AN ABSTRACT OF THE DISSERTATION OF

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Abstract approved:

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Andrew R. Blaustein

The earth is undergoing a "biodiversity crisis" characterized by loss of populations, species, genetic diversity, and ecosystem services. Part of this crisis consists of population declines, extinctions, and increased incidence of deformities in amphibians. It is unknown whether deformities contribute to these declines. Many cases of population declines in amphibians are associated with infectious disease. Water molds (Oomycota: Oomycetes: Saprolegniales) (e.g. *Achlya* and *Saprolegnia*) may contribute to amphibian population declines whereas parasitic flatworms (*Ribeiroia*) appear to be major causes of amphibian deformities. I found that *Saprolegnia* can kill larval and newly-metamorphosed juvenile amphibians. In addition, I investigated synergistic effects of *Saprolegnia* and the environmental contaminant nitrate on survival of amphibian larvae. No synergisms were found. However, there was a less-than-additive interaction affecting one frog species. *Saprolegnia* killed *Rana aurora* (red-legged frog) larvae, but only when nitrate was not added, consistent with nitrate preventing *Saprolegnia* from killing *R. aurora*. I also tested for possible interactions among the pathogenic water mold *A. flagellata*, the pathogenic fungus *Batrachochytrium dendrobatidis* (BD), and the parasitic

trematode *Ribeiroia*. No between-pathogen interactions were found, however, there were main effects of *Ribeiroia* and BD. *Ribeiroia* caused mortality and increased frequency of limb deformities. There were differences in the composition of deformity types between my study and previous work, suggesting that the relative frequencies of different deformity types produced by *Ribeiroia* may be context-dependent. Also, there was an overall effect of delayed development in *Ribeiroia*-exposed individuals. In addition, individuals that did not die or display limb deformities following *Ribeiroia* exposure had slower development than controls not exposed to *Ribeiroia*. Delayed development may contribute to the effects of *Ribeiroia* on amphibian populations. In contrast, BD sped up development, although there was no evidence of BD infection. These results are consistent with amphibian larvae responding to the presence of BD by increasing the rate of development. Hastened metamorphosis and dispersal from larval habitats may decrease the risk of BD infection or reduce BD infection load. Mortality of post-embryonic life stages from water molds and sublethal effects of *Ribeiroia* and BD on amphibian development may influence how these pathogens interact with amphibian populations.

The Effects of Multiple Pathogens on Amphibians in the Pacific Northwest

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

John M. Romansic, Author

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The Effects of Multiple Pathogens on Amphibians in the Pacific Northwest

Chapter 1. General Introduction

1.1 The "Biodiversity Crisis" and Infectious Diseases

Estimates of the number of species range from 5 million to greater than 50 million (May, 1988). Mass extinctions have occurred at least five times in our planet's history (Jablonski, 1991). However, it is clear that the current rate of species extinctions is far greater than in any of the other mass extinction events (Myers, 1993). Myers (1993) estimated that 50% or more of the species inhabiting earth would be lost to extinction by the end of the 21st century. Species extinctions, though, are only one component of the biodiversity crisis. Genetic diversity is declining in some species (e.g. Garner *et al.*, 2005, Gustafson, *et al.*, 2007) and ecosystem function, including essential ecosystem services, are in decline (Vitousek, 1997, Dobson *et al.*, 2006).

Habitat destruction by humans is the most important cause of this "biodiversity crisis", but over-harvesting, pollution and introduction of non-native species by humans also contribute (Vitousek, 1997, Singh, 2002). Increases in ultraviolet-B radiation due to stratospheric ozone depletion may also contribute (Cockell & Blaustein, 2001), and anthropogenic climate change is predicted to cause widespread species extinctions and range reductions (Thomas *et al.*, 2004, Lawler *et al.*, 2006). Emerging infectious diseases are also a significant threat to biodiversity (Daszak et *al.*, 2000, Harvell *et al.*, 2002, Altizer *et al.*, 2003, Scholthof, 2003). However, the degree to which infectious diseases have caused biodiversity loss and threaten remaining biodiversity is highly

unknown (Smith *et al.*, 2006). It is crucial that we learn more about infectious diseases and how they may be controlled so that they do not thwart management actions such as habitat conservation and restoration.

