AN ABSTRACT OF THE DISSERTATION OF

Trang D. Dang for the degree of Doctor of Philosophy in Integrative Biology presented on June 9, 2017.

Title: The Effects of Host and Pathogen Variation on Infection Dynamics in the Amphibian-Chytrid System.

Abstract approved:

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Andrew R. Blaustein

This dissertation uses manipulative experiments to explore amphibian-*Bd* infection dynamics. Although there has been almost two decades research since the discovery of *Bd*, many questions still remain regarding what conditions mediate chytridiomycosis virulence. My research shows how certain host and pathogen factors can influence disease virulence. Identifying how host and pathogen factors mediate disease virulence is important in order to understand, predict, and mitigate this infectious amphibian disease.

Worldwide biodiversity loss is occurring at unprecedented rates. Numerous factors are contributing to this loss, including infectious disease. Among vertebrate groups, amphibians are experiencing the highest rate of population declines and extinctions and are vulnerable to numerous infectious pathogens that appear to be contributing to amphibian biodiversity loss. A widespread infectious chytrid fungus, *Batrachochytrium dendrobatidis* (*Bd*), causing the disease, chytridiomycosis, is implicated in numerous amphibian population declines and extinctions and can

induce sublethal effects within individuals. My dissertation research examines host-pathogen factors that mediate infection outcomes in the amphibian-*Bd* system.

The severity of an infectious disease is the result of specific host-pathogen interactions. Thus, there may be large variation in disease outcome depending on host and pathogen related factors. In Chapter 2, I conducted a comparative experimental study in larval amphibians using multiple host species and *Bd* strains. I showed that host species varied in *Bd* susceptibility but that susceptibility was also contingent on *Bd* strain type. Thus, I showed chytridiomycosis virulence depended upon host and pathogen traits and that a sensitive host species could be robust to certain pathogen strains.

In Chapter 3, I experimentally investigated multiple pathogen interactions with amphibian three amphibian host species to examine how *Bd* infection dynamics might change under simultaneous coinfection with a common water mold, *Saprolegnia ferax* (*Sf*). Coinfecting pathogens might interact within a host in a synergistic and antagonistic manner. In two host species, *Bd* infection intensity was slightly higher in hosts that were exposed to both *Bd* and *Sf* compared to just *Bd* alone indicating a small synergistic interaction with *Sf*. However, the differences were not significant in either host species. Survival differences were only detected in one host species; hosts exposed to *Bd* only experienced lower survival than those in the coinfected group. Additionally, I found host weight and days survived were predictive of *Bd* infection level for some hosts species showing species variation in infection response.

Lastly, in Chapter 4, I experimentally examined age-dependent differences in *Bd* infection heterogeneity in two host species. I followed this with another experiment to test whether age-dependent differences in infection intensity of these hosts ('donors') influenced subsequent *Bd* transmission to a naïve conspecific host ('recipients'). I found *Bd* infection intensity differences among juvenile and adult donors of both species. However, trend directions were not consistent; in one host species, adults had significantly higher infection levels than juveniles while the opposite was true in the second host species. Regardless of donor infection intensity, recipients had comparable infection levels within each host species. Survival also differed among host species and age groups suggesting *Bd* susceptibility may change with age and is species specific.

The Effects of Host and Pathogen Variation on Infection Dynamics in the Amphibian-Chytrid System

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APPROVED:

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Dean of the Graduate School

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.

Trang D. Dang, Author

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CONTRIBUTION OF AUTHORS

Chapter 1: Dr. Andrew Blaustein helped edit the manuscript.

Chapter 2: Dr. Catherine Searle helped analyze the results and helped edit the manuscript. Dr. Andrew Blaustein helped edit the manuscript.

Chapter 3: Natalie Hambalek provided experimental assistance and helped edit the manuscript. Dr. Andrew Blaustein helped edit the manuscript.

Chapter 4: Dr. Paul W. Bradley helped design the experiment and edit the manuscript. Dr. Andrew Blaustein helped edit the manuscript.

Chapter 5: Dr. Andrew Blaustein helped edit the manuscript.

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CHAPTER 1 – INTRODUCTION

Trang D. Dang and Andrew R. Blaustein

Infectious Diseases

Parasitism is one of the most prevalent lifestyles and is documented in an estimated 30% of all species in 43% of animal phyla (de Meeûs and Renaud, 2002; Lafferty *et al.*, 2006; Dobson *et al.*, 2008; Weinstein and Kuris, 2016). Infectious disease agents comprise a diverse subset of organismal life that include microparasites such as bacteria, fungi, protozoa, and viruses and macroparasites such as worms, arthropods, and other metazoans. Parasites may harbor their own parasites, a condition called hyperparasitism (Gleason *et al.*, 2014). Likewise, non-parasitic organisms can be host to a variety of parasites simultaneously (Sousa, 1994; Cox, 2001; Pedersen and Fenton, 2007; Balmer and Tanner, 2011). Interactions between parasites within a host and between parasites and their hosts has resulted in complex coevolutionary dynamics that influence host immune systems (Toor and Best, 2016; van Houte *et al.*, 2016) and parasitic virulence (Rigaud *et al.*, 2010; Bryner and Rigling, 2012).

The role infectious diseases may have in shaping host and pathogen populations is especially apparent in emerging infectious disease (EID) systems. EIDs are those in which an infectious pathogen is spread to new host species, localities, and/or are growing in incidence (Morse, 1995; Daszak *et al.*, 2000; Jones *et al.*, 2008). Several high profile EIDs have emerged in wildlife including amphibian chytridiomycosis, Tasmanian devil facial tumor disease, and white nose syndrome in bats(Daszak *et al.*, 2000; Fisher *et al.*, 2012; Olson *et al.*, 2013). Wildlife EIDs pose a significant threat to global biodiversity (Tompkins *et al.*, 2015). Host species whose populations are in decline and with small population sizes are especially at risk from EIDs (McCallum, 2012; Heard *et al.*, 2013). Furthermore, it is proposed that host populations that encounter additional stressors such

as human-induced habitat loss and environmental contaminants are at even higher risk of population declines due to disease epidemics (Cunningham and Daszak, 1998; De Castro and Bolker, 2004; Heard *et al.*, 2013).

Amphibian population declines

Extant amphibians are a diverse group that includes three large clades that diverged ~ 360 mya during the Late Devonian: Anura (frogs and toads), Caudata (salamanders and newts), and Gymnophiona (caecilians) (Vitt and Caldwell, 2009). These early-diverging vertebrates are found in a wide range of aquatic habitats on all major landmasses except Antarctica. Amphibians generally have a biphasic lifecycle, with most typically inhabiting aquatic freshwater habitats as larvae and transitioning to terrestrial habitat upon metamorphosis into a four-limbed adult. As biphasic ectotherms that employ cutaneous respiration, amphibians thus experience a large range of ecological stressors and can subsequently be sensitive to many environmental perturbations such as contaminants and pathogens (Stuart, 2004; Wake and Vredenburg, 2008; Relyea, 2009; Blaustein *et al.*, 2012).

Of the 6000+ amphibian species, one-third are estimated to have declining populations (Wake and Vredenburg, 2008; Kilpatrick *et al.*, 2010; Olson *et al.*, 2013). Thus, among vertebrate groups, amphibians are cited as the most threatened taxa, declining at a faster rate than mammals, birds, and fishes (Stuart, 2004; Wake and Vredenburg, 2008; Kilpatrick *et al.*, 2010; Hof *et al.*, 2011). Several factors contribute to population declines including habitat loss/degradation, pollution, invasive species, diseases, the global wildlife trade, and climate and atmospheric change (Stuart, 2004;

Wake and Vredenburg, 2008; Blaustein *et al.*, 2011). Forty-seven percent of amphibian species with declining populations are classified by the IUCN as undergoing enigmatic declines, the case whereby adequate habitat exists but, for unknown reasons, population declines are occurring (Stuart, 2004; Olson *et al.*, 2013). In these instances, factors like infectious disease and climate change are proposed as causal factors for decline, though clear supporting evidence depends on more rigorous surveillance efforts (Blaustein *et al.*, 1994a; Wake and Vredenburg, 2008; Blaustein *et al.*, 2012; Olson *et al.*, 2013; Heard *et al.*, 2013). The degree to which multiple factors affect amphibian population survival, alone and in concert with one another, is an active field of conservation research. While habitat destruction is regarded to play the single largest role in amphibian population declines, infectious diseases appear to contribute significantly to amphibian population declines and extinction events especially in vulnerable populations experiencing enigmatic declines (Berger *et al.*, 1998; Retallick *et al.*, 2004; Schloegel *et al.*, 2006; Vredenburg *et al.*, 2010; Olson *et al.*, 2013; Heard *et al.*, 2013). A study on the contribution of disease in the decline of vulnerable species found that disease risk increased in all species in more threatened IUCN categories (Heard *et al.*, 2013). Additionally, species vulnerable to major non-disease threats such as habitat degradation and invasive species were more likely to also be threatened by disease than species that were not (Heard *et al.*, 2013). For amphibians already facing a number of biotic and abiotic threats, the specter of an emerging infectious disease is a particularly significant.

Amphibian chytridiomycosis

Amphibians can be infected with several parasites including trematodes, water molds, viruses, and bacteria (Stuart, 2004; Blaustein *et al.*, 2012). One parasite highly cited in causing amphibian population declines is the chytrid fungus *Batrachochytrium dendrobatidis* (hereafter *Bd*). First discovered in 1998, the infectious disease caused by *Bd*, chytridiomycosis, is attributed to play a large role in many amphibian population declines and extinctions worldwide (Berger *et al.*, 1998; Wake and Vredenburg, 2008; Blaustein *et al.*, 2012; Fisher *et al.*, 2012; Olson *et al.*, 2013). Current evidence for *Bd*-induced population declines or extinctions have been reported for amphibian populations in Australia, Central America, and in California, USA (Berger *et al.*, 1998; Daszak *et al.*, 2003; Briggs *et al.*, 2010; Vredenburg *et al.*, 2010; Kolby and Daszak, 2016). Due to climate change, it is projected that *Bd* will spread to new regions and potentially infect additional amphibian populations (Xie *et al.*, 2016).

Bd is a member of the phylum Chytridiomycota, an early-diverging clade of zoosporic, cosmopolitan fungi commonly found in aquatic and moist soil substrates (Webster and Weber, 2007; Gleason *et al.*, 2008). Many are saprotrophic, breaking down dead organic matter such as keratin, chitin, starch, and cellulose (Gleason *et al.*, 2008). Some species parasitize plants, invertebrates, algae, and fungi (Longcore *et al.*, 1999; Webster and Weber, 2007; Gleason *et al.*, 2008). *Bd* is unusual among chytrids because of its ability to parasitize live vertebrate hosts. Free-swimming *Bd* zoospores infect keratinized tissue which includes larval mouthparts and the entire epidermis of adult amphibians, though colonization primarily occurs on the ventral surface of the pelvic patch and legs, i.e. areas in contact with the substrate (Berger *et al.*, 1998; Van Rooij *et al.*, 2015). Upon encystment, a germ tube penetrates the host epidermis enabling

intracellular sporangium development (Van Rooij *et al.*, 2012). *Bd* sporangia grow within the epidermal layers producing several zoospores inside (Longcore *et al.*, 1999; Van Rooij *et al.*, 2015). Mature zoospores exit via a discharge papilla into the surrounding aquatic environment to infect a new host or to reinfect the original host (Van Rooij *et al.*, 2015). *Bd* infection induces skin thickening and sloughing and can disrupt electrolyte regulation impairing respiration, ultimately led to cardiac arrest (Voyles *et al.*, 2009; Van Rooij *et al.*, 2015). Moreover, it is likely that *Bd* produces a toxin that can kill or cause sublethal affects in amphibians (Blaustein *et al.*, 2005; McMahon *et al.*, 2012). Infection inhibits host immune gene expression, amphibian (and mammalian) lymphocyte proliferation, and induce apoptosis by a non-protein factor(s), most likely from the cell wall (Rosenblum *et al.*, 2009; Fites *et al.*, 2013; Ellison *et al.*, 2014).

Studies performed on multiple *Bd* strains demonstrate sensitivity to environmental parameters. Generally, *Bd* growth favors an optimal temperature range between 17-25°C (but can tolerate 4-25°C) and a pH range of 6-7 (Piotrowski *et al.*, 2004; Johnson and Speare, 2005). However, abiotic environmental tolerance ranges may depend on strain (Stevenson *et al.*, 2013). Zoospores can remain viable in moist substrate for up to 3 months (Johnson and Speare, 2005) and in lake water for 7 weeks (Johnson and Speare, 2003) . Though sensitive to desiccation (Johnson and Speare, 2003), zoospores can attach to bird feathers and survive 1-3 hours of drying hinting at the potential of wild avian vectors (Johnson and Speare, 2005). *Bd* invasion and spread may be due to human activities facilitating the movement of amphibians via the pet, food, bait, and research trade (Picco and Collins, 2008; Schloegel *et al.*, 2009). Amphibian species associated with the global wildlife trade include those that can tolerate high *Bd* infection burdens

such as *Xenopus laevis* (African clawed frog), *Rhinella marinus* (cane toad), and *Lithobates catesbeianus* (American bullfrog) (Daszak *et al.*, 2003; Hanselmann *et al.*, 2004; Garner *et al.*, 2006; Schloegel *et al.*, 2012). *L. catesbeianus* is frequently linked to *Bd* introductions with market samples observed carrying *Bd*-GPL strains in Brazil and Japan (Schloegel *et al.*, 2012). The extraordinary numbers of amphibians trafficked in the global amphibian trade increase the risk of invasive species and *Bd* strain spread.

Bd has a broad host range and infects over 500 amphibians species (Fisher *et al.*, 2009b; Olson *et al.*, 2013). A chief element of successful *Bd* infection is the ability to colonize amphibian epidermal tissue. This requires the destruction of major structural skin proteins responsible for maintaining epidermal integrity such as keratin and collagen (Burmester *et al.*, 2011). Comparative genomics studies with closely related chytrid relatives, have uncovered a vast expansion of protease gene families (metallo-, serine-, and aspartyl proteases) (Rosenblum *et al.*, 2008; Joneson *et al.*, 2011; Farrer *et al.*, 2017). Protease gene expression is significantly increased in *Bd* when exposed to host substrate confirming the importance of these putative virulence factors in amphibian infection (Rosenblum *et al.*, 2012; Farrer *et al.*, 2017). Evidence also suggests *Bd* can secrete a toxin that may contribute to host pathology (Blaustein *et al.*, 2005; McMahon *et al.*, 2012).

Although *Bd* has a worldwide distribution, evidence for chytridiomycosis leading to amphibian population declines has only been documented in 15% of IUCN Red Listed amphibian species reported to be under threat from this disease (Heard *et al.*, 2011). Certain populations in Central America, Australia, and the Sierra Nevada mountain range of California have experienced massive losses [but see recovery in some populations

(Knapp *et al.*, 2016; Scheele *et al.*, 2017)] associated with *Bd* epidemics yet others (particularly in Asia, Africa, and North America) have been found coexisting with low and high *Bd* infection levels along with no documented population losses (Ouellet *et al.*, 2005; Wake and Vredenburg, 2008; Pilliod *et al.*, 2010; Kielgast *et al.*, 2010; Swei *et al.*, 2011; Bai *et al.*, 2012; Bataille *et al.*, 2013; Rodriguez *et al.*, 2014). The variable nature of observed chytridiomycosis virulence may be due to a variety of factors in including those related to host and pathogen traits.

Bd susceptibility differs widely among amphibian host species (Blaustein *et al.*, 2005; Garcia *et al.*, 2006; Garner *et al.*, 2006; Searle *et al.*, 2011; Brannelly *et al.*, 2012; Ohmer *et al.*, 2013; Gervasi *et al.*, 2017). Susceptibility can also differ by other host traits such as age (Ortiz-Santaliestra *et al.*, 2013; Abu Bakar *et al.*, 2016), population (Bradley *et al.*, 2015), stress level (Gabor *et al.*, 2015), and immune system attributes or history (Gervasi *et al.*, 2014; McMahon *et al.*, 2014; Becker *et al.*, 2015; Bataille *et al.*, 2015). While *Bd* infection is generally regarded as being more deadly to postmetamorphic amphibians (Rollins-Smith, 1998; Rachowicz and Vredenburg, 2004), larval hosts can die from infection and may also experience a range of sublethal effects (e.g. smaller size at metamorphosis, slower development, altered feeding behavior) impacting future fitness (Blaustein *et al.*, 2005; Garner *et al.*, 2009; Venesky *et al.*, 2011). In adult hosts, pathological *Bd* infection can result in weight loss, lethargy, abnormal posture, and mortality (Van Rooij *et al.*, 2015). Though *Bd* infection generally represses host immune function, in some cases a robust immune response is achieved but may not be adequate in fighting infection (Ellison *et al.*, 2014).

