $BD^-$  treatments, *Bufo* survivorship decreased while *Pseudacris* survivorship increased compared to respective single-species treatments. In this context, *Pseudacris* was the superior competitor to *Bufo*. Competitive dominance of *Pseudacris* was amplified in  $BD^+$  treatments where dramatic increases in mass and length accompanied increased survivorship in *Pseudacris* that were combined with *Bufo*. Similarly, *Rana* survivorship, mass, and length increased when combined with *Bufo* in  $BD^+$  treatments, whereas this effect was absent in  $BD^-$  treatments. Thus, we observed competition mediated by the BD pathogen, where *Bufo* mortality increased in the presence of heterospecific competitors and the BD pathogen. We also observed pathogen-mediated mutualism in both *Pseudacris* and *Rana* when each species was combined with *Bufo*. This mutualism was evidenced by dramatic increases in mass and length for both species in  $BD^+$  treatments. These effects were likely the result of competitive release experienced by *Pseudacris* and *Rana* following high *Bufo* mortality from infection and interspecific competition working synergistically in mixed species combinations. Indeed, increased opportunistic cannibalism of dead *Bufo* by

heterospecifics likely explains the paucity of intact *Bufo* carcasses available for mass and length data in our study. Whether BD infection increases *Bufo* vulnerability to cannibalism or other forms of intraguild predation remains a potentially significant area for future work.

Empirical support for the importance of infectious pathogens in structuring communities and influencing biodiversity remains scarce in contemporary ecological literature (Hatcher 2006). Our results provide quantitative evidence that BD may significantly affect community composition and diversity through differential species susceptibility within a community. Furthermore, this study empirically illustrates how BD may promote species exclusion or coexistence by indirectly affecting species interactions through competition and mutualism mediated by a shared pathogen. Future studies incorporating more realistic communities (e.g., mesocosms studies), accompanied by well-designed and long-term field studies will help determine to what extent the patterns we have observed in the laboratory are corroborated in the field. Given the global decline of amphibian populations and the association of some of these declines with BD infection, future studies explicitly addressing interactions between coexisting host species will be critical for informing conservation and management efforts, and to gain a mechanistic understanding of infection dynamics occurring in the amphibian chytridiomycosis system at the community level. Since parasitism may be the most common life-history strategy (May 1988), it is plausible that parasites may influence nearly all aspects of host ecology and play a major role in structuring communities and whole ecosystems. Empirical examination of existing

theory via experimentally tractable multi-host systems will bring us closer to a mechanistic understanding of the phenomenological relationships between biodiversity and disease dynamics, and the role of parasite-mediated interactions in determining patterns of species occurrence and abundance in nature.

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Table 3.1. Mean proportion mortality, mass (mg), length (mm), days survived and log BD pathogen concentration  $\pm$  standard errors for three focal host species (P=*Pseudacris regilla*; B=*Bufo boreas*; R=*Rana cascadae*) in various combinations (1-, 2-, and 3-species combinations). BD<sup>+</sup> are infected, and BD<sup>-</sup> are pathogen-free control treatments. NA indicates treatments where excessive decomposition/cannibalism prevented adequate measurements.

Species combos	Focal species	BD <sup>-</sup>				BD <sup>+</sup>				
		Mortality	Mass	Length	Days	Mortality	Mass	Length	log [BD]	Days
P	P	0.64 $\pm$ 0.08	173.7 $\pm$ 59.0	7.9 $\pm$ 1.0	21.7 $\pm$ 3.2	0.53 $\pm$ 0.08	171.1 $\pm$ 13.7	8.9 $\pm$ 0.3	0.11 $\pm$ 0.04	27.6 $\pm$ 1.9
B	B	0.81 $\pm$ 0.06	166.1 $\pm$ 41.5	8.1 $\pm$ 0.6	18.4 $\pm$ 3.2	0.81 $\pm$ 0.06	87.6 $\pm$ 31.6	7.2 $\pm$ 0.7	0.53 $\pm$ 0.10	16.5 $\pm$ 2.5
R	R	0.44 $\pm$ 0.07	381.7 $\pm$ 83.2	11.9 $\pm$ 1.1	31.2 $\pm$ 1.7	0.46 $\pm$ 0.07	441.4 $\pm$ 64.3	12.1 $\pm$ 0.8	0.009 $\pm$ 0.007	29.4 $\pm$ 2.8
PB	P	0.33 $\pm$ 0.10	144.3 $\pm$ 45.2	7.9 $\pm$ 1.1	32.3 $\pm$ 3.1	0.38 $\pm$ 0.10	433.1 $\pm$ 102.9	11.5 $\pm$ 0.9	0.20 $\pm$ 0.10	33.3 $\pm$ 2.8
PB	B	0.88 $\pm$ 0.07	206.0 $\pm$ 49.3	8.7 $\pm$ 1.0	22.3 $\pm$ 3.2	0.96 $\pm$ 0.06	NA	NA	0.31 $\pm$ 0.12	10.5 $\pm$ 2.1
BR	B	1.00 $\pm$ 0.00	149.5 $\pm$ 117.9	7.3 $\pm$ 2.2	9.8 $\pm$ 2.6	0.92 $\pm$ 0.06	NA	NA	0.38 $\pm$ 0.24	11.2 $\pm$ 3.5
BR	R	0.43 $\pm$ 0.11	353.5 $\pm$ 116.6	9.0 $\pm$ 1.4	31.0 $\pm$ 3.3	0.42 $\pm$ 0.10	693.5 $\pm$ 110.4	14.6 $\pm$ 0.6	0.01 $\pm$ 0.01	29.4 $\pm$ 2.5
PR	P	0.64 $\pm$ 0.10	313.5 $\pm$ 171.4	7.7 $\pm$ 1.0	22.2 $\pm$ 4.5	0.67 $\pm$ 0.10	277.1 $\pm$ 74.2	10.2 $\pm$ 1.3	0.04 $\pm$ 0.03	23.0 $\pm$ 3.6
PR	R	0.48 $\pm$ 0.11	426.8 $\pm$ 126.2	11.2 $\pm$ 1.6	30.4 $\pm$ 3.9	0.38 $\pm$ 0.10	457.1 $\pm$ 81.9	13.0 $\pm$ 1.1	0.009 $\pm$ 0.006	32.6 $\pm$ 3.9
PBR	P	0.42 $\pm$ 0.12	172.4 $\pm$ 63.4	8.7 $\pm$ 1.6	31.9 $\pm$ 3.3	0.69 $\pm$ 0.12	200.0 $\pm$ 51.6	9.3 $\pm$ 0.8	0.12 $\pm$ 0.11	21.7 $\pm$ 2.4
PBR	B	0.94 $\pm$ 0.06	128.2 $\pm$ 35.19	8.3 $\pm$ 1.0	11.5 $\pm$ 4.3	1.0 $\pm$ 0.00	NA	NA	0.02 $\pm$ 0.01	13.9 $\pm$ 1.6
PBR	R	0.61 $\pm$ 0.12	212.8 $\pm$ 107.4	7.7 $\pm$ 1.5	27.0 $\pm$ 3.3	0.13 $\pm$ 0.09	868.8 $\pm$ 208.9	14.0 $\pm$ 1.2	0.004 $\pm$ 0.004	38.8 $\pm$ 2.4