1.2 The Potential Role of Pathogens in Amphibian Population Declines

As part of worldwide losses in biodiversity, many amphibian populations are declining and disappearing (Houlahan *et al.,* 2000, Stuart *et al.,* 2004). The same factors discussed above appear to be involved in causing these declines, including habitat destruction (Dodd & Smith, 2003), introduced species (Kiesecker & Blaustein 1998, Kiesecker *et al.,* 2001a, Kats & Ferrer, 2003, Vredenburg, 2004, Knapp *et al*., 2007), global climate change (Pounds & Crump, 1994, Pounds *et al.,* 1999), pollution (Beebee *et al.,* 1990), ultraviolet-B (UV-B) radiation (Blaustein *et al.,* 1994a, 1998), and infectious diseases (Daszak *et al.*, 1999, 2003).

Only a few pathogens have received a considerable amount of attention as possible contributors to amphibian population declines. These include viruses, the pathogenic fungus *Batrachochytrium dendrobatidis* (BD), and water molds of the genus *Saprolegnia*.

1.3 *Batrachochytrium dendrobatidis*

BD has been associated with population declines in amphibians in several locales around the world, including areas where large numbers of dead post-metamorphic individuals were found (Berger *et al.*, 1998, Longcore *et al.*, 1999, Bosch & Martinez-Solano, 2001, Bradley *et al.*, 2002, Lips *et al.*, 2006, Schloegel *et al.*, 2006, Rachowicz *et al.*, 2006, Bosch *et al.*, 2007). Depending upon the species and life stage, BD can kill

amphibians and may cause sublethal effects (Berger *et al.*, 1998, Parris, 2004, Parris & Baud, 2004, Parris & Beaudoin, 2004, Parris & Cornelius, 2004, Blaustein *et al.*, 2005). Complex interactions between BD and other factors may occur. For example, sublethal effects of BD on frog larvae may be diminished by copper contamination (Parris & Baud, 2004) and intensified by predators (Parris & Beaudoin, 2004) and competitors (Parris & Cornelius, 2004). The susceptibility of some frog species to BD decreases at temperatures close to 30º C, the thermal maximum of the fungus (Woodhams *et al.*, 2003, Berger *et al.*, 2004, Piotrowski *et al.*, 2004). Associations between BD-correlated population declines and climatic warming have been reported for *Atelopus* frogs in Central and South America and *Salamandra salamandra* (fire salamanders), *Alytes obstetricans* (midwife toads), and *Bufo bufo* (common toads) in Spain (Pounds *et al.*, 2006, Bosch *et al.,* 2007).

Skerratt *et al.* (2007) claim that BD is by far the most likely cause of most amphibian population declines not due to obvious causes such as habitat destruction. However, the strength of evidence for BD as a cause of widespread amphibian declines is questionable (McCallum, 2005). Even if BD has caused widespread losses in amphibian biodiversity, there are some amphibian populations that persist with BD infection in the host population without obvious adverse effects (Retallick, *et al.*, 2004, Daszak *et al.*, 2005, Kriger & Hero, 2006, 2007, Briggs *et al.*, 2005). These populations could be negatively affected by other threats, including other pathogens besides BD. Thus, these other pathogens should not be ignored as possible contributors to amphibian population declines.

1.4 Ranaviruses

Ranaviruses in the family Iridoviridae are associated with massive mortality in frogs and salamanders (Jancovich *et al.*, 1997, Bollinger *et al.*, 1999, Daszak *et al.*, 2000, 2003, Green *et al.*, 2002, Docherty *et al.*, 2003, Brunner *et al.*, 2004, Greer *et al.*, 2005, Cunningham *et al.*, 2006, Fox *et al.*, 2006). Association of these viruses with population-level declines in amphibians has not yet been reported. However, these viruses have the potential to contribute to amphibian population declines (Daszak *et al.*, 2003).