Additionally, *Bd* virulence is not consistent across all strains. Comparative strain studies reveal the broad range of genetic and phenotypic diversity among Bd isolates (Berger *et al.*, 2005; Retallick and Miera, 2007; Fisher *et al.*, 2009a; Farrer *et al.*, 2011; Gahl *et al.*, 2012; Becker *et al.*, 2017; Dang *et al.*, 2017). *Bd* has a complex evolutionary history indicating neither a completely novel nor endemic pathogen (Rosenblum *et al.*, 2013). The largest *Bd* lineage, the Global Panzootic Lineage (*Bd*-GPL) has members on 5 continents and is linked to the most severe amphibian population declines (Farrer *et al.*, 2011). However, even among strains in this putatively virulent group, great genetic and phenotypic diversity exists (Garner *et al.*, 2009; Farrer *et al.*, 2011; Rosenblum *et al.*, 2013; Becker *et al.*, 2017). The genetic profile of hypervirulent *Bd* strains differ from hypovirulent ones and are correlated to phenotypic traits such as size of sporangium and *in vivo* virulence (Fisher *et al.*, 2009a; Farrer *et al.*, 2011; but see Becker *et al.*, 2017 for inconsistencies). Its patchy worldwide distribution and variable phylogenetic topology suggests an ancient origin predating documented amphibian population declines as well as recent introduction and spread of some strains in certain geographic areas (Rosenblum *et al.*, 2013). Experimental *Bd* strain studies suggest strong local selection pressures may mediate virulence.

Amphibian-*Bd* dynamics can be highly context-dependent and affected by multiple stressors other than host and pathogen factors. *Bd* and amphibian sensitivities to other factors such as ultraviolet radiation (Blaustein *et al.*, 1994b), environmental contaminants (Relyea, 2009), climate change (Xie *et al.*, 2016), habitat loss (Gibbs, 2000) can interact in a myriad of ways ultimately influencing infection outcome (Blaustein *et al.*, 2011; Venesky *et al.*, 2013). Numerous lines of chytridiomycosis research are

devoted to investigating the effect of different environmental factors on host and pathogen outcomes.

Dissertation work

In the roughly two decades since *Bd* was discovered, empirical host-pathogen infection studies have provided much needed insight into the dynamics of this unique and important wildlife disease. However, assumptions linger and uncertainty remains. In my dissertation work I explored the mutability of infection virulence by manipulating host and pathogen variation.

Attempts to uncover consistent patterns associated with strain variation are difficult and often conflicting across studies when tested in different host species. Likewise, host species susceptibility studies are often done on only one *Bd* strain, with the resultant disease outcome designating that species' degree of *Bd* tolerance. A strong argument can be made for only testing local host species against local *Bd* strains, however, these constraints may restrain our ability to understand the diversity of *Bd* strain pathogenicity and host species susceptibility. Moreover, the likelihood of an amphibian host encountering novel *Bd* strains in the wild is highly probable due to the extent and magnitude of the live amphibian trade (Fisher and Garner, 2007; Schloegel *et al.*, 2009, 2012; Kolby *et al.*, 2014); and projections suggesting that climate change will influence the spread and range of *Bd* further supports this notion (Xie *et al.*, 2016). In Chapter 2, I explored virulence variation among three *Bd* strains and three host species. By employing a comparative experimental design using multiple hosts with a range of tolerances and

multiple *Bd* strains, I captured differential virulence under some host-pathogen combinations but not others.

In Chapter 3, I studied how *Bd* infection outcomes differed under a single pathogen infection with only *Bd* and under a coinfection with the common water mold *Saprolegnia ferax* in three host species. Organisms are exposed to multiple pathogen types in the wild and are frequently found to have coinfections yet most disease research focuses on host response to a single pathogen type. Though an overall trend of increased *Bd* infection was found under simultaneous infection with *S. ferax*, in general, coinfected hosts did not have significantly disease outcomes compared to those exposed to only *Bd*. These results show exposure to multiple pathogens may not always result in synergistic or antagonistic effects.

Conspecific host infection heterogeneity can affect a host's ability to acquire, tolerate, and transmit infectious pathogens. Recently metamorphosed amphibians are generally thought to be the most susceptible to *Bd* while older adults are believed to be more resistant and/or tolerant due to immune system maturation and increased energetic resources to fight infection (Rollins-Smith, 1998). To test whether *Bd* infection burden differed by age, in Chapter 4 I examined *Bd* susceptibility in juvenile and adults hosts of two host species and tested whether this affected transmission to a subsequent conspecific host. I found unexpected differences in *Bd* infection among host age and species groups. I also found age-related infection differences did not strongly affect transmission differences to naïve hosts and that transmission mainly differed by host species.

My dissertation highlights the dynamic nature of infectious diseases by experimentally manipulating and exploring variation in hosts and pathogens. This work

builds on almost two decades of amphibian chytridiomycosis research and expands our understanding of this complex and important disease.

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CHAPTER 2 – VIRULENCE VARIATION AMONG STRAINS OF THE EMERGING INFECTIOUS FUNGUS, *BATRACHOCHYTRIUM DENDROBATIDIS* (*BD*), IN MULTIPLE AMPHIBIAN HOST SPECIES

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Abstract

Emerging infectious diseases have been documented in numerous plant and animal populations. The infectious disease amphibian chytridiomycosis, caused by the fungus *Batrachochytrium dendrobatidis* (*Bd*), is associated with global amphibian population declines. While much *Bd*-amphibian research has centered on response variation in hosts, a paucity of information exists on how variation in the pathogen, such as strain differences, affects infection dynamics. To examine how different *Bd* strains may differentially impact multiple hosts, we conducted laboratory experiments to measure 2 infection outcomes, host survival and pathogen load, in 3 amphibian host species (Pacific treefrog, western toad, and Cascades frog) after exposure to 3 different *Bd* strains (an additional fourth *Bd* strain was tested in toads only). Our results confirm that the infection response differs among host species. Western toads experienced significant mortality, but Pacific treefrogs and Cascades frogs did not. Interestingly, our experiment also captured strain dependent virulence variation but only in 1 host species, the western toad. Increased mortality was observed in 2 of the 4 *Bd* strains tested in this host species. Toads were also the only host species found to have variable pathogen load dependent on strain type; individuals exposed to the Panama strain harbored significantly higher loads compared to all other strains. These findings underscore the dynamic nature of *Bd* infection, showing that virulence can vary contingent on host and strain type. We highlight the importance of both host- and pathogen-dependent factors in determining overall infection virulence and show the need for *in vivo* testing to fully assess pathogenicity.

Introduction

Emerging infectious diseases (EIDs) are increasing worldwide, threatening biodiversity and public health (Fisher *et al.*, 2012). The increase in disease emergence is often linked to human activities such as urbanization, land-use change, wildlife trade, pollution, and climate change (Tompkins *et al.*, 2011; Farrer *et al.*, 2011; Fisher *et al.*, 2012). Pathogenic fungi are responsible for many well-documented EIDs such as white nose syndrome in bats and wheat stem rust (Fisher *et al.*, 2012). One high profile fungal pathogen, the chytrid *Batrachochytrium dendrobatidis* (*Bd*), which causes the disease amphibian chytridiomycosis, has been found in over 500 amphibian species and is listed as a major contributor to global amphibian population declines (Fisher *et al.*, 2012; Tompkins *et al.*, 2015). *Bd* infection susceptibility and virulence are dynamic and can depend on both host- and pathogen-associated factors (Fisher *et al.*, 2009; Farrer *et al.*, 2011; Gervasi *et al.*, 2013; Bradley *et al.*, 2015). Additionally, the active global trade in live amphibians raises the risk of contact between nonnative host species and pathogen strains (Schloegel *et al.*, 2009). By investigating how infection virulence varies among different host and pathogen types, we can uncover virulence variation in the amphibian—*Bd* system that could help us understand the consequences of *Bd* strain introduction due to the live amphibian trade.

The effects of chytridiomycosis can vary among host species and life stages (Blaustein *et al.*, 2005; Garner *et al.*, 2009; Gervasi *et al.*, 2013) and can even vary between populations of the same species (Bradley *et al.*, 2015). *Bd* infects keratinized tissues in amphibians, i.e. the mouthparts in larvae and the skin in adults. Although *Bd* infection is thought to have the most profound effects on amphibians during and post-

metamorphosis, exposure during the aquatic larval stage can also result in high larval mortality (Blaustein *et al.*, 2005; Fisher *et al.*, 2009; Searle *et al.*, 2011; but see Gervasi *et al.*, 2013) and can influence post-metamorphic fitness(Garner *et al.*, 2009). Other host traits such as behavior and skin microbiota may also affect host susceptibility and survival (Venesky *et al.*, 2014; Becker *et al.*, 2015).

Additionally, infection dynamics may be driven by intrinsic pathogen related factors (Salvaudon *et al.*, 2005). Comparative genomics studies have identified a *Bd*-global panzootic lineage (GPL) containing North and Central American strains associated with high virulence and amphibian population declines (Farrer *et al.*, 2011; Schloegel *et al.*, 2012; Rosenblum *et al.*, 2013). Like other emerging parasites, the global spread of *Bd* strains, particularly those in the *Bd*-GPL, has been linked to the commercial amphibian trade (Schloegel *et al.*, 2009; Farrer *et al.*, 2011; Schloegel *et al.*, 2012). Amphibian trafficking activities increase the likelihood of contact between foreign hosts and strains via the release of live animals and/or housing water (Schloegel *et al.*, 2009, 2012). Furthermore, *Bd* strain introduction may facilitate a sexual recombination event resulting in a hybrid. Schloegel *et al*. (2012) first detected a hybrid *Bd* strain on a wild Brazilian bullfrog; a multi-locus sequence typing analysis suggested it originated from a GPL and an endemic Brazilian strain. Even so, research exploring virulence differences among strains is still a nascent endeavor. Although virulence variation due to host factors has been documented in the amphibian—*Bd* system, most *Bd* studies investigating the effects of strain differences have only focused on responses within a single host species (Berger *et al.*, 2005; Retallick and Miera, 2007; Farrer *et al.*, 2011; Piovia-Scott *et al.*, 2015; but see Gahl *et al.*, 2012). Due to the limited information on how infection virulence varies

with different *Bd* strains and different host species, we sought to investigate the dynamic nature of this disease by measuring host survival and pathogen load in a comparative experiment using different North and Central American *Bd* strains and North American host species.

Materials and methods

Bd culture

All host species were tested with the following *Bd* strains: JEL425, isolated in Panama; JEL630, isolated in Oregon, USA; and JEL646, isolated in California, USA. All but one *Bd* strain (JEL425) in this study have been used in phylogenetic analyses placing them in the putatively virulent GPL (Schloegel *et al.*, 2012; Rosenblum *et al.*, 2013; Table 2.1). JEL425 was isolated in Panama 2004, a period when this region was experiencing amphibian population losses, but has never been used in a comparative genomics analysis to establish membership in the GPL clade. An additional GPL strain, JEL627 (Rosenblum *et al.*, 2013), isolated in Maine, USA, was tested on western toads, a known sensitive species (Searle *et al.*, 2011). These strains represent novel isolates from a range of geographic distances from the collection site of the 3 North American focal host species used in this study (Table 2.1). Strains will be referred to by their geographic origin (e.g. 'Oregon *Bd'* for JEL630). *Bd* strains were obtained from initial cryogenic stock from the lab of Dr. Joyce Longcore (University of Maine). All strains were cultured at ~20°C; Maine, California, and Panama isolates underwent 2 passages through 1%

tryptone broth while the Oregon isolate went through 5 passages before plating for use. Before the experiment, all strains were plated on 1% tryptone-agar plates for 1 to 2 wk.

Animal husbandry

Host species in this study are commonly found in the US Pacific Northwest: Pacific treefrog *Pseudacris regilla*, Cascades frog *Rana cascadae,* and western toad *Anaxyrus boreas*. Cascades frog and Western toad population declines have been documented in the western USA and are both species of concern in this region (Muths *et al.,* 2003; Piovia-Scott *et al.,* 2015). Experimental evidence has demonstrated that Pacific treefrogs are able to tolerate relatively high *Bd* loads compared to sympatric species making them a possible reservoir host (Reeder *et al.,* 2012; Gervasi *et al.,* 2013). To make sure study animals did not have prior *Bd* infections, newly laid eggs of each species were collected from the Cascades Range of Oregon and reared in the laboratory until they reached Gosner developmental stages 26 to 34 (first emergence of limb bud to initial differentiation of digits; Gosner, 1960). Tadpoles were housed in 10 l glass aquaria at a density of 100 tadpoles tank^{-1} at ~15°C under a natural photoperiod. Tadpoles were fed every other day with a 3:1 ground mix of alfalfa pellets and fish flakes (Brine Shrimp Direct).

Experimental protocol

We examined the effects of *Bd* in the larval stage for all species. Once hatched, tadpoles were randomly assigned to different *Bd* strain treatment groups (Table 2.2). Individuals were housed in 1 l plastic cups filled with 600 ml of dechlorinated water and

allowed to acclimate for 24 h before pathogen exposure. *Bd*-agar plates (7–8 plates per *Bd* strain group) were flooded with 10 ml of dechlorinated water, and actively swimming zoospores were quantified with a hemocytometer after 10 min. Each *Bd*-treated tadpole received 10 ml of *Bd* broth at a concentration of 1×10^4 zoospores ml^{-1} for a total of 1×10^5 zoospores (a dose used previously tested in the same larval host species by Gervasi *et al.*, 2013). *Bd* broth was not filtered and thus contained both zoospore and sporangia structures. The process was repeated on sterile agar plates for the control group. Water was changed once a week for the duration of the experiment (20 d). Animals were monitored daily for survival, and deceased individuals were preserved in 95% ethanol (EtOH). At the end of the experiment, all remaining animals were euthanized in MS-222 and preserved in 95% EtOH. For all individuals, ending measurements of mass and snout–vent length (SVL) were taken.

DNA extraction and qPCR

A subsample of 15 *Bd*-exposed animals and 3 control animals from each species underwent quantitative PCR (qPCR) analysis to determine *Bd* infection load. Whole mouthparts were excised and processed following Boyle *et al.* (2004) except that we used 60 μl of Prepman Ultra (Applied Biosystems) instead of 40 μl in all DNA extractions. Each sample was run in triplicate, and an average genome equivalent (GE) for each sample was calculated against *Bd* standards made from transgenic *Escherichia coli* cultures carrying plasmids of the *Bd* internal transcribed spacer region with standard titrations from 10^{-1} to 10^2 (USGS, https://water.usgs.gov/nrp/microbiology/resources/resources.html#Bd_std). Quantitative

PCR was conducted on a StepOnePlus Real-Time PCR System (Applied Biosystems) using primers and probes developed by Boyle *et al.* (2004). A no-template control containing nanopure water was included in each qPCR plate and always tested negative. An animal was considered positive if 2 of the 3 qPCR replicates tested positive. There was never an instance where only 1 replicate tested positive. GEs were averaged from all positive qPCR replicates for each *Bd*-exposed animal.

Data analysis

Cox proportional hazards models were employed to compare rates of survival among *Bd* treatments for each host species (Cox, 1972). This test compares survival curves to analyze the probability of mortality from different variables (likelihood ratio test). The probability of mortality due to a factor is represented by a hazard ratio. Infection load data (in units of GE ind.$^{-1}$) were log-transformed to meet parametric assumptions (log-GE + 1) before conducting statistical tests (untransformed data Table 2.3). We conducted analyses of variance (ANOVA) with each amphibian host species to test *Bd* strain and experimental days alive as main effects. After log transformation, 3 extreme outliers were detected (>3× the inter-quartile range) and removed from load analysis in Pacific treefrogs to meet data assumptions of normality (*Bd* treatment groups California, Oregon, and Panama each had 1 individual removed; Oregon and Panama outliers had no detectable *Bd* load). Significant results were followed by a Tukey's HSD test. All analyses were performed in R with the package 'survival' (R Core Team 2014).

Results

Host survival

Western toad survival was not affected by exposure to Oregon and Maine *Bd* strains (Fig. 2.1A). However, western toad mortality increased significantly by a factor of 2.21 (95% CI: 1.02–4.79) and 2.51 (95% CI: 1.16–5.45) when exposed to the Panama and California *Bd* strains, respectively (Fig. 2.1A). No *Bd* strains affected survival of Pacific treefrog and Cascades frog tadpoles when compared to control groups ($p > 0.05$ in all cases, Fig. 2.1B,C).