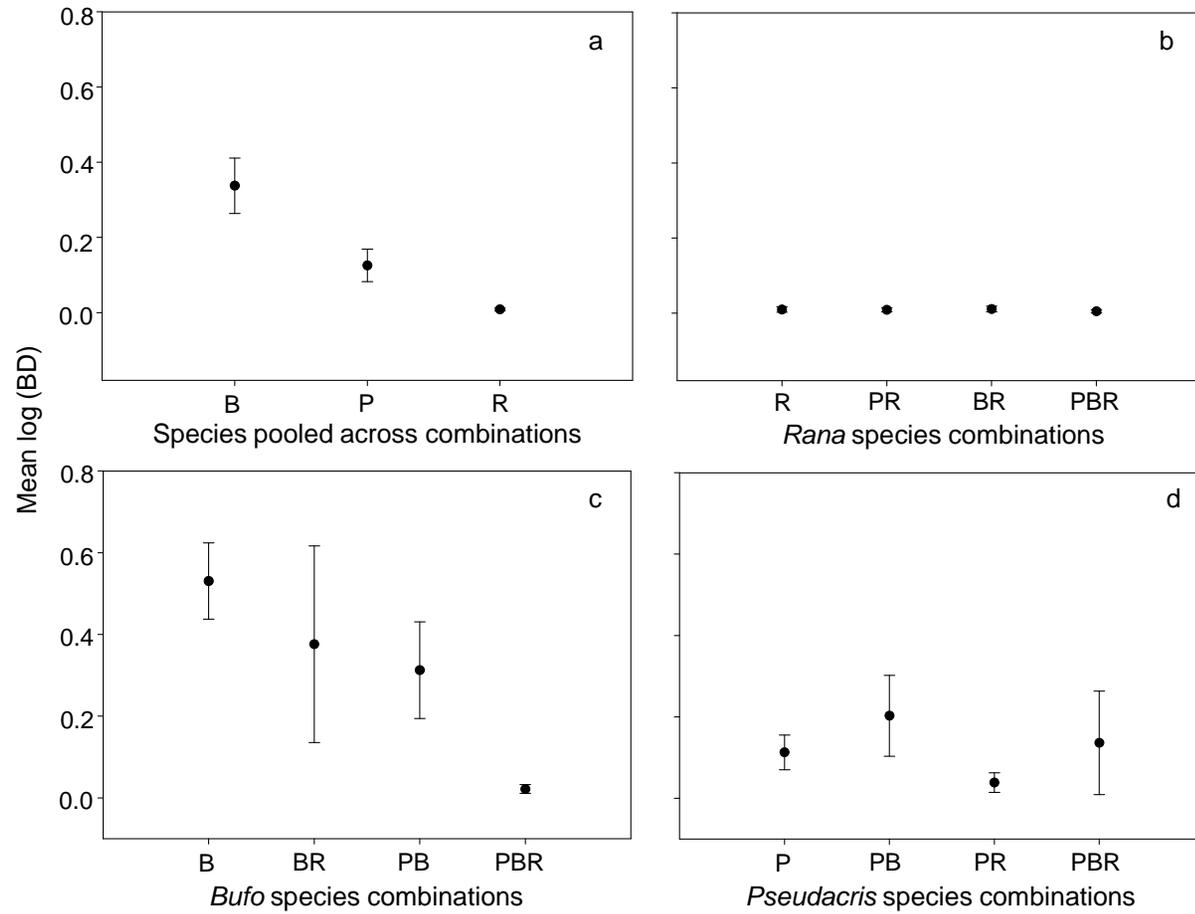


Figure 3.1. Mean log of genome equivalents for *Batrachochytrium dendrobatidis* (mean log(BD)) detected using real-time PCR. (a) The mean BD loads for three species across all species combinations where they occur; and by species combinations containing *Rana cascadae* (b), *Bufo boreas* (c), and *Pseudacris regilla* (d).

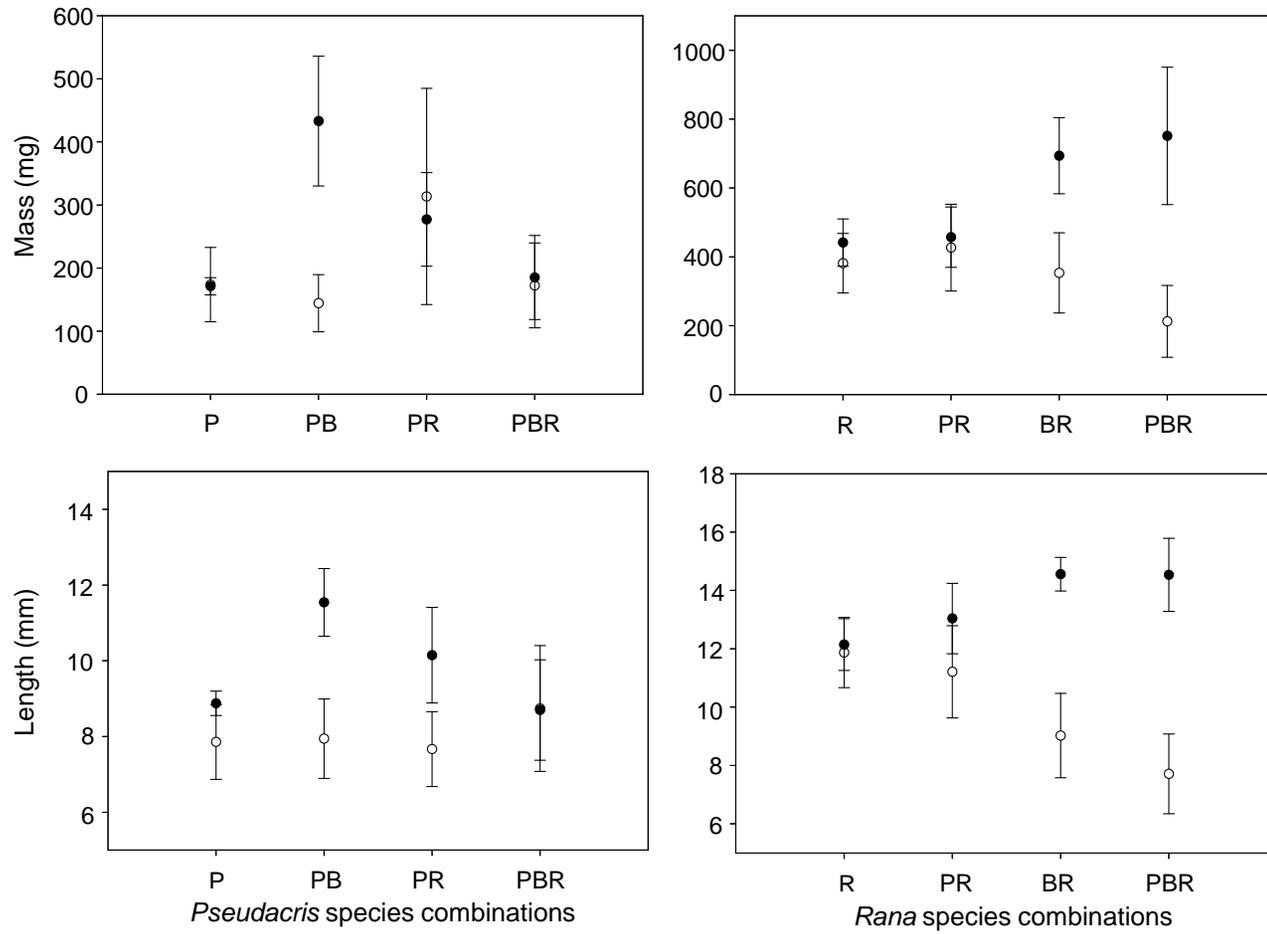


Figure 3.2. Mean mass (mg) and body length (mm) of tadpoles in BD<sup>+</sup> (●) and BD<sup>-</sup> treatments (○) across species combinations containing *Pseudacris regilla* (a,c) and *Rana cascadae* (b,d).