Ambystoma tigrinum virus (ATV), a ranavirus, infects salamanders and is associated with mass mortality in *Ambystoma tigrinum* (tiger salamander) larvae (Jancovich *et al.*, 1997, Jancovich *et al.*, 2001, Brunner *et al.*, 2004, Collins *et al.*, 2004). In laboratory experiments, ATV has caused mortality, reduced growth, and increased activity in *A. tigrinum* larvae (Parris *et al.*, 2004, 2005, Brunner *et al.*, 2005). Infection load and ATV-induced mortality in *A. tigrinum* larvae increased with decreased temperature (Rojas *et al.*, 2005). ATV infection caused an upward shift in thermal preference of *A. tigrinum* larvae, but temperatures close to the thermal growth maximum of the virus (31º C) were lethal to larvae of this species (Jancovich, 1999, Parris *et al.*, 2004). The herbicide atrazine decreased susceptibility to ATV infection and ATV-induced mortality in *A. macrodactylum* (long-toed salamander) larvae, but reduced peripheral leucocyte levels and increased susceptibility to ATV infection in *A. tigrinum* larvae (Forson & Storfer, 2006a,b). Phylogenetic patterns in ATV infecting *A. tigrinum* in western North America are consistent with introduction of non-native eastern North

American ATV genotypes via use of eastern *A. tigrinum* as fish bait in the west (Jancovich *et al.*, 2005).

1.5 Water Molds (Saprolegniales)

Water molds (Oomycota: Oomycetes: Saprolegniales) are fungus-like organisms found in aquatic systems and moist soils (Dick, 1990). Most aquatic water molds are saprobic and some water molds, including aquatic species, are parasitic (Dick 1990, Johnson *et al.*, 2002a). Water molds infect a variety of organisms, including insects (MacGregor, 1921, Kerwin & Washino, 1988), turtles (Tiffney, 1936), fish (Walpole & Huxley 1882), and amphibians (Blaustein *et al.,* 1994b). Water mold infection is associated with mass mortality in wild fish (Walpole & Huxley 1882, Schaefer *et al.,* 1981, Johansson *et al.,* 1982, Khulbe, 1992, Bisht *et. al.*, 1996) and fish in hatcheries (Tiffney & Wolf, 1939, Leaño, *et al.*, 1999).

Infection of amphibians has been documented in a few water mold species, including *Achlya flagellata*, *S. ferax, S. parasitica* and an *Aphanomyces*. (Tiffney & Wolf, 1939, Blaustein *et al.*, 1994b, Berger *et al.*, 2001, Lefcort *et al.*, 1997). However, many other species of water molds may also infect amphibians. *Achlya glomerata, A. racemosa, Pythiopsis humphreyana, S. diclina, S. megasperma,* and *S. monoica*, have been found on dead amphibian embryos (Green & Converse, 2005, Pilliod & Fronzuto, 2005), but infection of live amphibians has not been reported for these species. Water mold infections have been reported on amphibian embryos (Blaustein *et al.*, 1994b), larvae (Walls & Jaeger, 1987, Converse & Green, 2005), and post-metamorphs (Ford *et al.,* 2004). Koch's postulates (Pelczar & Reid, 1965) have been fulfilled for *S. ferax*

using *Bufo boreas* (western toad) embryos (Kiesecker *et al.,* 2001b). Multiple unidentified species of both *Achlya* and *Saprolegnia* have been found on dead embryos of *R. sylvatica* (wood frog) and *B. americanus* (American toad) in Massachusetts, USA (Touchon *et al.*, 2006, Gomez-Mestre *et al.*, 2006). Water mold cultures containing multiple species of both *Achlya* and *Saprolegnia* caused mortality, decreased time to hatching, decreased length and decreased developmental stage as hatchlings in embryos of *R. sylvatica* and *B. americanus* and in de-jellied embryos of *A. maculatum* (spotted salamander) (Touchon *et al.*, 2006, Gomez-Mestre *et al.*, 2006). In *R. sylvatica,* early hatching increased susceptibility to predatory notonectids (Gomez-Mestre *et al.*, 2006).

There is experimental evidence that invasion of water mold hyphae from adjacent embryos causes higher prevalence of infection and mortality than colonization from water mold zoospores (Green, 1999, Robinson & Griffiths, 2003). Green (1999) suggests that, in salamanders, oviposition of single embryos, in contrast to oviposition of embryo masses, may have evolved because of selection for avoidance of water mold infection. Green (1999) further suggests that selection for prevention of fungal infection may have contributed to the evolution of parental care of embryos in amphibians. In *B. boreas* and *R. cascadae*, communal oviposition increases embryo mortality, apparently because it increases the density of susceptible hosts (Kiesecker & Blaustein, 1997). Embryos of *Pseudacris regilla* (Pacific treefrog) are also susceptible to mortality from *S. ferax* (Kiesecker & Blaustein, 1995), but non-communal oviposition appears to protect the embryos of this species from high levels of mortality from this pathogen (Kiesecker & Blaustein, 1995).