Host pathogen load

We used ANOVAs followed by Tukey's HSD tests to determine infection load differences among treatments for each host species. *Bd* load was not predicted by number of days alive in any species. In Cascades frogs and Pacific treefrogs, strain type did not significantly affect pathogen load. However, *Bd* load did significantly differ among strain types in western toads ($F_{3,55} = 21.9$, $p < 0.001$). Toads exposed to Panama *Bd* had significantly higher loads compared to California, Maine, and Oregon *Bd* treatments (Tukey's HSD test $p \leq 0.001$ in all cases, Fig. 2.2A). *Bd* infection loads did not differ among the other strains. In Pacific treefrogs, California and Panama *Bd* treatment groups each had 1 animal that did not have detectable *Bd* infection following qPCR analysis. Weight and SVL were not associated with pathogen load. All control animals sampled for qPCR tested negative for *Bd* infection. Overall, Cascades frogs had lower loads compared to western toads and Pacific treefrogs (Fig. 2.2). Variation in *Bd* load due to strain type

was only apparent in western toads (i.e. we could not detect strain differences in other host species).

Discussion

We found that variation in host mortality and infection load was driven by differences among amphibian hosts and pathogen strain type. By comparing disease outcomes among different combinations of host species and pathogen strains, we found *in vivo* virulence variation that may not have been detected if we had only tested a single host species or pathogen strain. We also tested amphibian hosts reared from eggs to ensure that infection virulence was not affected by prior host infection history. Exposure to all tested *Bd* strains did not significantly impact survival of Pacific treefrog and Cascades frog hosts, supporting previous work that found larvae of these species to be relatively robust to *Bd* infection (Blaustein *et al.*, 2005; Reeder *et al.*, 2012; Gervasi *et al.*, 2013). Pacific treefrog adults have been previously named as an important reservoir host for *Bd* due to their ability to harbor high pathogen loads without incurring significant mortality (Reeder *et al.*, 2012). Our study suggests this may be true in the larval stages as well. Cascades frogs had the lowest pathogen loads across all *Bd* strains, concurring with prior studies showing this species to be relatively resistant to *Bd* infection (Searle *et al.*, 2011; Gervasi *et al.*, 2013). One explanation for this consistent pattern is pathogen consumption by the host, although this has yet to be definitively tested in this species (Keesing *et al.*, 2006; Venesky *et al.*, 2014)

Although western toads and Pacific treefrogs carried similar pathogen loads, toads displayed decreased infection tolerance as evidenced by significantly increased mortality

from 2 of the 4 strains (Fig. 2.1A). Since *Bd* infects larval mouthparts, impaired feeding ability is expected to be the main mechanism that reduces survival, although other factors have been suggested, such as mortality due to an inadequate or costly immune response (Garner *et al.*, 2009). While our results support research positing toads to be more vulnerable to *Bd*-induced mortality than other sympatric species (Carey *et al.*, 2006; Searle *et al.*, 2011), they also show that toad mortality can vary by *Bd* strain type. Toads exposed to *Bd* strains from Oregon, Maine, and California had similar infection loads but significant mortality was only seen in those exposed to the California strain. This difference in mortality may be due to underlying pathogen-related factors (e.g. strain-specific toxin production, local evolutionary history, growth rate, etc.). Our results emphasize the dynamic nature of infectious diseases, as virulence is an emergent property mediated here by the interactions between the host and pathogen.

Comparative intraspecific *Bd* research can also reveal the potential consequences of the global trade in amphibians for food, pets, bait, research, etc. The amphibian trade, especially in a highly *Bd*-tolerant host, the North American bullfrog *Lithobates catesbeianus*, is quickly facilitating the spread of *Bd* strains around the world (Schloegel *et al.*, 2009, 2012)A 6 yr, 3 city survey of live amphibian markets found an overall *Bd* infection prevalence of 62% in live frogs sold for human consumption. Amphibians sampled for this survey (Schloegel *et al.*, 2009) came from wild and captive populations in Asia and South America and did not include species involved in the pet trade. Another genomics study carried out by Schloegel *et al.* (2012) found Brazilian *Bd* strains present in a live bullfrog from a US market as well as in wild invasive bullfrogs in Japan. These studies provide evidence suggesting that global trafficking of amphibians can enable the

global spread of novel *Bd* strains. Although our study was a controlled laboratory experiment, the data indicate functional virulence variation among geographically disparate *Bd* strains and suggest that realistic scenarios resulting in exotic *Bd* strain introduction, such as via the amphibian trade, may hold adverse consequences to susceptible, naïve amphibian hosts. Comprehensive *Bd* strain research can be used to inform amphibian trade policy and regulations by identifying and monitoring geographic regions and host populations in danger of exposure to or currently with high-virulence *Bd* strains in circulation. Additional experimental research exploring genomic *Bd* strain diversity and disease pathology is needed to better understand the underlying mechanisms mediating virulence in *Bd* infections.

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Figures

Figure 2.1. Survival curves of *Batrachochytrium dendrobatidis* (*Bd*)-exposed animals and controls for each host species. (A) Western toad, *Anaxyrus boreas*, survival was significantly lowered in the Panama (JEL425) and California (JEL646) *Bd* treatment group compared to the control treatment group. (B) Pacific treefrog, *Pseudacris regilla*, survival was high and not affected by exposure to any *Bd* strain. The control survival curve is obscured behind the Oregon *Bd* survival curve; both treatments had 0 mortality. (C) Cascades frogs, *Rana cascadae*, did not show any significant survival differences among treatment groups. CA: California; ME: Maine; OR: Oregon; Pan: Panama

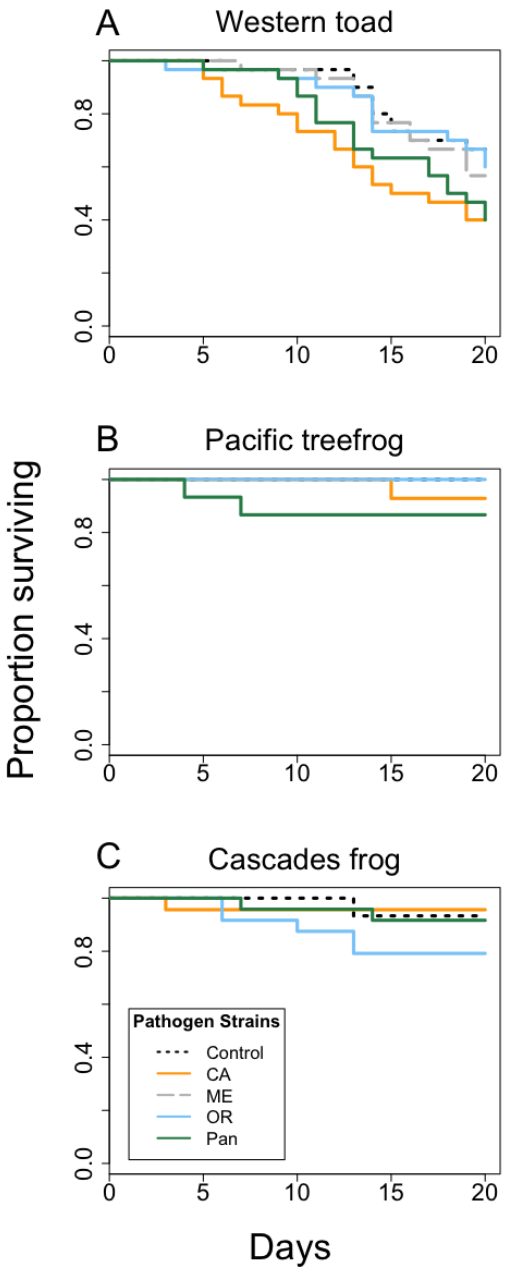
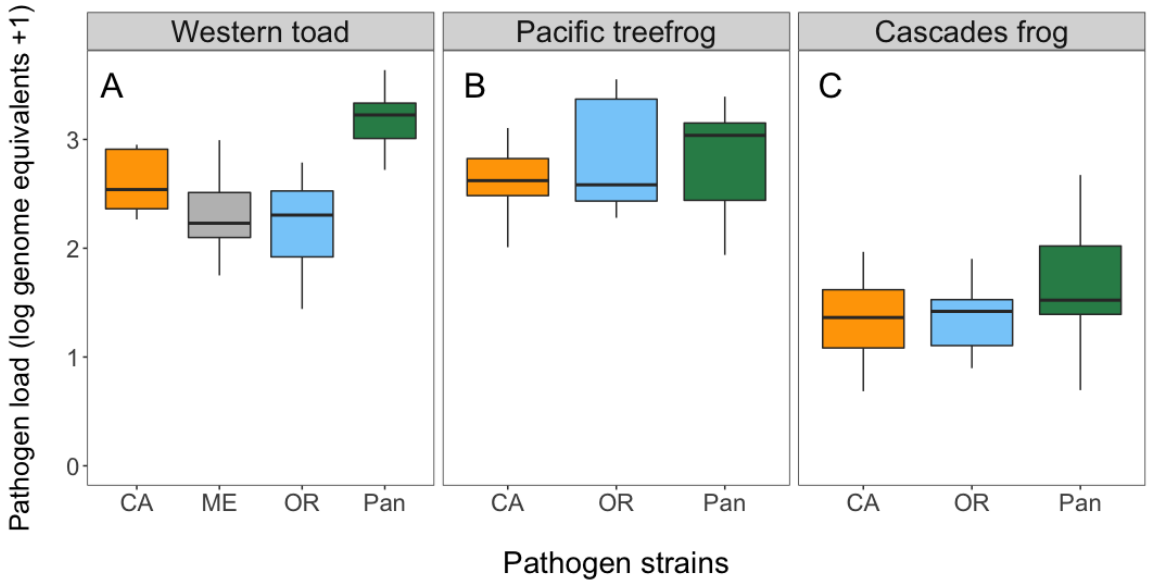


Figure 2.2. Average pathogen load (log genome equivalents + 1) results. (A) Western toad, *Anaxyrus boreas*, pathogen loads were significantly higher in the *Batrachochytrium dendrobatidis* (*Bd*) Panama (JEL425) strain treatment group compared to the California (JEL646), Maine (JEL627), and Oregon (JEL630) treatment groups. (B,C) For Pacific treefrogs, *Pseudacris regilla*, and Cascades frogs, *Rana cascadae*, there was no difference in average pathogen load among any *Bd* strain treatments. CA: California; ME: Maine; OR: Oregon; Pan: Panama. Thick horizontal lines: medians; boxes: inter-quartile range (IQR); whiskers: full data range within (±) 1.5 × IQR



Tables

Table 2.1. *Batrachochytrium dendrobatidis* (*Bd*) strains used in this experiment including isolate identity, geographic origin, year isolated, and host species

Isolate	Geographic origin	Year isolated	Amphibian host
JEL425	El Cope, Coclé (Panama)	2004	*Bufo haematiticus*
JEL627	Bethel, Maine (USA)	2009	*Lithobates catesbeianus*
JEL630	Finley National Wildlife Refuge, Oregon (USA)	2009	*Lithobates catesbeianus*
JEL646	Point Reyes, California (USA)	2010	*Pseudacris regilla*

Table 2.2. Experimental hosts and *Batrachochytrium dendrobatidis* (*Bd*) strain treatment groups with sample sizes; (–) no treatment

Host species	Strain treatments (sample size)				
	Control	California (JEL646)	Maine (JEL627)	Oregon (JEL630)	Panama (JEL425)
Pacific treefrog *Pseudacris regilla*	14	14	–	16	15
Western toad *Anaxyrus boreas*	30	29	30	30	30
Cascades frog *Rana cascadae*	15	23	–	24	24

Table 2.3. Range, median, and mean of untransformed pathogen loads (genome equivalents) for different strains of *Batrachochytrium dendrobatidis* (*Bd*)

Host Species	*Bd* strain	Range	Median	Mean
Pacific treefrog *Pseudacris regilla*	California (JEL646)	9.6-1273.3	417.3	493.9
	Oregon (JEL630)	0-3567.8	381.8	1170.5
	Panama (JEL425)	0-2471.5	1088.7	1032
Western toad *Anaxyrus boreas*	California (JEL646)	18.4-892.6	345.1	489.5
	Maine (JEL627)	55.3-984.0	168.7	268.3
	Oregon (JEL630)	26.7-612.1	200.7	227.3
	Panama (JEL425)	524.1-4338.3	1677.6	1760.2
Cascades frog *Rana cascadae*	California (JEL646)	3.8-406.6	22.1	54.4
	Oregon (JEL630)	1.4-78.8	25.3	28.4
	Panama (JEL425)	4-470.1	32.3	93.4

CHAPTER 3 – THE EFFECT OF A SIMULTANEOUS COINFECTION WITH THE CHYTRID FUNGUS, *BATRACHOCHYTRIUM DENDROBATIDIS*, AND A WATER MOLD, *SAPROLEGNIA FERAX*, ON MULTIPLE AMPHIBIAN HOST SPECIES

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Abstract

Beyond single pathogen infection studies, coinfection experiments investigate the complex interactions between multiple pathogens in one host. Hosts may encounter different pathogen types simultaneously that can interact synergistically or antagonistically, ultimately influencing host response however, our understanding of disease dynamics primarily relies on empirical data from single pathogen experiments. Our study explores amphibian chytridiomycosis under a single pathogen infection with the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*), and under a simultaneous coinfection with a water mold, *Saprolegnia ferax*. We studied the effect of coinfection in three amphibian host species with varying susceptibilities and found while coinfected hosts where infected with more *Bd*, the difference was not significant. Additionally, compared to controls, lower survival was only apparent in the *Bd* only infection treatment of one host species. Higher weight and longer survival time was associated with lower *Bd* infection, but these results were not consistent across all hosts species. These findings show coinfection dynamics may not always be strongly evident and indicate sublethal ways coinfecting pathogens may affect their hosts.

Introduction

Hosts are exposed to multiple pathogens in the wild and are commonly found infected by more than one infectious agent (Sousa, 1994; Cox, 2001; Telfer *et al.*, 2010; Ezenwa *et al.*, 2010; Balmer and Tanner, 2011; Warne *et al.*, 2016). Furthermore, coinfections can alter disease dynamics due to synergistic or antagonistic pathogen interactions (Holmes, 1961; Sousa, 1994; Telfer *et al.*, 2010; Ezenwa *et al.*, 2010). These

variable interactions may occur due to processes such as competition over host resources, alteration of host tissue affecting colonization, or induction of a host immune response, which may encourage or inhibit another pathogen (Cox, 2001; Janovy Jr, 2002; Pedersen and Fenton, 2015). The type, strength, and direction of these interactions within a host can affect host survival as well as each pathogens transmission in the host population. Research exploring concurrent infections can augment our understanding of infectious disease dynamics in complex systems with multiple hosts and pathogens (Sousa, 1994; Pedersen and Fenton, 2007; Telfer *et al.*, 2010; Buhnerkempe *et al.*, 2015; Johnson *et al.*, 2015).

Amphibians are an example of hosts that harbor multiple pathogens simultaneously and these infections seem to be contributing to some population declines and extinctions (Berger *et al.*, 1998; Densmore and Green, 2007; Vredenburg *et al.*, 2010; Blaustein *et al.*, 2012; Fisher *et al.*, 2012; Gleason *et al.*, 2014). One widespread pathogen, the aquatic zoosporic fungus, *Batrachochytrium dendrobatidis*, hereafter *Bd*, infects over 500 amphibian species worldwide (Berger *et al.*, 1998; Kilpatrick *et al.*, 2010; Olson *et al.*, 2013). *Bd* infects tissue of larval and adult amphibians (Kilpatrick *et al.*, 2010; Van Rooij *et al.*, 2015). Though most *Bd* research has focused on investigating its sole effects on hosts, amphibians encounter a diverse array of pathogens including bacteria, viruses, fungi, and protists in nature that can interact within one host (Densmore and Green, 2007; Prada-Salcedo *et al.*, 2011; Blaustein *et al.*, 2012; Johnson and Hoverman, 2012; Warne *et al.*, 2016). Additionally, many amphibian species and populations may harbor *Bd* without experiencing population declines (Ouellet *et al.*, 2005; Pilliod *et al.*, 2010). This suggests that *Bd*-linked amphibian population declines

are complex and may likely be influenced by other factors such coinfections (Heard *et al.*, 2011; Blaustein *et al.*, 2012; Johnson and Hoverman, 2012). The limited data exploring multiple infections renders an incomplete and potentially misleading framework to study amphibian disease (Blaustein *et al.*, 2012; Gleason *et al.*, 2014).

Another class of widespread aquatic amphibian pathogens are water molds. Similar to *Bd*, water molds are ubiquitous in freshwater ecosystems (Gleason *et al.*, 2014) and parasitize all stages of amphibians (Bragg, 1962; Blaustein *et al.*, 1994; Berger *et al.*, 2001; Warkentin *et al.*, 2001; Ault *et al.*, 2012). Water molds in the genus *Saprolegnia* are generally thought to be opportunistic pathogens (i.e. pathogens that infect a compromised host) infecting epidermal tissues of amphibian, fish, and gelatinous amphibian eggs (Blaustein *et al.*, 1994; Kiesecker *et al.*, 2001; Densmore and Green, 2007; van den Berg *et al.*, 2013). The common water mold *Saprolegnia ferax* (*Sf*), found in the US Pacific Northwest, can reduce survival in eggs, larvae, and adult stages of certain amphibian species (Blaustein *et al.*, 1994; Densmore and Green, 2007; Romansic *et al.*, 2007, 2009).