CHAPTER 4

INTERSPECIFIC DIFFERENCES IN ANTIPREDATOR BEHAVIOR OF  
AMPHIBIAN HOSTS EXPOSED TO THE FUNGAL PATHOGEN,  
*BATRACHOCHYTRIUM DENDROBATIDIS*.

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**Abstract**

Parasite-induced behavioral changes can have important consequences for ecological communities. The effects of parasitism on antipredator behaviors in hosts may be especially important since few behaviors are more crucial for ensuring immediate survival than the avoidance of lethal predators. We examined the effects of an emerging fungal pathogen of amphibians, *Batrachochytrium dendrobatidis* (BD), on antipredator behaviors of four anuran species at the larval stage. In two species, we also tested whether BD-induced changes in antipredator behaviors altered survivorship in the presence of lethal predators. Our results show that exposure to BD was sufficient to induce changes in activity rate and refuge use for western toad (*Bufo boreas*) larvae, but not in the other species tested. Predators consumed BD exposed and non-exposed prey at similar rates suggesting the possibility that predators may not differentiate between infected and uninfected prey. BD exposure may influence predator-prey and infection dynamics by inducing changes in antipredator behaviors of some host species.

## Introduction

Pathogens and parasites can influence animal behaviors with important consequences for the surrounding community. Some behavioral changes caused by parasitism, such as fearlessness towards predators and reversed geo- or phototaxis, can have lethal outcomes (Moore 2002) while other changes, such as altered aggregation or thermoregulatory behaviors (Han et al. in press; Ouedraogo et al. 2004) might appear less dramatic. Thus, parasites can exert substantial selection pressures on hosts by altering their behaviors and can contribute to the evolution of social and mating interactions in many systems (Altizer et al. 2003b; Freeland 1976; Loehle 1995). With the number of emerging infectious disease events increasing worldwide (Jones et al. 2008), studies examining the effects of parasitism on host behaviors will lend insight to the important and potentially increasing role of infectious agents in ecological communities.

One emerging fungal pathogen, *Batrachochytrium dendrobatidis* (BD, Berger et al. 1998; Longcore et al. 1999), infects many amphibian species at post-embryonic stages (larvae, juveniles and adults; (e.g. Blaustein et al. 2005; Garcia et al. 2006; Puschendorf et al. 2006). The resulting disease, chytridiomycosis, has been associated with declines and extinctions of several amphibian populations in North and Central America, and Australia (Lips et al. 2006; Rachowicz et al. 2006; Schloegel et al. 2006). Although BD infects only amphibians, it may be considered a generalist pathogen because it infects amphibians from taxonomically diverse groups across

multiple life history stages (salamanders, frogs and toads from different families; [www.spatalepidemiology.net](http://www.spatalepidemiology.net)). Severe infections can be lethal in some species (e.g. Rachowicz et al. 2006), but asymptomatic in others (e.g. Daszak et al. 2004; Sánchez et al. in press). BD infection can also negatively affect growth and development rates in host species (e.g. Parris and Beaudoin 2004). Exposure to the BD pathogen can also alter ecologically important host behaviors, including some social interactions (schooling) and thermoregulatory behaviors (Han et al. in press). Studies on the effects of BD on host behaviors remain scarce (see Han et al. in press; Parris et al. 2006; Rowley and Alford 2007).

Many behavioral decisions in the animal kingdom result from information transmitted via chemical and visual cues. In aquatic systems chemical cues are especially important for facilitating social interactions, mating, oviposition decisions, parental care and predator-prey interactions (e.g. Bentley and Day 1989; Blaustein and Waldman 1992; Kam and Yang 2002; Kats and Dill 1998; Schwab and Brockman 2007). For example, in larval amphibians, chemical cues from predators (predator cues) and chemical cues signaling alarm from injured conspecifics (alarm cues) can provide information about predation risk (Chivers and Smith 1998; Kats and Dill 1998). Potentially, parasitism may change the way infected hosts process or respond to unaltered chemical cues (Jog and Watve 2005). These changes can influence the chemically-mediated behavioral interactions in a community.

Behavioral responses to predator or alarm cues may have particularly important consequences for hosts, as few behaviors are more crucial for ensuring

immediate survival than avoidance of lethal predators (Lima and Dill 1990). Amphibians have many natural predators in the wild, including other amphibians (Morin 1983). In the Pacific Northwest, salamanders are among the most important vertebrate predators of amphibian larvae (e.g. Peterson and Blaustein 1991). In response to the risk of predation by salamanders, anuran larvae (tadpoles of frogs and toads) may exhibit various antipredator behaviors. For example, exposure to chemical cues from predators that depend on vision for prey capture may cause tadpoles to seek refuge; and exposure to cues from vibration/motion-detecting predators may cause tadpoles to drastically decrease activity levels (Lima and Dill 1990). Although the expression of antipredator behaviors is threat-sensitive and context-dependent, combinations of flight and refuge use are commonly exhibited by many amphibian species (Chivers and Smith 1998; Kats and Dill 1998).

Exposure to a pathogen that alters antipredator behaviors could have potentially important consequences for predator-prey dynamics through host survival (Parris et al. 2006). We conducted a series of experiments to determine whether activity rates and refuge use were affected by BD exposure in larvae of four anuran species using chemical cues from predators that co-occur with these species in natural amphibian communities. In addition, we conducted a feeding trial on two of these host species to determine if BD-induced changes in antipredator behavior altered survivorship when hosts are faced with lethal predators.

## Methods

### Tadpole hosts

BD does not infect amphibian embryos, presumably because they lack keratin which is required by BD for growth, reproduction, and survival (Longcore et al. 1999). To ensure infection-free animals, we collected partial clutches of several egg masses of *Rana aurora* from permanent ponds in Monmouth, OR (Polk County, elevation 61 m). Several whole egg masses of *Pseudacris regilla* were collected from temporary ponds and wetlands in Corvallis, OR (Benton County, elevation 87 m). Partial clutches of several *Rana cascadae* and *Bufo boreas* egg masses were collected from permanent water bodies in Deschutes County, OR (elevation 1951 m). Eggs were collected at early developmental stages 11-15 (Gosner 1960). Eggs were reared in 37 L aquaria containing filtered water with aeration. Upon hatching, conspecifics from separate clutches were mixed between multiple 37 L aquaria at densities of approximately 100 tadpoles per tank. Complete water changes were conducted approximately every 7 days. Tadpoles were fed a 2:1 ratio of ground alfalfa pellets to fish flakes *ad libitum*. All animals were kept at 14-16°C on a 14:10 photoperiod for the duration of the experiment. At Gosner developmental stage 28-29 (Gosner 1960), tadpoles were moved into 11 L aquaria at densities of 20 tadpoles for inoculation with BD or a control treatment.

### Inoculation regime

We used inoculation methods known to produce infection in larvae of these species (Han et al. in press). Culture plates (1% Tryptone and agar) containing BD (JEL strain 215) were flooded with 15 mL of filtered water for 20 minutes to allow the discharge of infectious zoospores from sporangia. The liquid contents from 2 flooded culture plates were added to each of six 11 L aquaria containing 20 tadpoles each. The culture plates were submerged for 1-2 seconds in water to ensure that zoospores were transferred from the culture plate to the aquarium. The procedure was repeated with control culture plates (1% Tryptone and agar without BD) for six additional 11 L aquaria containing 20 animals each. Using a hemacytometer we counted an average concentration of  $6.18 \times 10^6$  zoospores/mL for three flooded BD culture plates. Tadpoles were exposed to treatments for 10 days. Tadpoles used in these experiments were taken haphazardly from treatment aquaria to avoid pseudoreplication. For brevity, we refer to tadpoles exposed to BD as “BD<sup>+</sup>” and tadpoles exposed to the control agar wash as “BD<sup>-</sup>”. Tissues from animals used in these experiments were not retrospectively sampled to quantify infection severity, thus BD<sup>+</sup> and BD<sup>-</sup> designations refer only to exposure status. Since it is not possible to match BD infection severity in tadpoles *a priori* without sacrificing them, our hypotheses and results examine the effects of BD exposure only (not infection severity).

### Predators

We chose predators that co-occur with host species from two amphibian communities commonly found in the Pacific Northwest. From the valley region, we collected

rough-skinned newts (*Taricha granulosa*) as predators of *R. aurora* and *P. regilla* (Calef 1973). From high-elevation sites, we collected larval long-toed salamanders (*Ambystoma macrodactylum*), a common predator of *R. cascadae* and *B. boreas* (Bancroft et al. 2008). *Taricha* were held in 37 L aquaria, two individuals per tank. Six *Ambystoma* larvae were housed in each of several 37 L aquaria. *Ambystoma* were separated by mesh compartments (12 x 20 x 25 cm) within aquaria to prevent cannibalism (Wildy et al. 1998). Predators were fed a single species diet of tadpoles *ad libitum* for approximately one month prior to experiments to accumulate species-specific chemical cues in the water. For example, *Ambystoma* larvae were fed a diet of only *P. regilla* tadpoles for one month prior to trials on *P. regilla*. Water used for behavior trials contained a combination of predator cues, and alarm cues derived from predators consuming tadpoles. We refer to this mixture of predator and tadpole alarm cues as “predator cue” hereafter. Neutral (control) cues were harvested from two 37 L aquaria containing approximately 28 grams of *Tubifex* worms in each tank (*Tubifex tubifex*) for all species except *R. aurora*. The neutral cue for *R. aurora* trials was filtered water (Hews and Blaustein 1985) because *Tubifex* worms were unavailable during trials for *R. aurora*. *Tubifex* worms are not consumed by tadpoles, and are non-predatory detritivores that occur in many freshwater ecosystems (Gilbert and Granath Jr. 2003). Predators were starved for 5 days prior to feeding trials to standardize hunger levels.

#### Activity rates and refuge use of tadpoles

To examine activity and refuge use, we filled opaque plastic chambers (31.5 x 11 x 20 cm) with 1 L filtered water. The chambers were placed atop grids visible through the bottom of the chambers (grid squares = 25 mm<sup>2</sup>). Refuges were constructed using black polyvinyl chloride pipe cut into segments and fitted inside 20 mL glass beakers to form a dark tunnel. A single refuge was placed haphazardly in one corner of each chamber. Chambers containing 1 refuge and 1 tadpole from the appropriate treatment (BD<sup>+</sup> or BD<sup>-</sup>) were left overnight so tadpoles could acclimate. Our experiment employed a 2x2 factorial design with four treatment combinations for each host species tested separately: BD<sup>+</sup> with predator cue, BD<sup>+</sup> with neutral cue, BD<sup>-</sup> with predator cue, BD<sup>-</sup> with neutral cue. Treatments were randomly assigned to chambers and replicated 30 times for each species except *Rana aurora* (20 reps for predator cue treatments, 25 reps for neutral cue treatments). 500 mL of predator or neutral cue was added to chambers. Tadpoles experienced an acclimation period of 60 minutes prior to data collection. Prior to trials, tadpoles of each species were observed near, underneath, or inside of refuges (pers. obs.) ensuring that tadpoles were not repelled by the presence of constructed refuges.

We recorded the number of gridlines crossed and the number of times each tadpole used refuge for 30-second intervals for 4 hours. To ensure independence of activity data from refuge use data, we counted the number of times the tadpole used refuge during the 30 second period and recorded the number of gridlines crossed by the tadpole when it was not using refuge. Black curtains surrounding test chambers eliminated visual cues from observers during trials. All trials were conducted between

1000 and 1400 hours. We used 2-way analyses of variance for statistical analyses of the mean activity rate and refuge use of tadpoles from  $BD^+$  and  $BD^-$  treatments for each species.

### Feeding trials

We tested whether BD-exposure altered survivorship for *R. cascadae* and *R. aurora* in the presence of lethal predators. Rectangular plastic tubs (capacity: 39.7 L, size: 0.16 x 0.86 x 0.42 m) were filled with approximately 7.6 L filtered dechlorinated water and acclimated to cold-room temperature (14-16°C). 10 tadpoles from either  $BD^+$  or  $BD^-$  treatments were added to each tub and allowed to acclimate overnight. At 0900 the next morning a single predator was added to each tub (*A. macrodactylum* larvae for *R. cascadae* trials, and adult *T. granulosa* for *R. aurora* trials). We recorded the number of tadpoles remaining in each tub after 60 minutes. Each predator was used only once and there were 7 replicates per treatment. Data on the proportion of consumed tadpoles were arcsine root transformed, and analyzed using a Student's t-test.

## **Results**

There were no differences in activity rate (mean number of gridlines crossed in 30 seconds) or refuge use (mean number of times observed using refuge in 30 seconds) between BD or cue treatments for *R. cascadae* and *P. regilla* (Table 4.1).

*Rana aurora* showed a main effect of cue treatment on activity rates (Figure 4.1a) but there was no effect of either BD exposure or cue treatment on refuge use (Figure 4.1b). Post-hoc tests using Tukey HSD revealed that *R. aurora* tadpoles exposed to predator cues were less active than those exposed to neutral cues (Figure 4.1a). In *B. boreas*, there were significant main effects of both BD and cue treatments in activity and refuge use (Table 4.1). The Tukey HSD procedure revealed that BD<sup>+</sup> *Bufo* tadpoles in predator cue treatments were more active and used refuge more often (Figure 4.2a, b).