Our study specifically explores how the presence of *Sf* impacts *Bd* infection in a larval host. A *synergistic* interaction might occur if *Bd* invasion is greatly facilitated by *Sf* colonization leading to higher *Bd* infection in the host. This coinfection interaction is highly detrimental to the host as the activities of both pathogens increase damage to the host more than one singular infection of either. Alternatively, an *antagonistic* interaction may occur if *Bd* and *Sf* compete for shared host resources (dermal tissue), thus decreasing *Bd* infection. By conducting research on the interactive coinfection relationship between *Bd* and *Sf*, we explored the *Bd*-amphibian disease system in a manner that more closely

mirrors conditions in the wild where amphibian hosts are exposed to a barrage of different pathogens (Romansic *et al.*, 2011; Blaustein *et al.*, 2012; Gleason *et al.*, 2014; Romansic *et al.*, 2017).

To test the effects of the syntopic pathogens *Bd* and *Sf* within hosts, we paired tadpoles of three amphibian host species with three different pathogen treatments and measured host survival, *Bd* infection prevalence, and *Bd* infection intensity. Since *Bd* and *Sf* infect dermal tissue, we expected that the coinfection treatment will result in a synergistic *Bd*/*Sf* interaction yielding higher *Bd* infection levels thus reducing host survival in all host species. Alternatively, since these two pathogens colonize dermal tissue, there might be an antagonistic interaction due to pathogen competition for host resources in the coinfection treatment groups. If *Sf* inhibited *Bd* colonization, lower *Bd* infection intensity would be detected in the coinfection treatment versus *Bd* alone.

Materials and Methods

Animal husbandry

Host species used in this research are found in the US Pacific Northwest: *Pseudacris regilla* (Pacific tree frog), *Anaxyrus boreas* (western toad), and *Rana pretiosa* (Oregon spotted frog) (Table A1). *Bd* has been detected in wild PNW populations of all host species (Pearl *et al.*, 2007, 2009; Adams *et al.*, 2010). *P. regilla* tolerate high levels of infection compared to other species and may serve as reservoir hosts for *Bd* in the wild (Reeder *et al.*, 2012; Searle *et al.*, 2013). *A. boreas* is a sensitive species in the western US with declining populations (Blaustein *et al.*, 1994, 2005; Carey *et al.*, 2006). Previous

Bd research on juvenile *R. pretiosa* have found this species to be relatively robust to *Bd* infection (Padgett-Flohr and Hayes, 2011), but experimental studies and field observations suggest there may be sublethal effects resulting in suboptimal body condition associated with individuals harboring *Bd* infection (Pearl *et al.*, 2009; Padgett-Flohr and Hayes, 2011). *R. pretiosa* belong to family Ranidae, many of which are relatively resistant to *Bd* infection (Gervasi *et al.*, 2017) putatively due to their production of diverse antimicrobial skin peptides (Rollins-Smith *et al.*, 2002; Conlon *et al.*, 2011, 2013; Holden *et al.*, 2015). Antimicrobial peptides produced by metamorphosed *R. pretiosa* are diverse compared to related ranid species and have been shown to inhibit *Bd* growth (Conlon *et al.*, 2011, 2013).

R. pretiosa populations historically spanned southwestern British Columbia to Puget Sound, south-central Washington, and the Cascades Range but are now primarily found in south-central Washington and the east side of the Cascades Range in Oregon (Green *et al.*, 1997; Blaustein *et al.*, 1999; Conlon *et al.*, 2011). As of August 2014, *R. pretiosa* is now listed as threatened by the US Fish and Wildlife Service (https://www.fws.gov/oregonfwo/articles.cfm?id=149489458). Although habitat loss is generally thought to be the largest single contributor to their population declines, compounding stressors such as invasive species, pollution, and pathogens can act synergistically to impair individual immune function and ultimately reduce population viability (Pearl *et al.*, 2009). A previous survey found low *Bd* prevalence in *R. pretiosa* larvae compared to other life stages (Pearl *et al.*, 2009), however this may be an artifact of swabbing. In hosts with low *Bd* loads, superficial swabbing can under estimate

pathogen burdens (Retallick *et al.*, 2006; Shin *et al.*, 2014). To our knowledge, no experimental study has assessed the susceptibility of *R. pretiosa* larvae to *Bd*.

To ensure animals did not have prior infection histories with *Bd*, eggs were collected from the Cascades Range, OR and reared in controlled laboratory conditions until Gosner stages 26-30 (hind limbs buds beginning to emerge) (Gosner, 1960). Larvae were house in 40 L aquaria at a density of ~100 larvae per aquaria under a constant temperature, ~15°C, and natural photoperiod. A 3:1 ground mixture of alfalfa pellets and fish flakes (Brine Shrimp Direct) were fed to tadpole aquaria *ad libitum* every other day. A full water change was performed every 7 days. Eggs were collected under Oregon Department of Fish and Wildlife permits and the Oregon State University Institutional Animal Care and Use Committee approved the research protocol.

Bd culture

For all host species, we cultured *Bd* strain JEL646 isolated from *P. regilla* from CA, USA (obtained from Dr. J. Longcore, University of Maine) following methods of Searle *et al.* (2011b). This *Bd* inoculate had undergone 4 passages through 1% tryptone broth before plating on 1% tryptone-agar media. *Bd* plates were incubated at ~ 22°C for 11-14 days before use. To create *Bd* inoculate for each host, *Bd* plates were flooded with 10 mL of dechlorinated water. After 10 min, plates were gently scrapped with a rubber policeman to dislodge *Bd* colonies. Inoculate from flooded plates was pooled and *Bd* concentration was calculated using a hemocytometer. The pooled inoculate was diluted with dechlorinated water to a final concentration of $5x10^2$ zoospores/mL. Each *Bd*-exposed host received 10 mL of *Bd* inoculate for a total of $5x10^4$ zoospores in 610 mL of

water per individual housing unit. Relative to comparable larval challenge experiments, this low dose was chosen to not overwhelm individuals in the coinfection exposure group (Gervasi *et al.*, 2013). Sham *Bd* inoculate was created using the same *Bd* culturing procedure but with sterile tryptone-agar plates.

Sf culture

Sf was cultured from a previously identified and maintained isolate from the Oregon Cascade Range following methods of Romansic et al. (2009). Briefly, 20-30 sterile (autoclaved) hemp seeds were placed on yeast-glucose agar plates containing *Sf*. After 7 days, seeds had become inoculated with *Sf* and were removed and placed in sterile petri dishes containing 15 mL nanopure water. These plates were incubated at ~22°C for 7 days to allow *Sf* mycelia to grow and inoculate hemp seeds. Every host exposed to *Sf* received three *Sf*-laden hemp seeds from these plates. Sham hemp seeds were treated in the same manner (with no *Sf*) and given to control hosts and hosts receiving only *Bd*.

Experimental protocol

To test the effect of a *Bd*-*Sf* coinfection on larval host response and *Bd* infection, all individuals of a host species were randomly assigned to be exposed to *Bd* only (*Bd*+/*Sf*-), coinfection with *Bd* and *Sf* (*Bd*+/*Sf*+), or a control (*Bd*-/*Sf*-) pathogen treatment (Table 3.1). *P. regilla* had an additional treatment group of only *Sf*-exposed (Table 3.1). The experimental protocol was the same for all three host species except *P. regilla* experiments were carried out in October 2013 and *A. boreas* and *R. pretiosa* experiments

were conducted in January 2014. Analyses were conducted separately for each host species. After random assignment to experimental pathogen treatments, larvae were housed individually in 1L plastic cups filled with 600 mL of dechlorinated water and allowed to acclimate to laboratory conditions for 48 hours. At the start of the experiment, 10 mL of inoculate (*Bd* or sham) and three hemp seeds (*Sf*-laden or sterile) were added to appropriate experimental units. Animals were fed three times a week and a full water change was conducted once every 7 days for the duration of the experiment (30 days). Survival was monitored daily and deceased individuals were preserved in 95% ethanol. To limit handling stress, weight (g) measurements were taken only upon death for all animals. All animals were preserved in 95% ethanol for later infection intensity analysis.

Quantifying Bd intensity

All *A. boreas* and *R. pretiosa* hosts were sampled for *Bd* infection while a randomly chosen subset of 20 individuals was chosen for each *Bd*-exposed treatment group in *P. regilla* (Table 3.2). *Bd* infection intensity was measured using quantitative PCR (qPCR) (Boyle *et al*., 2004). DNA was isolated from whole mouthparts and processed following methods of Boyle *et al*. (2004) except for the use of 60 µl of Prepman Ultra (Applied Biosystems, Life Technologies) instead of 40 µl. DNA concentration was quantified using a spectrophotometer (Nanodrop Technologies). qPCR was conducted on an Applied Biosystems® 7500 FAST machine following protocol of Boyle *et al*. (2004). A negative-nanopure water control was included in all qPCR plate runs with serially diluted *Bd* standards (10^{-1}, 10^{0}, 10^{1}, 10^{2}). Each *Bd* sample was analyzed in triplicate; if two of the three qPCR replicates had detectable *Bd*, that sample was

considered *Bd* positive and an average *Bd* genome equivalent (GE) was calculated. *Bd* intensity was measured as the average amount of *Bd* GE per nanogram (ng) of extracted mouthpart DNA. This measure of *Bd* infection accounts for differences in individual host size which may influence the amount of mouthpart tissue extracted from each host (Han *et al.*, 2011; Searle *et al.*, 2011a; Han *et al.*, 2015).

Data analysis

Differences in *Bd* infection prevalence was compared between *Bd*+ and *Bd*+/*Sf*+ treatment groups in each host species by a N-1 Chi-Square test which is suited for comparing proportions in samples sizes (Campbell, 2007). *Bd* infection intensity was analyzed separately for each species using generalized linear models (GLM) with quasipoisson errors and pathogen treatment, days survived, and weight as predictor variables. Days survived could not be included in the analysis for *P. regilla* because only three individuals died in this species throughout the experimental run and one treatment group (*Sf*+) had zero deaths. We analyzed differences in host survival among different pathogen treatments groups for each host species using Kaplan-Meier curves and Cox-proportional hazards (Cox-ph) models (Cox, 1972). Cox-ph models compare survival curves to estimate the probability of mortality from different variables (likelihood ratio test). The effect of a factor on mortality probability is represented by a hazard ratio. All analyses were conducted in R version 3.3.3; survival analyses were done with the R package *survival* version 2.40-1 (R Core Team 2017).

Results

Host Bd intensity

Bd infection prevalence was lowest in *P. regilla* but did not differ between hosts in the *Bd*+ (55% infected) and *Bd*+/*Sf*+ (65% infected) pathogen treatment groups ($p = 0.52$; Table 3.2). *A. boreas* and *R. pretiosa* hosts had high *Bd* infection prevalence in all *Bd*-exposed treatment groups (percent positive range 76.9%-92.9%) but there were no differences between pathogen treatments in both species (both $p > 0.25$; Table 3.2). *Bd* infection intensity did not differ among pathogen treatment groups in *P. regilla* hosts ($p = 0.88$; Table 3.3, Figure 1); however, higher weight was associated with increased *Bd* intensity in this species ($F = 17.09$, $p < 0.001$; Table 3.3). *A. boreas* hosts that died earlier in the experiment had higher infection intensities than those that died later ($F = 31.74$, $p < 0.001$; Table 3.3). *Bd* intensity did not significantly differ between *Bd*+ and *Bd*+/*Sf*+ coinfection treatment groups but there was a slight trend for higher *Bd* infection level in the *Bd*+/*Sf*+ coinfection group ($p = 0.09$; Table 3.3, Figure 3.1). Host weight was not predictive of *Bd* intensity ($p = 0.44$; Table 3.3). For *R. pretiosa* hosts, higher weight and more days survived was associated with lower *Bd* infection intensity (weight $F = 16.15$, $p < 0.001$; days survived $F = 23.04$, $p < 0.001$; Table 3.3). *Bd* infection did not significantly differ among pathogen treatment groups but there was a marginal trend for higher *Bd* intensity in *R. pretiosa* hosts in the *Bd*+/*Sf*+ coinfection treatment ($p = 0.07$; Table 3.3, Figure 3.1).

Host survival

There was no effect of any pathogen treatment on *P. regilla* survival (Figure 2). All but three *P. regilla* survived until the end of the experiment; pathogen treatment groups *Bd*+, *Bd*+/*Sf*+, and control each had one tadpole die during the experiment. There was no mortality difference between any pathogen treatment group compared to control in *A. boreas* hosts (log-rank test p = 0.9; Figure 3.2A). In *R. pretiosa*, only the *Bd*+ treatment group had significantly decreased survival compared to control (hazard ratio = 2.7, p = 0.02; Figure 3.2B). Individuals in the coinfection treatment group, *Bd*+/S+, had marginally lower survival compared to those from the control treatment (hazard ratio = 2.1, p = 0.07; Figure 3.2B).

Discussion

We found little evidence for a *Sf* coinfection effect on *Bd* infection outcomes. *Bd* infection dynamics mostly varied by host species, host end weight, and days survived. *P. regilla* hosts had high survival in all pathogen treatment groups compared to control further supporting their designation as a putative reservoir host, especially in the larval stage (Blaustein *et al.*, 2005; Padgett-Flohr and Hopkins II, 2009; Reeder *et al.*, 2012; Searle *et al.*, 2013). *A. boreas* hosts did not experience increased mortality in either pathogen treatment group compared to control though it was found individuals surviving longer had lower *Bd* infection intensity. Survival differences were detected in only one species, *R. pretiosa*. Although there was a tendency for higher *Bd* infection intensity in *R. pretiosa* hosts exposed to a coinfection of *Bd* and *Sf* compared to just *Bd*, this trend was not significantly different. And while both pathogen treatments in this hosts species resulted in lower survival compared to the control group, only the *Bd*+ treatment group

was statistically significant. Higher host weight was associated with lower *Bd* infection level in *P. regilla* and *R. pretiosa* but not *A. boreas*. Lower *Bd* intensity was also correlated with hosts that survived longer in *A. boreas* and *P. pretiosa*; days survived could not predict *Bd* infection intensity in *P. regilla* due to very high host survival in all treatment groups.

Our hypothesis of lower host survival and higher *Bd* infection intensity in the *Bd*+/*Sf*+ coinfection treatment groups compared to *Bd*+ only groups was not strongly supported. In all species, we did not detect differences in *Bd* infection prevalence due to pathogen exposure. *Bd* infection intensity was statistically similar between pathogen treatment groups, however there was marginal evidence for higher *Bd* infection intensity in coinfected individuals compared to those exposed to only *Bd* in *A. boreas* and *R. pretiosa* host species (Table 3.3). This synergistic effect suggests *Bd* colonization may be amplified in the presence of another dermis-infecting zoosporic pathogen. Our ability to detect significant differences in this study may have been limited due to small sample sizes. Romansic *et al*. (2017) found *P. regilla* larvae exposed to a coinfection exposure of *Bd* and the water mold *Achlya* had higher *Bd* infection prevalence suggesting presence of water mold facilitated *Bd* infection. Mechanical facilitation is a common mechanism underlying synergistic interactions between coinfecting pathogens, especially those degrading dermal tissues (Evans *et al.*, 2007; Cutuli *et al.*, 2015); bacteria can exploit *Bd* infected tissues although this has not been linked to increased host mortality (Berger *et al.*, 2005). Continued research into amphibian disease cofactors could investigate conditions that might facilitate *Bd* infection in the field.

In two host species, *P. regilla*, and *A. boreas*, survival differences were not detected with any pathogen treatment compared to control. While larval *P. regilla* was expected to be robust to *Bd* infection, previous research found larvae of this species to be sensitive of *Sf* (Romansic *et al.*, 2009). We did not observe survival differences in *P. regilla* hosts exposed to *Sf* only compared to control. *A. boreas* is thought to be particularly sensitive to *Bd* infection (Blaustein *et al.*, 2005; Carey *et al.*, 2006; Searle *et al.*, 2011a) although high larval survival with *Bd* exposure has been documented in this species before (Gervasi *et al.*, 2013). Lower survival was detected in *R. pretiosa* but only in the *Bd*+ treatment group (the coinfection treatment group also experienced lower survival but this was not statistically different from control). *Bd* infection intensity, being comparable between both pathogen treatment groups in this species, suggests some pathogen interaction effect between *Bd* and *Sf* resulting in higher host survival. *Bd* susceptibility has been tested in *R. pretiosa* before but only in recently metamorphosed hosts (Padgett-Flohr and Hayes, 2011). Though this study shows juvenile *R. pretiosa* are robust to *Bd* infection, we show this is not true for the larval stage. Future species conservation efforts should take into account larval *Bd* susceptibility and plan interventions to mitigate *Bd*-induced mortality at this life stage. Overall, these results show *Sf* coinfection does not dramatically influence *Bd* infection dynamics. This supports recent coinfection research in larval amphibians using *Bd* and the water mold *Achlya* (Romansic *et al.*, 2011, 2017).