In feeding trials using lethal predators, there were no differences between BD<sup>+</sup> and BD<sup>-</sup> treatments in the proportion of tadpoles consumed by predators after 60 minutes for either prey species (*R. cascadae*,  $t=-0.61$ ,  $p=0.55$ ; *R. aurora*,  $t=-1.02$ ,  $p=0.33$ ). *Ambystoma* predators consumed a mean proportion of  $0.5\pm 0.05$  *R. cascadae* tadpoles in a 60 minute period in BD<sup>+</sup> treatments, and a mean proportion of  $0.5\pm 0.05$  tadpoles in BD<sup>-</sup> treatments. *Taricha* predators consumed a mean proportion of  $0.3\pm 0.1$  BD<sup>+</sup> *R. aurora* tadpoles and  $0.1\pm 0.1$  BD<sup>-</sup> *R. aurora* tadpoles in 60 minutes. To ensure that data from feeding trials were not affected by predators becoming satiated within 60 minutes, we continued observations until predators ceased consuming tadpoles for three successive data collections (30 minutes). All predators continued consuming tadpoles for another 180 minutes before ceasing foraging and attack behavior on remaining tadpoles.

## Discussion

Parasites can alter key behaviors in hosts which can have important consequences for the interactions between species in a community. Our results show interspecific differences in antipredator behaviors of amphibian larvae in response to chemical cues of predators. In addition, we found that BD exposure caused strong reactions to predator cues in *Bufo boreas*, but did not alter antipredator behaviors in tadpoles of three other species tested (*R. aurora*, *P. regilla*, *R. cascadae*).

*Rana aurora* in *Taricha*-derived predator cue treatments drastically reduced their activity regardless of BD exposure status. In *R. aurora* it seems that predator cues alone were sufficient to induce antipredator behaviors (reduced activity). Three of the species tested (*P. regilla*, *R. cascadae*, and *R. aurora*) showed no differences in activity rate or refuge use in response to predator cues. Decreased activity and increased refuge use may impact growth and development rates by hampering foraging and active thermoregulation by tadpoles (Dupré and Petranka 1985; Martin and Lopez 1999). Thus, tadpoles from these three species may require more concentrated predator cues or additional cues (such as visual stimuli) to elicit antipredator behaviors. It is possible that behavioral responses to added predator cues occurred instantaneously, and tadpoles resumed typical behaviors when there were no visual cues or additional predator cues during the trials. Reduced activity may lead to decreased foraging and thermoregulation, which may have important fitness

consequences, especially for amphibian larvae developing in ephemeral habitats (e.g. *P. regilla* and *R. aurora*).

BD<sup>+</sup> *Bufo* tadpoles reacted strongly to introduced predator cues by increasing activity rates and refuge use compared to BD<sup>-</sup> tadpoles. Hyperactive BD<sup>+</sup> *Bufo* that frequently utilize available refuge may experience decreased predation by making capture mechanically more difficult for predators (Hews 1988; Niell and Cullen 1974). Moreover, *B. boreas* typically occur in large aggregations in nature (O'Hara and Blaustein 1982). Large groups of hyperactive BD<sup>+</sup> *Bufo* could add to a confusion effect that helps to evade predation (reviewed in Hoff et al. 1999; Watt et al. 1997), though group behavior may differ from that of individuals observed in a laboratory setting. Increased activity of BD<sup>+</sup> *Bufo* individuals may make them more conspicuous to predators, thereby increasing predation rates. However, the adaptive values of forming a group likely outweigh the predation risk for individuals. For example, aggregating *Bufo* may be more effective at filter feeding and thermoregulating than individuals (Beiswenger 1977; Hoff et al. 1999; Watt et al. 1997). Thus, even though a large aggregation of hyperactive BD<sup>+</sup> *Bufo* may be more conspicuous to predators, any increases in predation rates are likely offset by increased rates of development, survival, and fitness in remaining group members. The adaptive values of aggregation may explain why this behavior persists in nature even though large groups of infected conspecifics could also lead to increased BD transmission rates and infection severity of conspecifics in close proximity to each other (Han et al. in press). Incidentally, *B. boreas* tadpoles have several invertebrate predators (e.g., Hews 1988) but few known

vertebrate predators because they are noxious and unpalatable (Kats et al. 1988; Peterson and Blaustein 1991). In the laboratory we observed that *Ambystoma* larvae are voracious predators of *Bufo* tadpoles (pers. obs.), and should be considered an important predator in future studies of *Bufo boreas* during larval stages.

Predator-prey interactions in aquatic habitats are presumed to be facilitated by chemical cues in combination with visual cues (Mathis and Vincent 2000; Parris et al. 2006). Interestingly,  $BD^+$  *Bufo* showed significant antipredator behaviors in response to chemical predator cues alone (no visual cues) while  $BD^-$  tadpoles did not show any difference in behavior between predator cue treatments. It is possible that  $BD^-$  exposure may cause *Bufo* to make behavioral decisions more quickly and based on less information, while  $BD^-$  tadpoles require either stronger chemical cues, or a combination of chemical and visual cues from predators before responding with antipredator behaviors (e.g. Parris et al. 2006).

Although we did not examine the physiological basis of altered behavior in tadpole hosts, it is possible that infection alters the ability of hosts to receive and process chemical cues. Among the host species tested, *Bufo* larvae typically experience the most severe infections (Blaustein et al. 2005; Han et al. in press) and in this study, *Bufo* was the only species whose behaviors were altered in response to  $BD$  exposure. Thus, it is possible that the degree of behavioral change we observed in  $BD^+$  *Bufo* may correlate with infection severity. The relationships between infection severity and degree of behavioral change in hosts are an interesting area for future work.

Results of feeding trials showed no difference in the number of  $BD^+$  vs.  $BD^-$  tadpoles consumed by predators for either of the two species examined (*R. cascadae* and *R. aurora*). Since *R. cascadae* showed no detectable change in activity or refuge use as a result of BD exposure, it is not surprising there were also no differences in the number of tadpoles consumed by *Ambystoma* predators between BD treatments. Similarly, feeding trials with *R. aurora* showed no differences in survivorship between BD treatments. Encounter rate, attack rate, and capture rate are all important components contributing to overall rate of predation (Hews 1988). Feeding trials in this study were limited to observing the capture rates of predators when prey were in close proximity without access to refuge. Thus, although *R. aurora* showed significant drops in activity levels in response to predator cues in behavior trials, decreased activity levels may be more effective in reducing encounter rate with predators and may have little effect on curbing attack or capture rate once prey have already been detected by the predator.

Results from the feeding trials also suggest that neither *Taricha* nor *Ambystoma* differentiated between  $BD^+$  and  $BD^-$  hosts, consuming tadpoles from both treatments with similar frequency. Selective predation on infected or uninfected prey has been shown both empirically and theoretically to affect prey population dynamics (Packer et al. 2003 and references therein). Non-selective predation on infected and susceptible tadpoles may offer a general benefit for host populations by reducing the lifespan of infected individuals (Packer et al. 2003), and thereby decreasing BD transmission to susceptible hosts. In addition, many amphibian predators (including

*Ambystoma* and *Taricha* spp.) are themselves susceptible to BD infection (Davidson et al. 2003; Padgett-Flohr and Longcore 2005; Padgett-Flohr and Longcore 2007). Thus, altered transmission dynamics as a result of consuming infected prey or cannibalizing infected conspecifics (Pfennig et al. 1998) remains an unexplored possibility that could influence infection dynamics in both predator and prey populations.

While our study did not quantify pathogen load in host tadpoles, our results confirm that exposure to BD was sufficient to induce changes in host behaviors in *Bufo*. Much of the literature surrounding this pathogen has focused on the implications of direct, infection-induced mortality for amphibian communities. In our study, BD exposure did not cause notable mortality in any of the species, highlighting the possibility that sublethal BD infection can have ecological consequences for amphibian communities by inducing changes in antipredator behaviors.

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Table 4.1. The mean number of gridlines crossed (a) and the mean number of times animals were observed using refuge (b) in 30 seconds for *Rana aurora* in a 2x2 factorial design with two chemical cue treatments (predator, neutral), and two BD exposure treatments (BD<sup>+</sup>, BD<sup>-</sup>).

Species	Source	Activity 2-way ANOVA				Refuge use 2-way ANOVA			
		Df	MS	F	<i>p</i>	Df	MS	F	<i>p</i>
<i>R. cascadae</i>	BD	1	0.48	0.02	0.89	1	0.02	0.39	0.54
	Cue	1	0.12	0.01	0.94	1	0.19	3.27	0.07
	BD*Cue	1	6.96	0.30	0.58	1	0.02	0.33	0.57
	Residuals	108	23.00			109	0.06		
<i>R. aurora</i>	BD	1	4.16	0.99	0.32	1	0.00	0.01	0.91
	Cue	1	112.19	26.77	<b>0.00</b>	1	0.00	0.10	0.75
	BD*Cue	1	2.70	0.64	0.42	1	0.01	0.22	0.64
	Residuals	116	4.19			86	0.03		
<i>B. boreas</i>	BD	1	25.48	0.24	0.63	1	0.23	4.71	<b>0.03</b>
	Cue	1	280.66	2.61	0.11	1	0.41	8.37	<b>0.00</b>
	BD*Cue	1	464.62	4.32	<b>0.04</b>	1	0.10	2.09	0.15
	Residuals	116	107.49			116	0.05		
<i>P. regilla</i>	BD	1	8.04	0.96	0.33	1	0.00	0.00	0.96
	Cue	1	13.93	1.67	0.20	1	0.02	0.27	0.61
	BD*Cue	1	0.02	0.00	0.96	1	0.03	0.49	0.48
	Residuals	114	8.36			116	0.06		

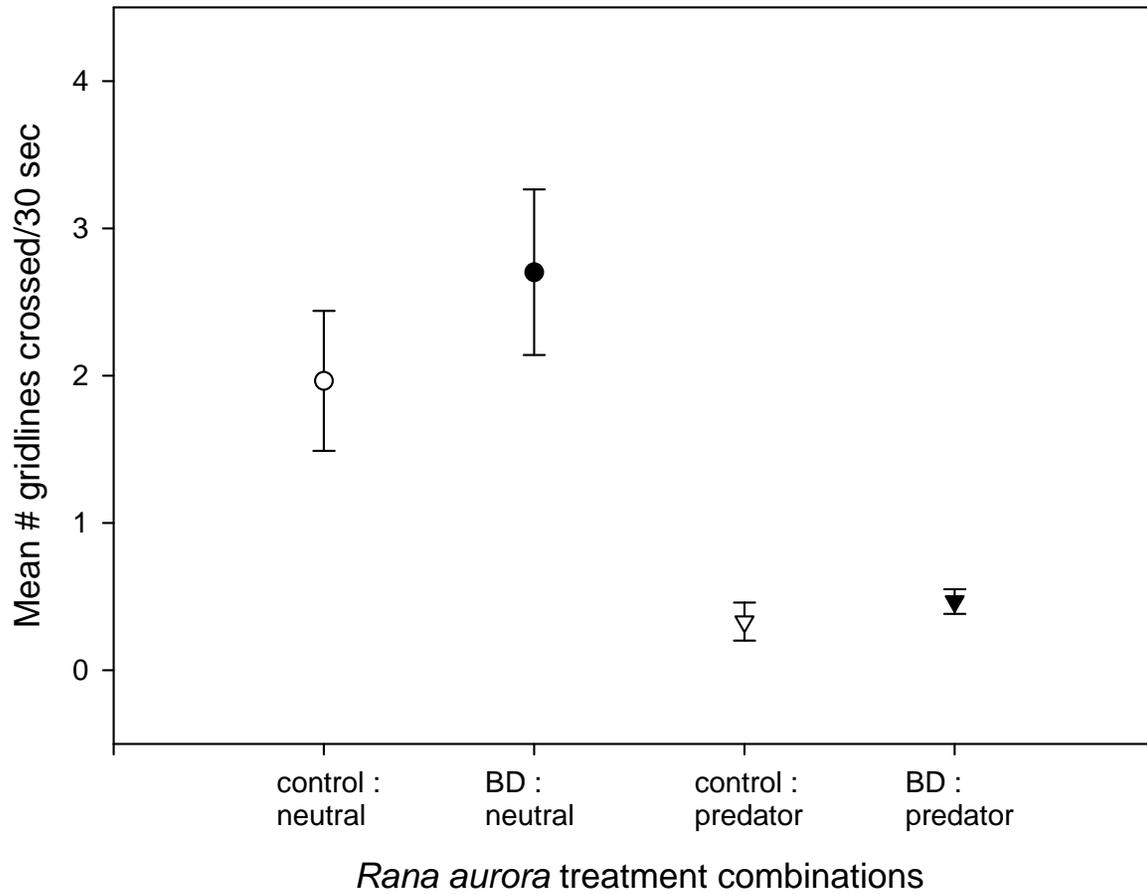


Figure 4.1a. The mean number of gridlines crossed in 30 seconds for *Rana aurora* in a 2x2 factorial design with two chemical cue treatments (predator, neutral), and two BD exposure treatments (BD<sup>+</sup>, BD<sup>-</sup>).