Amphibian coinfection research is crucial in light of present-day amphibian population declines (Daszak *et al.*, 2000; Stuart, 2004; Wake and Vredenburg, 2008; Kilpatrick *et al.*, 2010; Blaustein *et al.*, 2012). The degree to which *Bd* can contribute to

amphibian population declines is strongly supported in some population declines and uncertain in others (Berger *et al.*, 1998; Vredenburg *et al.*, 2010; Heard *et al.*, 2011). The enigmatic nature of many amphibian population declines strongly point to multifactorial causes. Research that aims to test multiple cofactors have found pathogen interactions can result in antagonistic or synergistic effects which may subsequently mediate host response (Sousa, 1994; Janovy Jr, 2002; Evans *et al.*, 2007; Ezenwa *et al.*, 2010). Because of the ubiquity of chytrid and oomycete species in aquatic systems (Gleason *et al.*, 2008; Freeman *et al.*, 2009; Sarowar *et al.*, 2013; Comeau *et al.*, 2016) and their ability to infected many different amphibian host species (Densmore and Green, 2007; Kilpatrick *et al.*, 2010; Derevnina *et al.*, 2016; Gervasi *et al.*, 2017), *Bd* and water mold coinfection research is needed to better understand how these common pathogens interact with each other to affect amphibian hosts. With advancements in amphibian microbial pathogen research and molecular technology, we are only now beginning to appreciate the diversity of naturally-occurring amphibian pathogens (Rowley *et al.*, 2013; Gleason *et al.*, 2014; Chambouvet *et al.*, 2015). The presence of many different amphibian-infecting aquatic zoosporic pathogens warrants future coinfection research to untangle their interactive roles in amphibian population declines.

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Figures

Figure 3.1. Violin plot (with boxplot overlay) of *Bd* infection intensity in A) *P. regilla*, B) *A. boreas*, and C) *R. pretiosa* hosts. B = *Bd* inoculate and sterile hemp seeds; BS = *Bd* inoculate + *Sf*-inoculated hemp seeds. Boxplot: horizontal line = median, box = interquartile range (IQR), whiskers: full data range within 1.5*IQR, black dots: outliers.

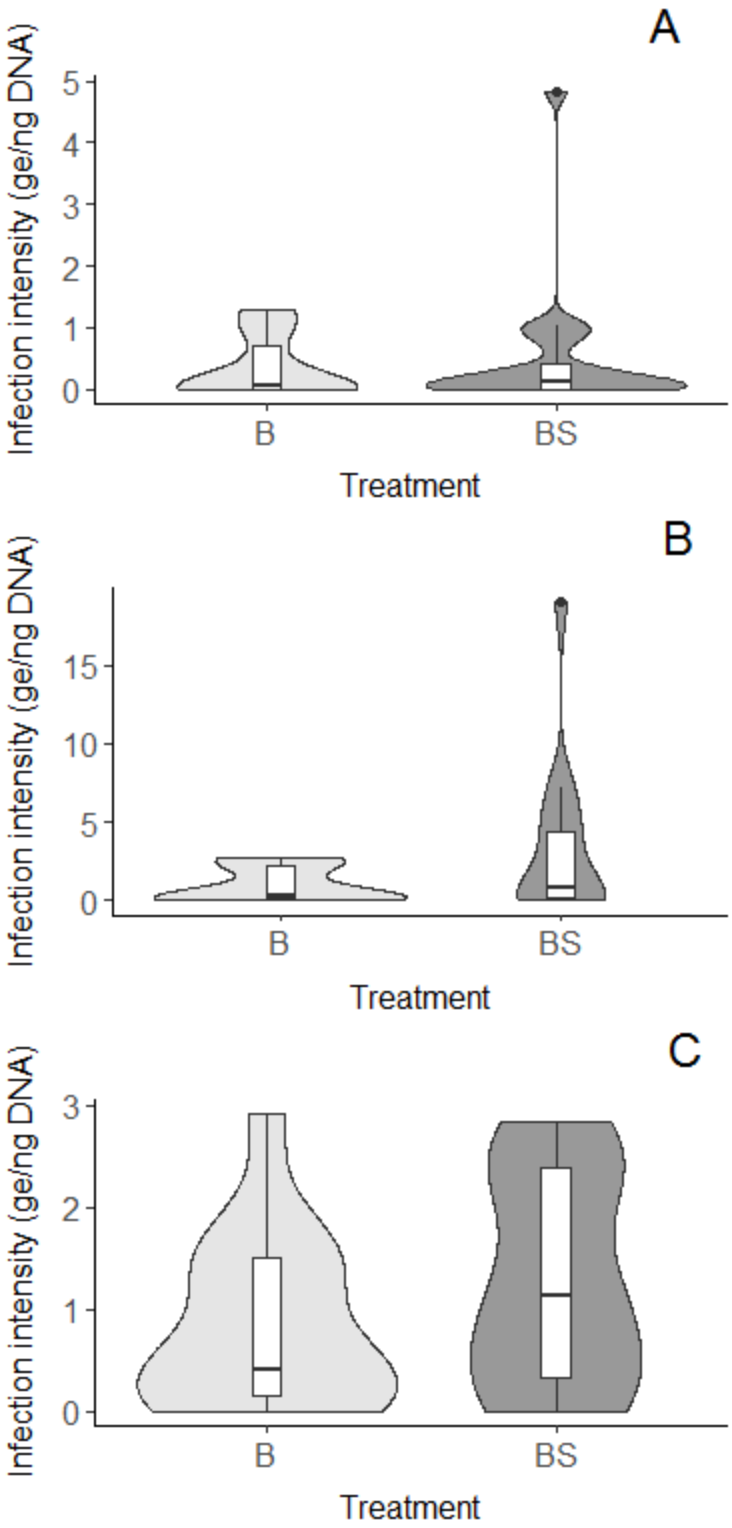
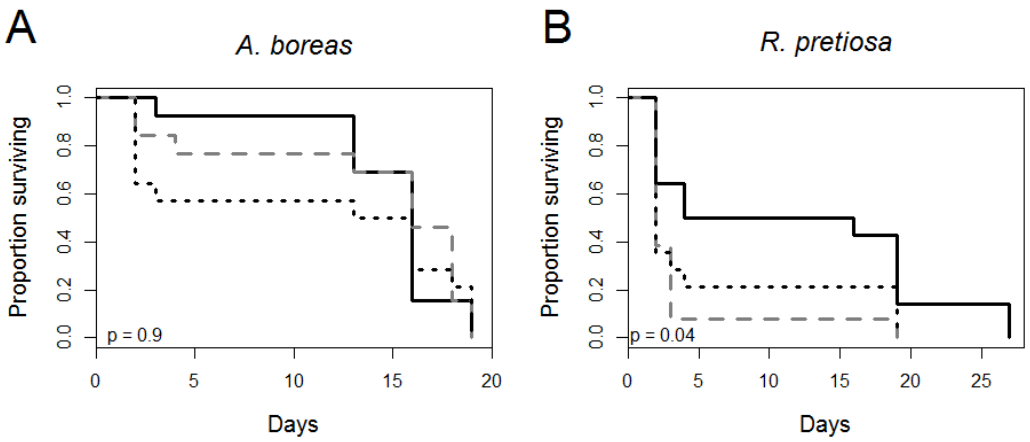


Figure 3.2. Survival curves for A) *A. boreas* and B) *R. pretiosa*. Control group = solid black line; *Bd*+ exposed group = long-dash gray line; *Bd*+/*Sf*+ = short-dash black line.



Tables

Table 3.1. Experimental design with parasite treatment groups and number of hosts per species per group. S+, *Sf* inoculated hemp seeds; B+, *Bd* inoculate; S-, sterile hemp seeds; B-, sham *Bd* inoculate.

Host species	Parasite treatments			
	B- / S- (control)	B+ / S+	B+ / S-	B- / S+
P. regilla	36	38	37	37
A. boreas	13	14	13	-
R. pretiosa	14	14	13	-

Table 3.2. Proportion of *Bd* positive hosts from *Bd* only and *Bd* with *Sf* coinfection treatments. B+/S+, *Bd* inoculate and *Sf* inoculated hemp seeds; B+/S-, *Bd* inoculate and sterile hemp seeds. *Bd* detection via qPCR was conducted on all *Bd* exposed individuals from *A. boreas* and *R. pretiosa* and 20 individuals from each *Bd* treated group in *P. regilla*.

Host species	Parasite treatments					
	B+ / S+			B+ / S-		
	N sampled	N positive	% positive	N sampled	N positive	% positive
P. regilla	20	13	65%	20	11	55%
A. boreas	14	13	92.9%	13	10	76.9%
R. pretiosa	14	12	85.7%	13	12	92.3%

Table 3.3. Summary of *Bd* infection intensity statistical analyses. *Bd* intensity was analyzed using a GLM with weight, pathogen treatment, and days survived as predictors.

Species	Predictor	F	*p* - value	Pattern
*P. regilla**	Weight	17.09	< **0.001**	Lower *Bd* infection intensity with increased host weight
	Pathogen treatment	0.02	0.88	-
A. boreas	Weight	0.62	0.44	-
	Pathogen treatment	3.03	0.09	Trend for higher *Bd* infection intensity in *Bd*+/*Sf*+ treatment compared to *Bd*+ only
	Days survived	31.74	< **0.001**	Lower *Bd* infection intensity in hosts that survived longer
R. pretiosa	Weight	16.15	< **0.001**	Lower *Bd* infection intensity with increased host weight
	Pathogen treatment	3.69	0.07	Trends for higher *Bd* infection intensity in *Bd*+/*Sf*+ treatment compared to *Bd*+ only
	Days survived	23.04	< **0.001**	Lower *Bd* infection intensity in hosts that survived longer

* *P. regilla* analysis did not include “days survived” due to low number of deaths (n=3)

CHAPTER 4 – THE DYNAMICS OF HOST AGE AND PATHOGEN TRANSMISSION IN THE AMPHIBIAN—CHYTRID FUNGUS SYSTEM

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In review: Parasitology

Abstract

Infection heterogeneity results in highly skewed parasite burdens among conspecific hosts. This may be driven by factors affecting host susceptibility such as age, sex, behavior, or immune function. Differential host susceptibility can subsequently influence parasite transmission to other hosts and may ultimately effect host population dynamics. We tested age-dependent host susceptibility (measured as infection intensity) to an emerging fungal parasite of amphibians, *Batrachochytrium dendrobatidis* (*Bd*), in juvenile and adult post-metamorphic hosts of two species, *Pseudacris regilla* and *Rana aurora*. We found age and species differences in infection susceptibility. *P. regilla* adults had higher infection intensities than juveniles, however the opposite trend was found in *R. aurora*. We further assessed whether age-dependent infection intensity of these hosts (donors) influenced between-host *Bd* transmission by pairing donors with uninfected conspecific juveniles (recipients). All recipients had species-specific infection levels regardless of donor age and infection intensity. Our findings revealed host age can drive heterogeneous infection patterns but these trends may not be generalizable across different species. We also found age-dependent infection intensity did not affect subsequent *Bd* transmission to recipient hosts. Our results highlight the importance of comparative empirical studies to address complexity in *Bd* infection dynamics.

Introduction

Disease susceptibility can depend on intrinsic host traits such as species, age, sex, size, body condition, immune function, and behavior (Wilson *et al*., 2001; Streicker *et al*., 2013; Johnson and Hoverman, 2014; Bielby *et al*., 2015; Abu Bakar *et al*., 2016; Gervasi

et al., 2015, 2017) as well as parasite specific traits, e.g. strain, passage history, transmission route (Franzot *et al*., 1998; Bouklas *et al*., 2015; Stephenson *et al*., 2017). Additionally, disease patterns, especially host-parasite contact rate, can be mediated by environmental factors such as climate, seasonality, and spatio-temporal variation of hosts and parasites (Wilson *et al*., 2001; Altizer *et al*., 2006; Levi *et al*., 2015; Paull *et al*., 2017). The extent to which heterogeneities in a host population affect downstream patterns of parasite transmission and aggregation is an active field of research in disease ecology (Streicker *et al*., 2013; Johnson and Hoverman, 2014; Auld *et al*., 2017; Stephenson *et al*., 2017). Much research on disease susceptibility research focuses on intrinsic host traits because host species are often of agricultural or conservation importance and most disease interventions operate at the host level, e.g. immunizing certain hosts, captive rearing programs, etc. (Altizer *et al*., 2003; Bletz *et al*., 2013; Scheele *et al*., 2014; Langwig *et al*., 2015). Furthermore, understanding host-associated susceptibility is necessary to predict disease spread in a heterogeneous host population and to forecast future risk to naïve hosts (Han *et al*., 2015*b*; Langwig *et al*., 2015; Xie *et al*. 2016; Agrawal *et al*., 2017).

We investigated heterogeneity in infection intensity due to host age and its effect on transmission in the emerging amphibian fungal disease chytridiomycosis, a disease implicated in the declines of numerous amphibian populations worldwide (Berger *et al*., 1998; Daszak *et al*., 2003; Wake and Vredenburg 2008; Kilpatrick *et al*., 2010; Olson *et al*., 2013; James *et al*., 2015). The causal parasite, the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*), is an amphibian generalist parasite, infecting ~500 species of amphibians worldwide (Olson *et al*., 2013; James *et al*., 2015). Infection occurs when a

free-swimming aquatic zoospore encysts in keratinized host tissue. In larval amphibian hosts, this consists largely of the mouthparts while the entire epidermis is vulnerable in metamorphosed amphibians (Voyles *et al*. 2009; Rollins-Smith *et al*., 2011; Van Rooij, *et al*. 2015). Chytridiomycosis in adult amphibians has been shown to lead to impaired electrolyte transport in the skin causing death by cardiac arrest (Voyles *et al*. 2009). *Bd* susceptibility and mortality can differ among hosts by species, life stage, behavior, immune history, skin microbiota, and populations (Blaustein *et al*. 2005; Venesky *et al*., 2011; Peterson *et al*., 2013; Bataille, *et al*., 2015; Bradley, 2015*a*; Bradley *et al*., 2015*b*; Kueneman *et al*., 2016; Dang *et al*., 2017; Gervasi *et al*., 2013, 2017)

Within amphibian hosts, age is a major factor in *Bd* susceptibility (Kriger and Hero 2006; Rollins-Smith *et al*., 2011). Because larvae only contain keratinized tissue in their mouthparts, they are thought to be relatively robust to negative effects of *Bd* infection compared to amphibians that have completed metamorphosis (Rachowicz and Vredenburg, 2004; Voyles et al. 2009; Gervasi *et al*. 2013), though larval mortality after exposure to *Bd* has been observed in some experimental studies (e.g. Blaustein *et al*., 2005; Fisher *et al*., 2009; Garner *et al*., 2009; Dang *et al*., 2017). In metamorphosed amphibians, *Bd* infection is most often thought to be most fatal to hosts that have recently undergone metamorphosis (Rollins-Smith *et al*., 2011; Ortiz-Santaliestra *et al*., 2013; Abu Bakar *et al*., 2016). The disruptive reorganization of the body during and after metamorphosis results in small body size and an immature immune system and skin microbiome, key factors linked to increased *Bd* susceptibility (Carey *et al*., 2006; Kriger and Hero 2006; Rollins-Smith *et al*., 2011; Ortiz-Santaliestra *et al*., 2013; Abu Bakar *et al*., 2016; Kueneman *et al*., 2016). As body size increases and the immune system of

metamorphosed amphibians mature with age, hosts are hypothesized to be more robust to *Bd* infection (see Ortiz-Santaliestra *et al*., 2013 and Abu Bakar *et al*., 2016). Consequently, if older adult frogs are able to tolerate *Bd* infection more than younger frogs, they may serve as *Bd* reservoirs that transmit zoospores to other individuals. However, recent empirical work by Bradley (2015*a*) in two host species found older amphibian hosts have higher mortality rates than younger hosts when experimentally exposed to *Bd*. These findings highlight the complex relationship between host age and *Bd* susceptibility. In natural populations susceptibility may differ drastically among hosts of different ages leading to complex disease dynamics. Additionally, heterogeneous infection patterns may arise due to the effect of susceptibility differences on overall parasite transmission and persistence in the environment.

Our study explored the effects of age-related susceptibility on infection intensity and whether this later mediates between-host transmission. We tested this in two co-occurring amphibian host species in the Pacific Northwest, USA: *Pseudacris regilla* (Pacific chorus frog) and *Rana aurora* (red-legged frog). Following terminology used by Stephenson *et al*. (2017), our study addressed the following questions:

1) Does heterogeneity in infection prevalence and intensity differ by host (donor) age? Because recently metamorphosed amphibians are thought to be the most vulnerable life stage to *Bd* infection, thus we expected juvenile (2 months post-metamorphosis) hosts to be more susceptible to *Bd* infection compared with adults (1 year post-metamorphosis) in both host species (i.e. less heterogeneity in infection prevalence/intensity and overall higher infection intensity will be observed in juvenile hosts).

2) Will infection heterogeneity in donors result in equivalent patterns in transmission to conspecific juvenile recipients? We expected donors with the highest infection intensity (juveniles) to transmit more *Bd* zoospores compared to donors with less infection intensity (adults).

Materials and Methods

Amphibian husbandry

We tested for age differences in *Bd* susceptibility (infection prevalence and intensity) using adult and juvenile donors of two Pacific Northwest amphibian species, *P. regilla* and *R. aurora*. We then paired each donor with a naïve conspecific juvenile recipient to determine whether donor age-mediated susceptibility influenced *Bd* transmission to another host.

To ensure hosts did not have prior infection history, freshly laid eggs were collected because *Bd* has never been found to penetrate the gelatinous egg casing (World Organisation for Animal Health, 2016). Eggs of *P. regilla* and *R. aurora* were collected in Spring 2013 (adult donor cohort) and 2014 (juvenile donor and juvenile recipient cohort) from the same sites in the Coast Range (*R. aurora*) and Cascades Range (*P. regilla*) of Oregon and reared in the laboratory until hatching. Adult and juvenile conspecifics were collected from the same sites to reduce site-population differences in infection response (Bradley *et al*., 2015*b*). Tadpoles were housed at a constant density of ~100 individuals per 40 L tank in a temperature regulated room held at ~15°C and under a natural photoperiod. Tadpoles were fed every other day ad libitum with a 3:1 ground

mix of rabbit chow and fish flakes. After hatching, animals were transferred to outdoor mesocosms, which were 320 L screen-covered plastic cattle drinking tanks filled with well water, at the Lewis Brown Horticulture Farm at Oregon State University. Each tank was inoculated two weeks prior with 90 g dried oak leaves, 1 L pond water with zooplankton and algae, and 5 g rabbit chow. Upon forelimb emergence, metamorphs were transferred to wet moss-covered mesocosms nearby (120 L plastic tanks with screen lids). Metamorphs were fed crickets *ad libitum* until ~1 month post-metamorphosis at which point animals were transferred inside to a temperature controlled room (~15°C) under natural photoperiod. Metamorphs were kept in moss-covered aquaria at a density of 30 individuals per tank. Frogs were fed crickets once a week and tanks cleaned once a month. At the start of the experiment, all adult hosts were over one year post-metamorphosis and all juvenile hosts were ~ two months post-metamorphosis (juveniles had been acclimated to laboratory conditions for >1 month).

Parasite culture

Bd (strain JEL646 isolated from *P. regilla* in Point Reyes, CA USA) was grown on 1% tryptone-agar plates for 13 days. Nine plates were flooded with 10 mL of water and sat for 10 min before gently scrapping with a rubber policeman to dislodge growing *Bd* colonies. Active, swimming zoospores were quantified with a hemocytometer. Each experimentally infected host received 10 mL of *Bd* broth at a concentration of 10,000 zoospores/mL for a total of 100,000 zoospores per petri dish (total water volume of 25 mL). This dose is consistent with Bradley (2015*a*) who used the same host species but lower than other post-metamorphic experiments conducted under similar laboratory

conditions (Searle *et al*., 2011; Bradley *et al*., 2015*b*; Gervasi *et al*., 2013, 2017). Sham *Bd* plates for the control group were made in the same way using sterile tryptone plates.

Experimental procedure

Each host species had three experimental treatment groups consisting of two hosts per replicate, a donor and a recipient, to test infection susceptibility of the donor and later transmission to the recipient (Table 4.1). Donors were exposed to *Bd* first for three days to assess infection susceptibility before pairing with recipients. The focal host to assess transmission, the recipient, was always an uninfected conspecific juvenile host. Thus each replicate of a *Bd* treatment group had a recipient paired with an infected adult donor or an infected juvenile donor. The control group paired an uninfected adult with an uninfected juvenile.

Donors exposed to *Bd* were weighed and measured before being randomly assigned to a treatment group (Table B1). Each donor was housed individually in large plastic petri dishes (140 x 33 mm) with holes drilled in the lids, 15 mL of dechlorinated water, and were allowed to acclimate for one day. The following day, *Bd* and control (water) inoculate was prepared and 10 mL delivered to each donor host; *Bd*-treated individuals received approximately 100,000 zoospores in 10 mL of broth and control individuals received 10 mL of sham broth. Donors remained in their individual dishes for three days before being paired with their randomly chosen juvenile recipient for the start of the experiment. On Day 0, each donor was lightly rinsed with dechlorinated water to remove any liquid that may have non-encysted *Bd* and swabbed 10 times with a sterile, rayon-tipped swab (Medical Wire and Equipment, UK) on the ventral surface of their

lower right limb (vent to toes). This initial sampling served as a measure of initial donor infection intensity before being paired with their uninfected partner (recipient). Once the donor and recipient were paired in a new dish, 25 mL of dechlorinated water was added to provide a thin layer of water to facilitate *Bd* zoospore transmission. The experiment ran for 20 days with daily checks conducted to record survival. Animals were fed crickets twice a week based on average species body weight (one cricket per 0.1 g body mass) and full water changes were conducted once a week. If one host in a pair died during the experiment, the other host was euthanized (immersion in MS-222) and ending body measurements and leg skin swabs were taken for both. Animals were preserved in 95% EtOH. Preserving both partners at this time point was done to measure the peak level of parasite transmission. If the remaining host were left for the remainder of the experiment, *Bd* infection levels might decrease or clear, complicating transmission detection and results. At the end of the experiment, any remaining hosts were euthanized in an overdose of MS-222 and preserved in 95% EtOH. Ending body measurements and skin swabs were also taken at this time for each host.

Infection prevalence and intensity

Donors were swabbed twice for Bd infection intensity; initial intensity was taken after a three-day exposure to Bd and before pairing with recipient partner and ending infection intensity was taken upon death or termination of the experiment. All recipients were swabbed upon death or experimental end. Skin swabs from all individuals in *Bd*-exposed treatment groups and three randomly chosen control hosts were used to measure *Bd* infection via quantitative PCR (qPCR). DNA was extracted following the protocol of

Boyle *et al*. (2004) except for the use of 60 µl of Prepman Ultra (Applied Biosystems, Life Technologies) instead of 40 µl for extractions. Each sample was analyzed in triplicate on an Applied Biosystems® 7500 FAST machine using primers and probes developed by Boyle *et al*. (2004) and tested against known *Bd* standards (USGS) of 100, 10, 1, and 0.1 genome equivalents. The average genome equivalent was recorded across the three replicates; samples were considered positive if two of the three qPCR replicates detected *Bd*.

The concentration of DNA per sample was quantified with a Qubit® high-sensitivity fluorescent dye assay. Infection intensity was calculated by dividing genome equivalents (ge) from qPCR by the amount of DNA in nanograms (ng) of each sample. By standardizing infection burden of each host this way we eliminate the potential effect of individual size, a confounder when comparing hosts of differing ages (Searle *et al*., 2011; Han *et al*. 2015*a*).

Statistical Analysis

Initial infection intensity difference among age was analyzed using a non-parametric Wilcoxon Rank Sum (Mann Whitney U) test on experimentally infected hosts of both species. Direction of change in infection intensity (gain or loss) was analyzed with a binomial generalized linear model (GLM) with species, age, and day of death as predictors. Ratio of infection intensity was analyzed separately for each species with a Wilcoxon Rank Sum test. Degree of heterogeneity (i.e. degree of parasite aggregation) in infection intensity was analyzed using two common indices, the variance-to-mean ratio and the corrected parameter k (an overdispersion parameter of negative binomial

distributions adjusted to account for sample size (Wilson *et al*., 2001)). Parasite aggregation increases with higher variance-to-mean ratios and lower k values. Infection heterogeneity is a common feature in many macroparasite systems and have k values <1 (Wilson *et al*., 2001). Lastly, a Wilcoxon Rank Sum test was used to assess whether there was a relationship between initial infection load and survival.

For all juvenile recipients, we analyzed infection intensity using generalized linear models (GLM) with quasipoisson errors. Covariates in the model included host species, donor age, donor initial infection intensity, recipient weight, and the interaction of species and donor age. In *R. aurora*, two recipients were cannibalized during the experiment so no infection intensity could be quantified from these samples.

Survival analysis was not the objective of the study, which sought to investigate heterogeneity in *Bd* infection and transmission among hosts, thus not all survival pairwise comparisons can be made because not all host combinations were tested for survival differences. For example, in *R. aurora*, the survival of juvenile recipients paired with juvenile donors cannot be compared to the control treatment because only a juvenile-adult uninfected pairing was made, not a juvenile-juvenile pairing. Survival comparisons are only stated where appropriate comparisons could be made.

Survival was analyzed using Kaplan-Meier survival curves, survival log-rank tests, and Cox-proportional hazards models. Survival curves were compared separately for each species and exposure group (donors and recipients) to assess survival differences among groups (for each comparison, the explanatory variable was donor age). If the initial log-rank test was significant, between group differences were analyzed using a pairwise log-rank test with Bonferroni adjustments for multiple comparisons. For

recipients, a Cox-proportional hazards (Cox-ph) model was used to estimate the effect of donor age and initial weight on survival. Survival analyses were conducted using the R packages "survival." In *R. aurora*, three juvenile recipients paired with control adults and two juvenile recipients paired with adult donors were cannibalized. Recipient survival analysis was conducted on all recipients and excluding cannibalized hosts. Conclusions were similar and we report the results from the analysis with the full dataset. All statistical analyses were performed in R version 3.3.2 (R Core Team, 2016).

Results

Prevalence in donors

All experimentally-infected donors had detectable initial levels of *Bd* before pairing with a recipient except for one juvenile *P. regilla* donor. This host likely had a low level of *Bd* infection that was below the assay detection limit. This host tested positive for *Bd* infection at the last sampling time point.

Infection intensity of donors

P. regilla initial infection intensity differed among age groups with adult donors having higher intensity compared to juvenile donors ($p = 0.03$; Figure 4.1A). The opposite trend was found in *R. aurora* – juvenile donors had higher initial infection intensity than adult donors ($p = 0.001$; Figure 4.1B). The two methods used to measure degree of parasite aggregation (mean-to-variance ratio and correct parameter k) also

confirm more highly skewed distributions in infection intensity in *P. regilla* adult donors compared to juveniles and in *R. aurora* juvenile donors compared to adults (Table 4.2).

Direction of change in infection intensity was predicted by host species with *R. aurora* more likely to lose infection compared *P. regilla* ($\chi^2 = 38.53$, $p < 0.001$; Figure 4.3). There was some evidence for a trend of juvenile *P. regilla* donors having a larger relative change in infection intensity ratio compared to adults ($p = 0.06$; Figure 4.2; Table B2). *R. aurora* juveniles and adults did not differ in their relative infection change ratio ($p = 0.15$; Figure 4.2; Table B2).

Between-host transmission from donors to recipients

Recipients were susceptible to *Bd* transmitted from donors, yet infection prevalence differed in both host species (Table 4.3). 100% of *P. regilla* recipients were infected while 57.1% (8 of 14) and 78.6% (11 of 14) *R. aurora* recipients were infected when exposed to adults and juvenile donors respectively (Table 4.3). Infection intensity differed between species but not any other predictor tested including initial infection intensity of donors (Figure B2); *R. aurora* recipients had significantly lower infection intensity than *P. regilla* recipients ($F = 36.1$, $p < 0.001$; Figure 4.4).

Survival

In *P. regilla*, adult donors had significantly lower survival compared to control adults and juvenile donors ($p = 0.0004$ and 0.03 respectively; Figure 4.5A). There was a marginally significant trend for lower survival in juvenile *R. aurora* donors compared to

control adults (Cox-ph hazard ratio = 6.86, p = 0.07) but overall, survival did not differ among age groups (Figure 4.5B).

P. regilla recipients did not experience any significant mortality among donor age groups (p = 0.44; Figure 4.6A). *R. aurora* recipients paired with juvenile donors had higher survival than those paired with adult donors (p = 0.02) and control adults (p = 0.001); *R. aurora* recipients paired with uninfected adults (control) and adult donors had comparably low survival (p = 0.43) (Figure 4.6B). *R. aurora* recipients with heavier initial weight had higher survival than smaller hosts (Cox-ph hazard ratio = 0.01, p = 0.005).

Discussion

Our study revealed host heterogeneity drives *Bd* infection dynamics under some circumstances but not in others. We found differences in infection level (measured as infection intensity) between juvenile and adult hosts in both species but with opposite trends between species. In *R. aurora*, juvenile donors carried higher infection intensity than adults, although survival did not significantly differ between juveniles and adults. Interestingly, in *P. regilla*, a putative reservoir host for *Bd* (Reeder *et al*., 2012), adults carried higher infection intensity compared to juveniles; additionally, adult *P. regilla* donors experienced reduced survival compared to adult controls and juvenile donors. This finding concurs, in part, with a recent study from Bradley (2015*a*) that assessed *Bd* susceptibility and survival using the same amphibian species across a wide age range of post-metamorphic hosts (1 week to 9 months). But while the results for *P. regilla* in both studies were similar, Bradley (2015*a*) found higher *Bd* levels and increased mortality in

older *R. aurora* whereas we found higher infection levels with younger hosts. The inconsistent patterns between the present study and Bradley (2015*a*) may stem from differences in experimental protocol between the two studies. Our study and the study of Bradley (2015*a*) used different *Bd* strains and eggs were collected from different sites. It is important to acknowledge the context-dependency of infection dynamics when conducting experimental research; conclusions may not be generalizable under different study conditions as several studies show. Abu Bakar *et al*. (2016) tested age susceptibility differences to *Bd* in *Litoria aurea* and found recently metamorphosed frogs experienced higher infection levels and higher morality compared to subadults and adults. Another study found different results with age between two species. Ortiz-Santaliestra *et al*. (2013) analyzed *Bd* susceptibility between post-metamorphic amphibian hosts of two age classes, the end of metamorphosis and ~ 4 weeks post-metamorphosis. In one host species (*Anaxyrus americanus*), younger hosts incurred higher mortality than older hosts, supporting the notion younger adult amphibians are more vulnerable to *Bd* infection. However, this trend was not found in a second, more tolerant host species (*Lithobates pipiens*). Again, differences in host source, age interval, species tested, rearing conditions, and *Bd* strain were not comparable between studies. Regardless of experiment-specific logistical differences, age-related infection outcomes were detected in all studies. These findings, taken together, show how the intrinsic host trait age can significantly affect *Bd* survival and infection.

Parasite susceptibility may be very dynamic in hosts with multiple life stages such as amphibians and insects (Brunner *et al*. 2004; Searle *et al*. 2013; Khan *et al*. 2016). Amphibians are unique among vertebrates in their biphasic lifecycle, encompassing an

aquatic larval stage followed a dramatic metamorphic transformation into a terrestrial or semiterrestrial adult stage (Vitt and Caldwell 2009). Transformation to a more terrestrial life involves many physiological alterations including development of dermal structures important in *Bd* susceptibility such as keratinized skin layers (Wells 2007; Van Rooij *et al*. 2015). Reliance on water, and thus dermal structure, varies among the adult stage of amphibian families (Wells 2007). Because *Bd* is a skin-colonizing aquatic parasite, comparative studies often highlight reliance on water as a major predicting factor of *Bd* susceptibility (Bancroft *et al*. 2011; Gervasi *et al*. 2017). This concurs with empirical research suggesting aquatic frogs in the family Ranidae (*R. aurora* in this study) to be generally robust to *Bd* infection (but see Vredenburg *et al*. 2010); a major factor is thought be their diverse mucosal skin microbiome, the first line of defense in amphibians (Rollins-Smith et al. 2002; Kilpatrick *et al*. 2010; Bletz *et al.* 2013; Kueneman *et al*. 2016; Gervasi *et al*. 2017). A comparative study by Gervasi et al. (2017) tested multiple species of true frogs (Ranidae species), treefrogs (family Hylidae which include *P. regilla*), and toads and found Ranidae host species generally had lower *Bd* infection levels compared to those of the more terrestrial families of treefrogs and toads. Traits of a more aquatic lifestyle include smooth skin, less ventral skin vascularization, and a diverse mucosal skin microbiota (Rollins-Smith *et al*. 2002; Wells 2007; Vitt and Caldwell 2009). Terrestrial frogs however, such as treefrogs (*P. regilla*) and toads, are generally more susceptible to *Bd* infection (Carey *et al*. 2006; Searle *et al*. 2011; Gahl *et al.* 2012; Reeder *et al*. 2012; Ellison *et al*. 2015; Gervasi *et al*. 2017). One factor may be a key terrestrial amphibian trait such as granular ventral skin, which is highly vascularized, and functions to quickly and efficiently absorb water (Wells 2007; Vitt and Caldwell 2009).