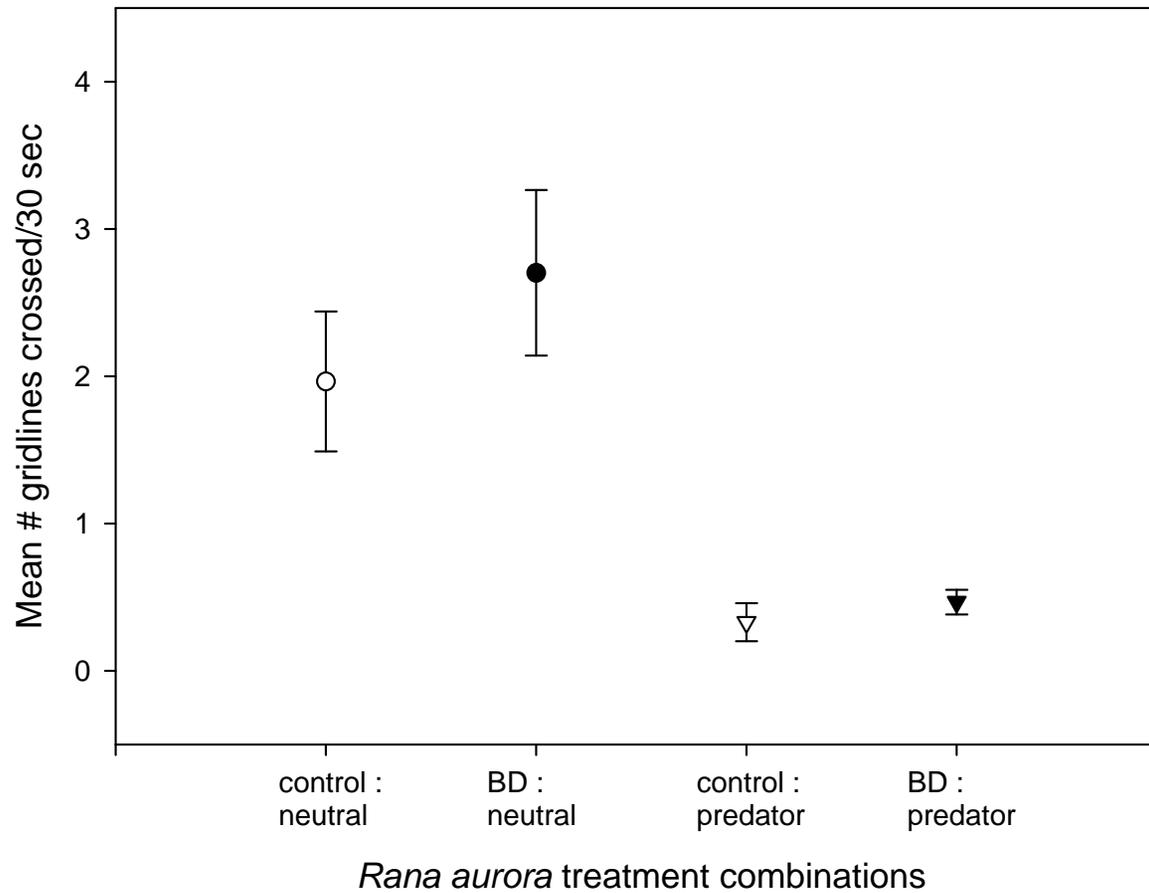


Figure 4.1b. The mean number of gridlines crossed (a) and the mean number of times animals were observed using refuge (b) in 30 seconds for *Rana aurora* in a 2x2 factorial design with two chemical cue treatments (predator, neutral), and two BD exposure treatments (BD<sup>+</sup>, BD<sup>-</sup>).

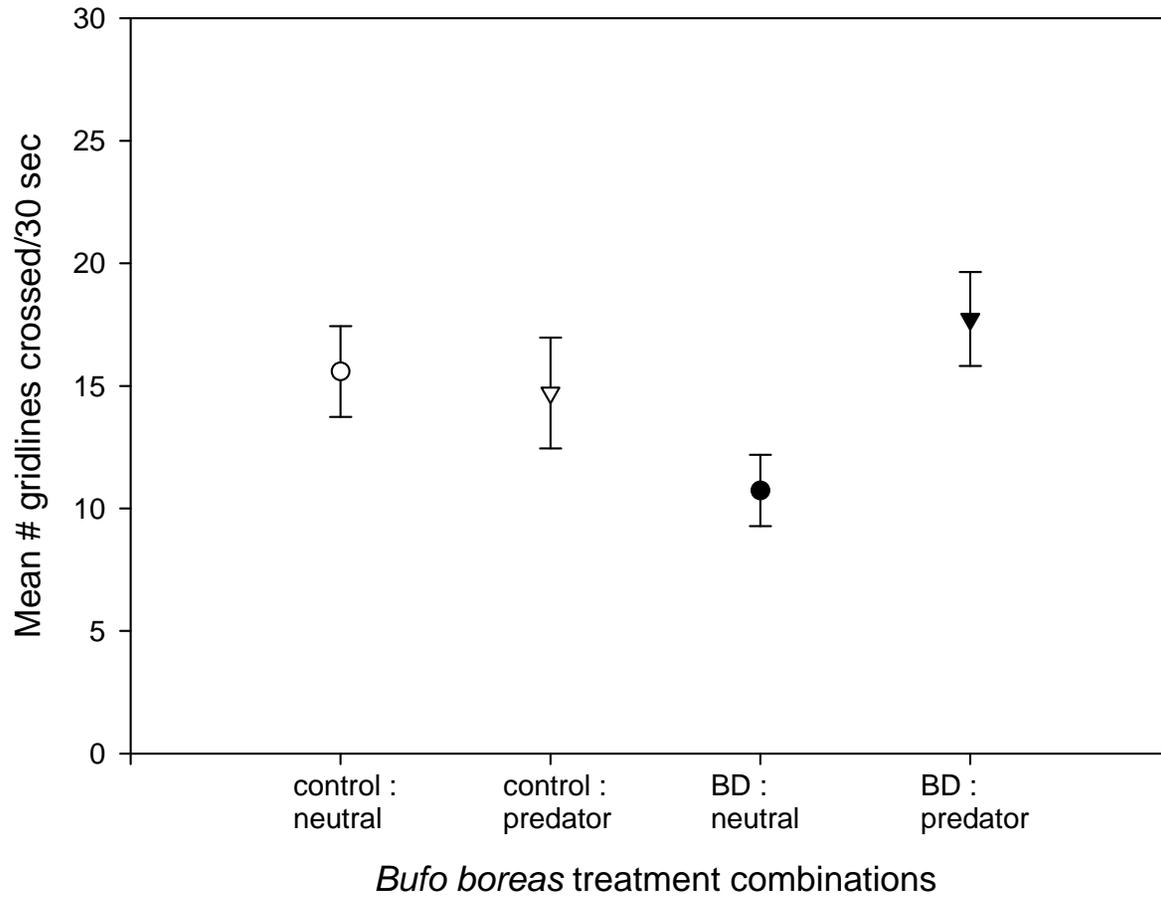


Figure 4.2a. The mean number of gridlines crossed in 30 seconds for *Bufo boreas* in a 2x2 factorial design with two chemical cue treatments (predator, neutral), and two BD exposure treatments (BD<sup>+</sup>, BD<sup>-</sup>).