Although *P. regilla* is known to be a tolerant host species, harboring high levels of and even amplifying *Bd* infection (Searle *et al*. 2011), our results show there are limits to tolerance. For adult *P. regilla* in our study, the higher infection intensity they carried compared to juveniles may have contributed to their increased mortality.

Adverse infection outcomes (e.g. increased mortality, higher infection levels) may also occur due to excessive immune responses (Lochmiller and Deerenberg 2000). Costly immune responses can stem from the resource intensive process of response induction or the self-harming pathological effects of immune response (Graham *et al*. 2009). Several lab and field studies have highlighted the costs of immunity. Strong immune responses to infection have been linked to higher mortality during migration (Hanssen *et al.* 2004), reduced reproductive success (Bonneaud *et al*. 2003; Uller *et al.* 2006), lowered growth (Uller *et al*. 2006) and impaired metabolic function in a variety of animals (Demas *et al*. 1997). Some amphibian-*Bd* research has shown circumstances where hosts do clear *Bd* infection yet experience sublethal and/or lethal effects (Bielby *et al*. 2015), this outcome is posited to be due to large and costly immune responses (Ellison et al. 2015). In this study, *R. aurora* juvenile and adults both reduced *Bd* infection levels however juveniles had higher initial infection intensity that may have taxed their underdeveloped immune system as there was a trend for more juvenile mortality.

Lastly, we did not detect transmission heterogeneity in our study. Although there was age-related initial infection heterogeneity, it did not predict transmission patterns to recipients in either species. All juvenile recipients had species-specific infection intensity regardless of donor age and degree of initial donor infection level. *P. regilla* recipients, had high infection intensity and no death from any donor treatment whereas *R. aurora*

recipients experience lower survival when paired with adult donors and uninfected adult control hosts. *R. aurora* recipients with juvenile donors did not experience significant mortality suggesting the effect of agonistic interactions from older, and thus larger, conspecifics is more fatal than exposure to a *Bd*-infected juvenile donor. In the field, encounters between juvenile and adult frogs pose a significant predation risk for smaller individuals, especially in Ranidae species (Wells 2007).

In conclusion, our results show infection heterogeneity exists due to host age in two amphibian species. We also show that infection intensity does not necessarily predict between-host transmission. We extend previous research elucidating variation in *Bd* susceptibility between multiple host species (Gahl *et al*., 2010; Searle *et al*., 2011; Venesky *et al*. 2011; Bielby *et al*. 2015; Gervasi *et al*., 2013) and show species differences can strongly influence infection dynamics (e.g. initial infection intensity, gain/loss of infection intensity, survival), but reveal these dynamics can change with age and may be due to differences in species lifestyle. We found *Bd* tolerance in *P. regilla* decreases with age, suggesting only larval and younger adult life stages might serve as community reservoir hosts. In another species, *R. aurora*, we showed host age was associated with lower risk of mortality and that agonistic interactions have a strong effect on survival as juvenile *R. aurora* recipients experienced higher mortality from adult conspecifics, regardless of infection status, than juvenile conspecific donors.

Differential disease susceptibility among life stages can alter natural demographic relationships between individuals of different age classes (Brunner *et al*. 2004; Rachowicz and Vredenburg 2004). Amphibian populations can fluctuate greatly and are largely dependent on viability of individuals in post-metamorphic age classes to

reproduce (Biek *et al*. 2002; Vonesh and De La Cruz 2002), thus increased *Bd* susceptibility in adult amphibians can significantly affect long-term population survival. Our results show host age can be a factor underlying some aspects of infection heterogeneity such as initial infection intensity and survival. Future research is needed to explore trade-offs involving amphibian *Bd* resistance and immune function with age.

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Figures

Figure 4.1. Distribution of initial infection intensity (*Bd* genome equivalents/ng DNA) of adult and juvenile donors of A) *P. regilla* and B) *R. aurora*.

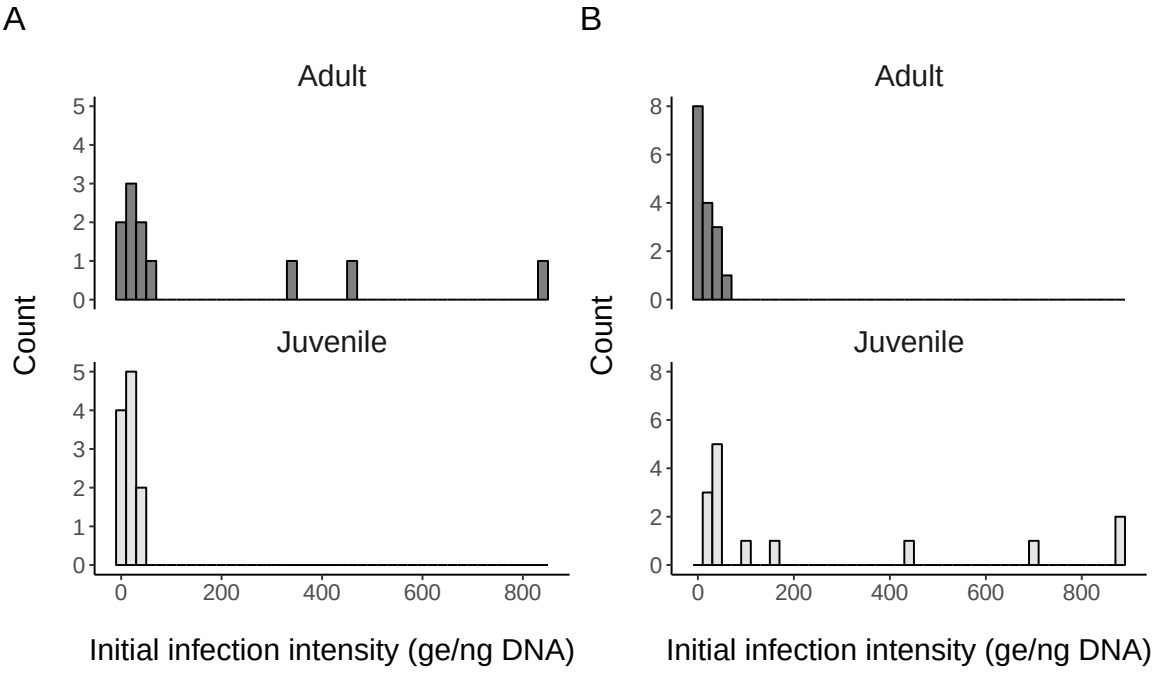


Figure 4.2. Direction and magnitude of change in infection intensity (gain or loss) of donors, calculated as the difference between the ending and initial infection intensity for A) *P. regilla* and B) *R. aurora*.

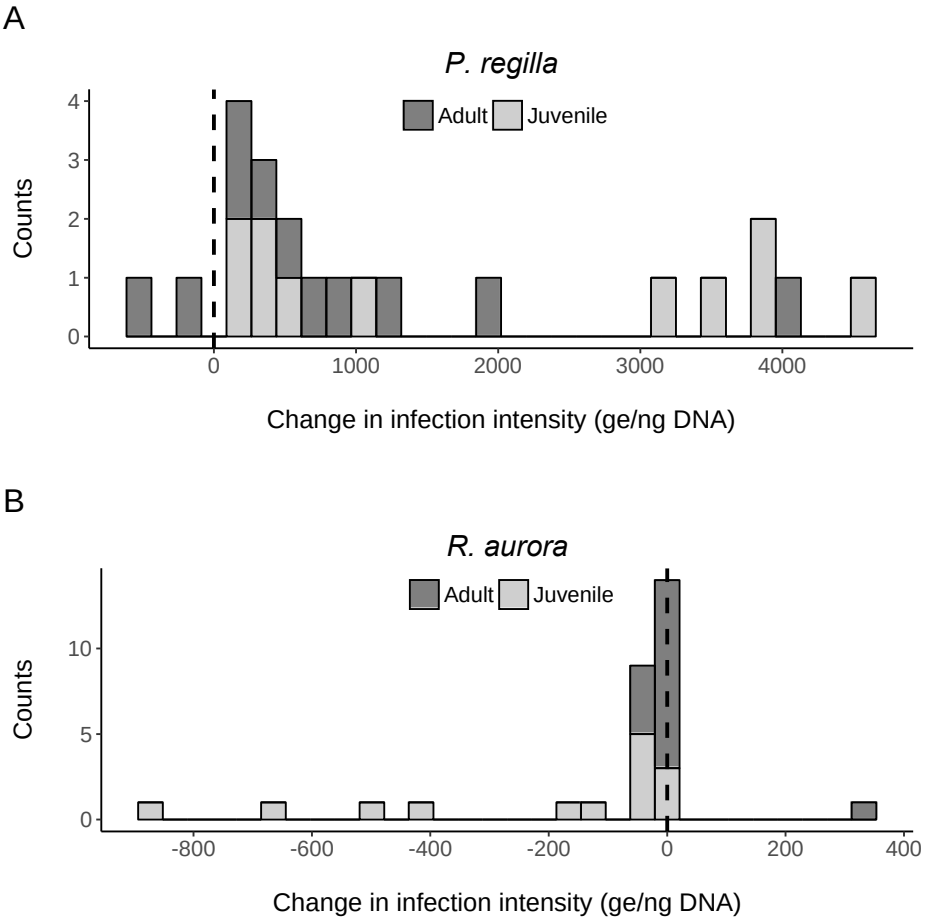


Figure 4.3. Change in infection intensity of donors through time. To see infection change lines of only hosts that died during the experiment see Supplementary Figure B1.

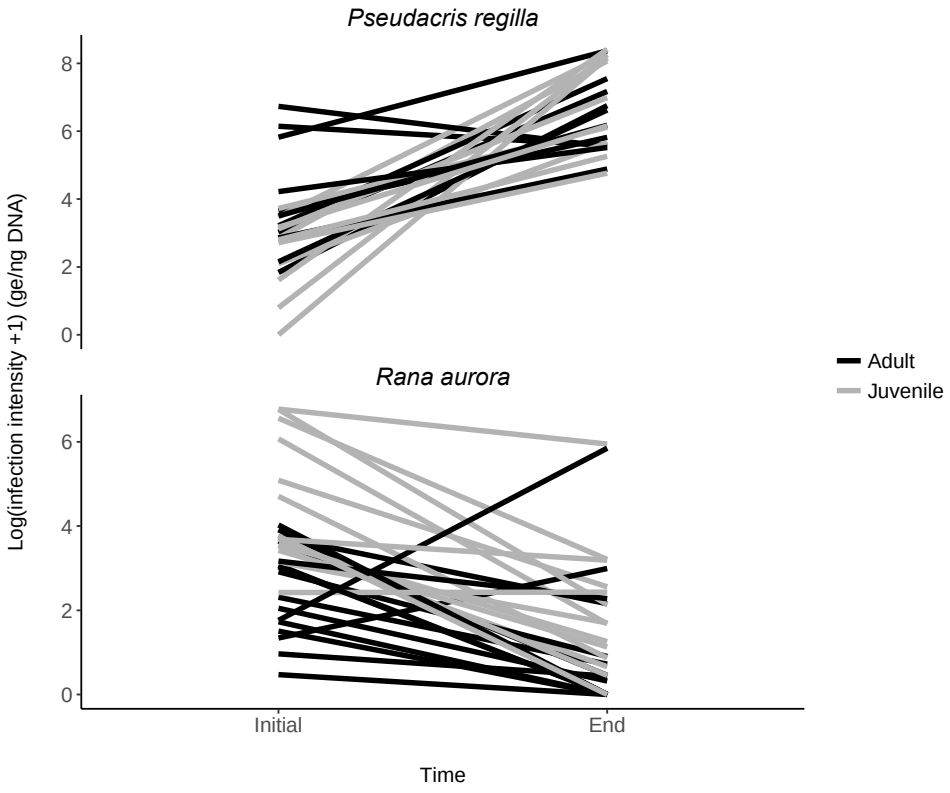


Figure 4.4. Distribution of infection intensity for recipients exposed to donors (pooled adult and juvenile donor groups).

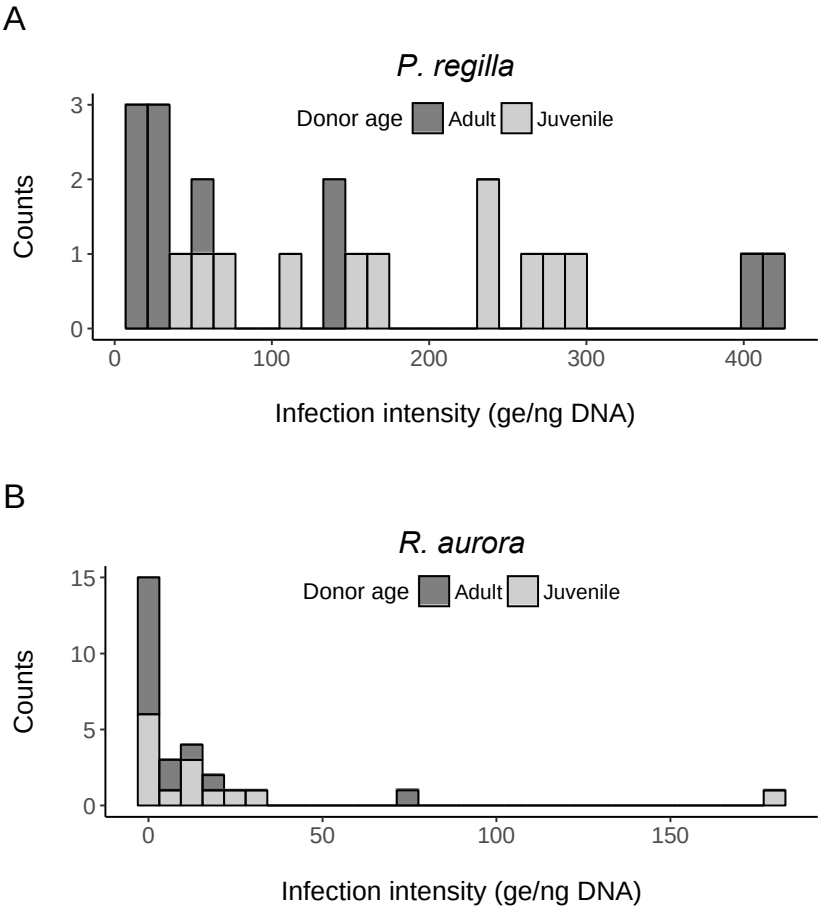


Figure 4.5. Kaplan-Meier survival curves for adult and juvenile donors of A) *P. regilla* and B) *R. aurora*.

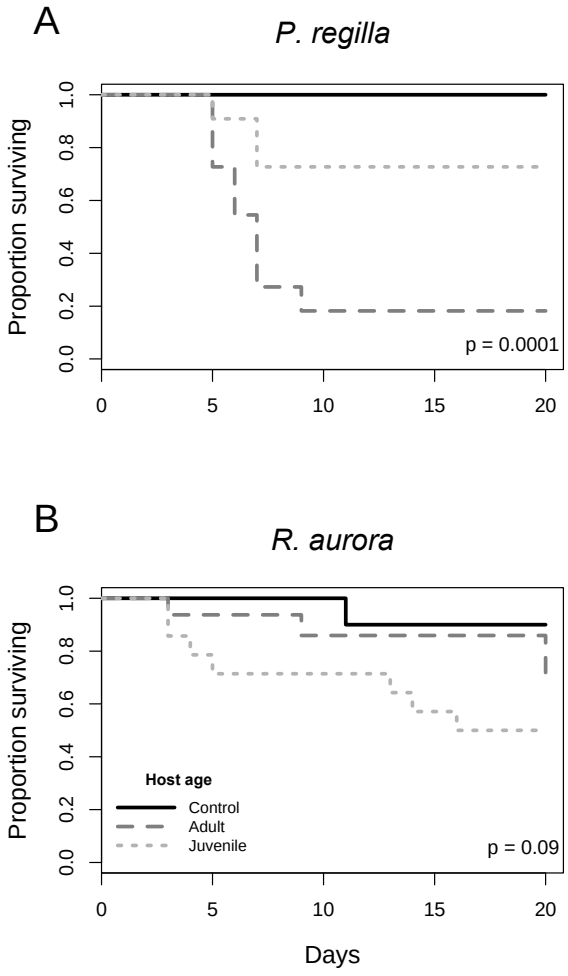
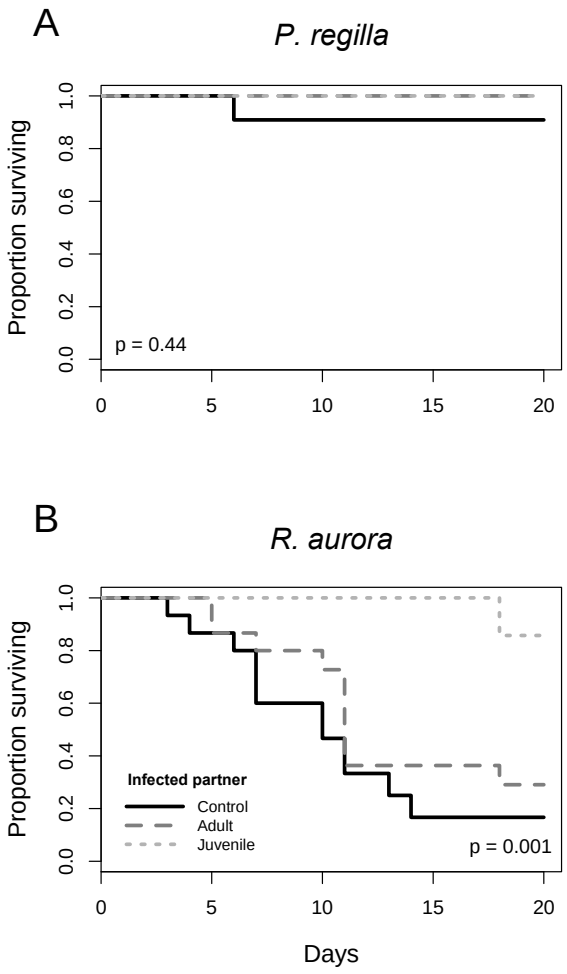


Figure 4.6. Kaplan-Meier survival curves for recipients paired with adult and juvenile donors of A) *P. regilla* and B) *R. aurora*. *R. aurora* juvenile predation by adult conspecifics was observed in three Control group individuals and two individuals in the adult donor group.



Tables

Table 4.1. Experimental design of donor-recipient transmission treatments with age of donor for both host species, *P. regilla* and *R. aurora*. Each treatment group replicate had a donor and recipient pair. Recipients in all treatment groups were always naïve conspecific juveniles.

Host species	*Bd* Treatment	Donor age	N
P. regilla	Infected adult	Adult	11
	Infected juvenile	Juvenile	11
	Uninfected control	Adult	11
R. aurora	Infected adult	Adult	16
	Infected juvenile	Juvenile	14
	Uninfected control	Adult	15

Table 4.2. Summary statistics for initial infection intensity (*Bd* genome equivalents/ng DNA) of donors. Parasite aggregation increases with higher variance-to-mean ratios and lower k values.

Host species	N	Mean	Median	Variance-to-mean ratio	Corrected parameter k
P. regilla					
Adult	11	168.3	32.19	434.28	0.30
Juvenile	11	15.85	15.46	10.12	1.64
R. aurora					
Adult	16	18.57	13.28	17.41	1.07
Juvenile	14	243.4	41.04	451.8	0.47

Table 4.3. Infection prevalence of juvenile recipients paired with conspecific adult and juvenile donors.

Host species	Adult donor		Juvenile donor	
	N	N infection detected (%)	N	N infection detected (%)
P. regilla	11	11 (100)	11	11 (100)
R. aurora	14	8 (57.1)	14	11 (78.6)

CHAPTER 6 – GENERAL CONCLUSIONS

Trang D. Dang and Andrew R. Blaustein

The outcome of an infection is a dynamic variable and is strongly dependent on host and pathogen characteristics. The aquatic chytrid fungus, *Batrachochytrium dendrobatidis* (*Bd*), has a global distribution, can infect a wide range of amphibian host species and is linked to worldwide amphibian population declines making it a pressing and unique emerging disease system to study host-pathogen dynamics (Olson *et al.*, 2013; James *et al.*, 2015). Various host-pathogen factors can alter infection outcomes including the broad range of *Bd* susceptibility among host species and life stage, wide phenotypic and genetic diversity among *Bd* strains, and within-host pathogen-pathogen interactions under coinfection conditions (Van Rooij *et al.*, 2015). To increase our understanding of this dynamic disease system, my dissertation utilized manipulative experiments to determine how key host and pathogen characteristics affected chytridiomycosis outcomes.

Bd lineage evolution is hypothesized to be strongly influenced by local selection pressures (Retallick and Miera, 2007; Doddington *et al.*, 2013; Rosenblum *et al.*, 2013), thus movement by local amphibians, nonnative amphibians via the wildlife trade, or non-amphibian vectors can likely introduce novel strains to a new host population (Fisher and Garner, 2007; Schloegel *et al.*, 2009, 2012; Burrowes and De la Riva, 2017). Multifactorial experiments comparing multiple pathogen strains and host species are needed to better capture *in vivo* virulence variation. In Chapter 2, I experimentally examined virulence variation in multiple pathogen strains within a reportedly hyper-virulent clade and multiple host species with varying susceptibilities. This study captured virulence variation dependent on both major predictors, host and strain type. Much of our amphibian-*Bd* infection knowledge is drawn from studies testing a single host species and

Bd strain. By conducting comparative experiments in multiple host species and pathogen strains I demonstrated differential disease outcomes for a known sensitive amphibian species, *Anaxyrus boreas*, showing that while exposure to some *Bd* strains resulted in significant mortality and infection load, exposure to other strains did not. Individuals of the other host species, *Pseudacris regilla* and *Rana cascadae*, did not experience altered survival or pathogen load differences with any *Bd* strain tested.

In Chapter 3, I investigated how *Bd* infection changes under a simultaneous coinfection with another dermis-targeting pathogen, the water mold *Saprolegnia ferax* (*Sf*). I tested this dynamic in three amphibian host species (*P. regilla, A. boreas and Rana pretiosa*). Though I found *Bd* infection intensity was higher in *A. boreas* and *R. pretiosa* hosts exposed to both *Bd* and *Sf*, the difference was not statistically significant compared to hosts exposed to only *Bd*. Lower *Bd* infection intensity was associated with higher host weight in *P. regilla* and *R. pretiosa* but not *A. boreas*. *R. pretiosa* and *A. boreas* hosts that survived longer also carried lower Bd intensity. Host survival was lower in *R. pretiosa* hosts exposed to *Bd* only compared to the coinfected group. This result is noteworthy since most *R. pretiosa-Bd* research is conducted on adult hosts and larvae are not thought to experience severe *Bd* infections (Pearl *et al.*, 2009). These findings indicate this species is susceptible to *Bd* in the larval stage and we emphasize future species conservation efforts incorporate *Bd* exposure risk.

In Chapter 4, I experimentally examined age-dependent *Bd* infection heterogeneity between two host life stages (in 'donor' hosts) and also tested whether this affected subsequent transmission to a naïve conspecific host ('recipient'). Age-related *Bd* dynamics were assessed in postmetamorphic donors of two species, *P. regilla* and *R.*

aurora. I showed *Bd* infection susceptibility varies by host age and is not consistent across host species. In *P. regilla*, juvenile donors had lower infection intensity compared to adult donors; however, the opposite trend was found in *R. aurora*. Additionally, I show differential infection intensity due to host age does not predict pathogen transmission in a subsequent conspecific host. All naïve conspecific hosts had comparable infection levels regardless of initial donor age and *Bd* intensity level. These results are striking because older postmetamorphic hosts are believed to be more robust to *Bd* than younger hosts due to higher energetic resources and a mature immune system (Rollins-Smith, 1998; Kriger and Hero, 2006; Ortiz-Santaliestra *et al.*, 2013; Abu Bakar *et al.*, 2016). While this may be the case for *R. aurora*, these results are surprising for *P. regilla*, a putative reservoir host known to be highly tolerant to *Bd* infection (Reeder *et al.*, 2012; Gervasi *et al.*, 2013). These findings show amphibian-*Bd* infection outcomes can differ among postmetamorphic life stages in unpredicted ways. Future research in host species differences in skin architecture and immune development may explain some of the processes behind these observed patterns.

Disease biology is inherently complex due to the dynamic relationship of hosts and pathogens. By conducting manipulative experiments, I provide insight into how host-pathogen factors mediate *Bd* infection outcomes. In all chapters, I used multiple host species and found varying *Bd* infection outcomes among host species. This supports previous research showing host species differences and broadens the scope of each study by demonstrating host species-dependent disease variation. One surprising finding is the increased sensitivity to *Bd* infection in adult *P. regilla* compared to juvenile and larval host stages. I tested *Bd* susceptibility in juvenile and adult *P. regilla* in Chapter 4 and

larval *P. regilla* in Chapters 2 and 3 using the same *Bd* strain. Larval and postmetamorphic juvenile *P. regilla* were consistently tolerant to *Bd* infection, harboring high *Bd* infection levels without experiencing significant mortality. This tolerance was not apparent adult hosts (Chapter 4). My research suggests that the designation of *P. regilla* as a reservoir host may only be applicable to the larval and juvenile stages or that some populations of this species may differ significantly in susceptibility at different life stages. These results warrant further study to determine what physiological changes occur with age to make some hosts gain and others lose *Bd* susceptibility. Dependence on water may predict adult amphibian *Bd* susceptibility as this is linked to changes in skin architecture and mucosal properties that might mediate *Bd* colonization (Vitt and Caldwell, 2009). I also found the susceptibility of *A. boreas,* a sensitive western species, varies with *Bd* strain. This finding may be useful in *Bd* immunization efforts. Vaccination studies have shown prior infection may immunize hosts in some instances (Ramsey *et al.*, 2010; McMahon *et al.*, 2014) but not others (Stice and Briggs, 2010; Cashins *et al.*, 2013). Use of a hypovirulent strain may be more effective than using heat-treated zoospores and safer than exposing frogs to *Bd* and then clearing infection with itraconazole (Venesky *et al.*, 2013).

The experiments presented in my dissertation show how mutable chytridiomycosis virulence can be under variable host-pathogen conditions. This work advances our understanding of this critical disease system by providing experimental evidence amphibian-*Bd* infection outcomes are highly context-dependent and that disease virulence is an emergent quality and cannot be solely predicted from host or pathogen attributes alone. Moving forward, experimental chytridiomycosis research that

incorporates multiple *Bd* strains, host species, and environmental conditions is needed to better understand and predict the effect of this emerging disease on amphibian populations.

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APPENDICES

Appendix A – Chapter 3 Supplementary Table

Table A1. Egg collection locations for host species used in this study.

Host species	Latitude	Longitude	Elevation (ft)
P. regilla	44°31’19.37” N	122°01’54.12” W	3652
A. boreas	44°06’02.7” N	121°37’39” W	8536
R. pretiosa	43°52’17.16” N	121°26’44.43” W	4160

Appendix B – Chapter 4 Supplementary Figures and Tables

Table B1. Initial mass (g) and SVL (mm) of donor hosts exposed to *Bd*.

Species	Age	Mass (g)	SVL (mm)
P. regilla	Adult	0.734	21.3
		1.101	22.5
		1.248	24.9
		1.414	23.5
		1.567	26.2
		1.602	26.4
		1.683	27.3
		1.809	27.3
		1.934	28.2
		2.126	27
		2.228	29.5
	Juvenile	0.356	15.4
		0.379	16.7
		0.426	17.6
		0.461	17.4
		0.48	19.2
		0.514	18.3
		0.522	18.2
		0.538	18.5
		0.544	18.6
		0.56	19.5
		0.568	19.5
R. aurora	Adult	1.839	26.7
		1.856	28.5
		1.956	27.3
		2.981	31.5
		3.106	30.3
		3.158	31
		3.17	34.4
		3.419	32.8
		3.517	34.5
		3.543	32.1
		4.032	36.2
		4.035	35.5
		5.099	36.9
		5.523	38.6
		5.553	38.1
		5.736	37.8
	Juvenile	0.287	15.4

Species	Age	Mass (g)	SVL (mm)
R. aurora	Juvenile (*continued)*	0.321	15.9
		0.334	15.2
		0.35	16.6
		0.357	16.3
		0.395	18.1
		0.412	17.1
		0.423	17.5
		0.599	18.3
		0.626	20.2
		0.655	20.1
		0.691	20.7
		0.723	19.7
		0.847	20.3

Table B2. Ratio of change (end/initial infection intensity) in donor infection intensity (*Bd* genome equivalents/ng DNA). A ratio of < 1 signals a decrease, while > 1 signals an increase in infection intensity; a value of 1 means infection level was neither lowered nor raised and a zero value represents individuals that cleared their infection or reduced infection to undetectable levels.

Host species	Host age	
	Adult	Juvenile
P. regilla	0.304	8.269
	0.569	11.261
	3.711	12.419
	8.031	20.626
	8.980	40.420
	12.791	50.133
	14.955	124.803
	67.049	196.194
	79.675	1,119.656
	99.019	3,152.110
	162.967	70,117.468
R. aurora	0.000	0.000
	0.000	0.009
	0.000	0.010
	0.000	0.013
	0.000	0.014
	0.000	0.028
	0.000	0.034
	0.016	0.071
	0.056	0.073
	0.084	0.074
	0.117	0.201
	0.185	0.436
	0.340	0.600
	0.384	1.011
	6.694	
	71.820	

Figure B1. Change in infection intensity of donors that died during the experiment.

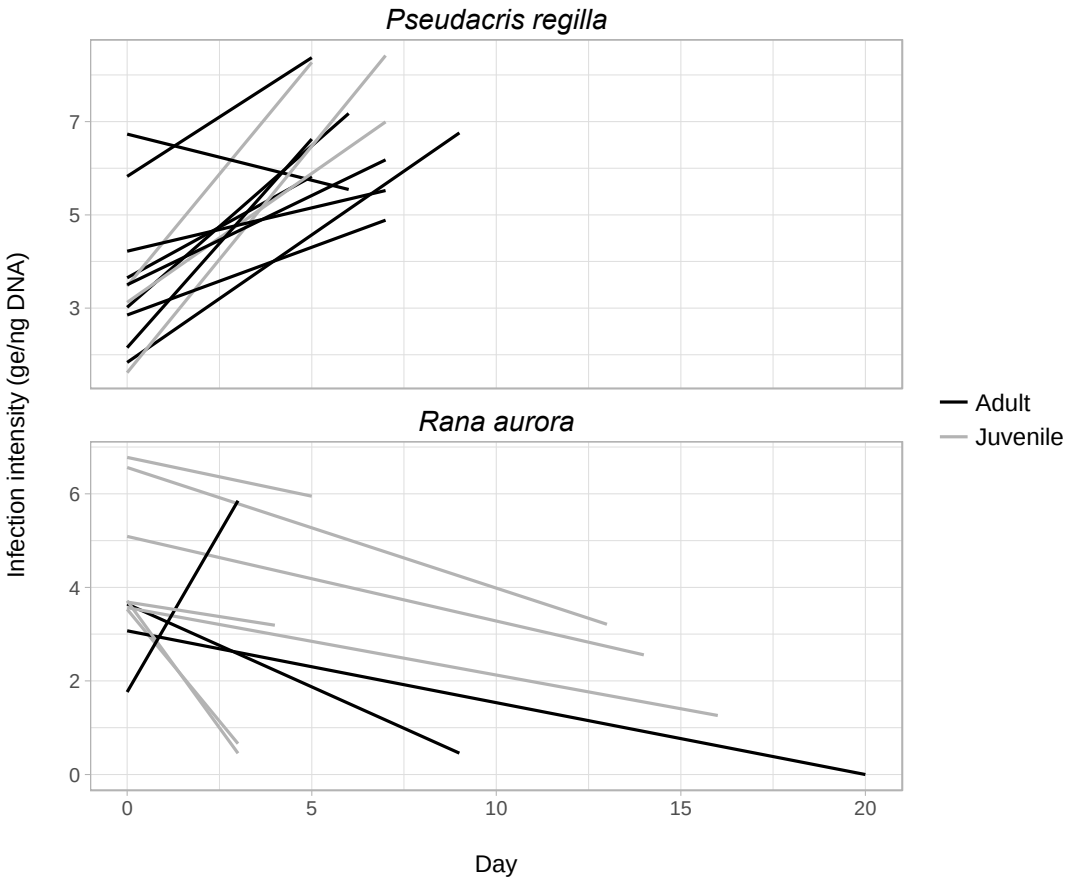

Figure B2. Relationship between log-transformed donor initial infection intensity and log-transformed recipient infection intensity of A) *P. regilla* and B) *R. aurora*. Dark gray circles represent recipients paired with adult donors while light gray circles represent recipients paired with juvenile donors.