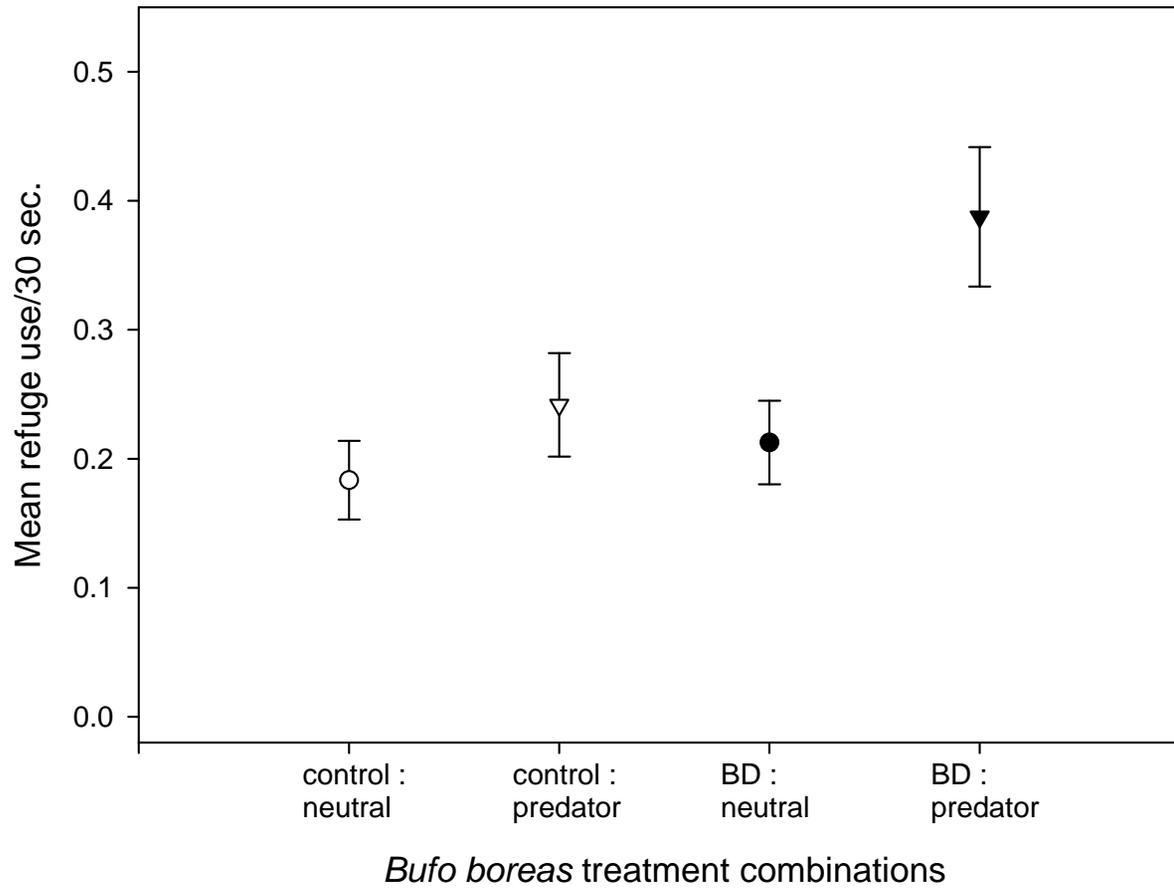


Figure 4.2b. The mean number of times animals were observed using refuge (b) in 30 seconds for *Bufo boreas* in a 2x2 factorial design with two chemical cue treatments (predator, neutral), and two BD exposure treatments (BD<sup>+</sup>, BD<sup>-</sup>).

CHAPTER 5.

CONCLUSIONS

Behavioral change can lead to important consequences for animal ecology. The recent emergence of *Batrachochytrium dendrobatidis* (BD) affords a unique opportunity to study the effects of a novel, globally distributed amphibian pathogen on the behavior and interactions between several native amphibian species in which BD virulence remains relatively low. My thesis explored how several familiar behavioral interactions in larval amphibians change in response to this novel pathogen as a first step towards understanding how infection-induced behavioral changes could influence host and pathogen ecology in natural systems.

In two experiments, I considered how two evolutionarily persistent amphibian behaviors, aggregation and thermoregulation, change with BD infection status (Chapter 2). Results from these experiments suggest that social amphibians may experience increased infection risk because aggregation behaviors change in response to BD exposure. I found that healthy *Bufo boreas* tadpoles schooled more frequently with BD<sup>+</sup> compared to uninfected groups of conspecifics. I also found evidence that healthy *Rana cascadae* tadpoles that had not been exposed to BD were attracted to groups of BD<sup>+</sup> conspecifics. Neither species avoided BD<sup>+</sup> groups of conspecifics, suggesting that some hosts may not be able to distinguish infection status in conspecifics. In experiments on thermoregulatory behavior I found that tadpoles of the four tested species did not induce behavioral fever in response to BD exposure. Even though warm water treatments in the laboratory and fortuitous encounters with thermal springs can effectively clear BD infection, my results suggest that amphibians may not actively seek these high temperatures to mediate BD infection. Rather than changing

thermoregulatory behaviors to clear a potentially lethal infection, larvae of some amphibians may continue seeking temperatures that are optimal for growth and development, as they have for millions of years. In this scenario, even if a minority of hosts actively avoid infected aggregations or periodically seek high temperatures to induce behavioral fever, they may also be highly conspicuous against a majority backdrop of hosts that do not behave similarly. This conspicuousness would select against avoidance or fever behaviors that could mediate BD-infection risk in the wild (e.g., removal via predation or decreased resource allocation through solitary foraging). This chapter emphasizes the importance of considering the relative roles of behavioral plasticity and behavioral inertia in interpreting host responses to selection pressures such as infectious pathogens in nature.

A third experiment (Chapter 3) explored how co-occurring host species can interact with one another to indirectly influence infection dynamics. My results showed strong interactions between species combinations and BD-infection leading to apparent mutualism and apparent competition between species. Specifically, *R. cascadae* and *P. regilla* showed increased growth (mass and length) and survivorship as a result of competitive release from high *B. boreas* mortality. *Bufo boreas* combined with other species experienced decreased infection severity but increased interspecific competition leading indirectly to increased mortality in mixed-species combinations. Though BD infection has received global notoriety because it causes lethal infections and mass mortalities, these results show how indirect interactions mediated by sublethal infection could influence patterns of species coexistence and

species exclusion in amphibian habitats. In this study I also observed a negative correlation between pathogen load and species richness for certain species combinations. However, pathogen load varied between species combinations of equal richness and density, suggesting that both species richness and species identity are factors moderating a dilution effect in this system. Empirical evidence for the dilution effect in disease ecology remains scarce in part because it is difficult to separate the effect of host density from species richness in natural systems. In the amphibian-BD system, a dilution effect could be operating on a context-dependent basis that depends on both the susceptibility and competence of component species. In addition to host species composition, differences in virulence between BD strains observed in recent studies indicate that infection patterns will also depend on differences in pathogen biology between host communities. Overall, by experimentally demonstrating the influence of indirect interspecific interactions on infection dynamics, Chapter 3 provides a preliminary examination of how BD could be affecting assemblages of multiple host species. Long-term field sampling of sites where multiple species coexist is an obvious next step to clarifying experimental results.

Larval amphibians that coexist and compete with one another in a guild must also contend with predators in a community setting. Results from a study examining antipredator behaviors of BD-infected hosts (Chapter 4) suggest that exposure to BD can cause interspecific differences in common antipredator behaviors. Activity and refuge-use increased in *B. boreas* in the presence of predator chemical cues, but this effect was absent in the other three species tested. Results from feeding trials also

show that altered behaviors in response to predator cues may not always translate to increased survival when prey are faced with lethal predators. Predators consumed BD<sup>+</sup> and BD<sup>-</sup> prey of two species (*R. cascadae* and *R. aurora*) at similar rates. This apparent lack of selective predation between BD-exposed and non-exposed tadpoles also shows that predators (e.g., *A. macrodactylum* and *T. granulosa*) may not (or do not) differentiate between infected and uninfected prey. Since many amphibian predators are also susceptible to BD infection, BD transmission to predators preying upon infected hosts remains an unexplored area for future work.

The experiments presented in this thesis observe familiar behaviors and interactions in an unfamiliar context of a recently described pathogen. It is unknown whether BD is novel in the Pacific Northwest, or whether it has been infecting native amphibians without detection until recently. In either case, differences in behaviors between infected and uninfected hosts could help explain current patterns of infection. Results from my dissertation suggest that BD infection propagates complex dynamics in natural systems through species-specific and context-dependent influences on host behaviors. Infection-induced behavioral changes may affect host and pathogen ecology in the wild by influencing contact patterns between susceptible and infected hosts, by affecting indirect interactions between multiple coexisting host species, and by shifting predator-prey dynamics in nature. Host behavior remains a crucial yet understudied determinant of disease ecology. Results from my dissertation highlight the general importance of host behaviors in determining infection patterns and dynamics in natural populations.

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