There are only limited data on the effects of water molds on post-embryonic amphibians. There is experimental evidence that *Saprolegnia* influences competitive interactions between larvae of *R. cascadae* and *P. regilla* in the Oregon Cascades Range, apparently by mediating density of larvae through differential effects on embryos of the competing species (Kiesecker & Blaustein, 1999). Berger *et al.*, (2001) reported massive mortality of *Bufo marinus* (cane toad) larvae associated with *Aphanomyces* infection in Australia. *Saprolegnia* has been found on dead amphibian larvae after mass mortality events (Bragg & Bragg, 1958, Bragg, 1962). *Saprolegnia*-associated mortality of salamander larvae in experiments has been associated with bite wounds and silt (Walls & Jaeger, 1987, Lefcort *et al.*, 1997). In addition, *Saprolegnia* infection was associated with red-leg syndrome and death in captive *Xenopus laevis* (African clawed frog) adults that had undergone oocyte-harvesting surgery (Ford *et al.,* 2004).

Models suggest that mortality of post-embryonic life stages contributes significantly to population declines and may affect populations more than mortality of embryos (Vonesh & de la Cruz, 2002a,b, Biek *et al.,* 2002). Thus, to evaluate the importance of water molds in contributing to amphibian population declines, we need knowledge of how these pathogens affect post-embryonic amphibians.

Saprolegnia ferax and other water mold species may contribute to population declines in amphibians (Blaustein & Kiesecker, 2002, Daszak *et al.*, 2003). The widespread introduction of hatchery-raised fish, which carry water molds, to many freshwater aquatic habitats may have spread non-native pathogenic water molds and

water mold genotypes with higher virulence than native genotypes to many amphibian populations (Hatai *et al.*, 1990, Kiesecker et al., 2001b).

1.6 Other Pathogens

Other amphibian pathogens associated with morbidity or mortality of wild amphibians include various species of virus, bacteria, fungi, mesomycetozoa, trematodes, and copepods (e.g. Worthylake & Hovingh, 1989, Gruia-Gray & Desser, 1992, Bennati *et al.*, 1994, Cunningham *et al.*, 1996, Speare *et al.*, 1997, Green *et al.*, 2002, Beasley *et al.*, 2005). None of these pathogens have been linked to amphibian population declines.

1.7 Amphibian Malformations

In addition to population declines in amphibians, anatomical deformities in amphibians appear to be increasing. A low (0-5%) prevalence of deformities should be expected in amphibian populations and therefore is not by itself a reason for special concern (Johnson & Lunde, 2005). However, many relatively recent reports of amphibian deformities describe a higher prevalence (e.g. Flax & Borkin, 1997, Oullet *et al.*, 1997, Helgen *et al.*, 1998, Johnson *et al.*, 2002b). These reports have led to concern that the prevalence of deformities in amphibian populations has increased over historic levels (Wake, 1998).

There is experimental evidence that several agents can cause deformities in amphibians, including, BD (Berger *et al.*, 1998, Rachowicz & Vredenburg, 2004, Blaustein *et al.*, 2004), low pH (Pough & Wilson, 1977, Hermosilla-B *et al.*, 1992) environmental contaminants (Hayes *et al.,* 2002, 2003) and ultraviolet-B radiation (UV-B) (Worrest & Kimeldorf, 1976, Blaustein *et al.* 1997, Pahkala *et al.*, 2001). In addition,

Bridges *et al.* (2003) found that UV-B interacted synergistically with pond water from a site with high prevalence of deformities in northern leopard frogs (*Rana pipiens*) to cause deformities in *R. pipiens*. A trematode, *Ribeiroia,* may be playing a crucial role in the apparent increase in amphibian deformities. *Ribeiroia* appears to be a large contributor to the high prevalence of limb deformities observed in many amphibian populations in North America (Johnson *et al.*, Johnson *et al.*, 1999, 2001a,b, 2002b) and there is qualitative evidence that *Ribeiroia*-associated limb deformities have recently increased in western North America (Johnson *et al*., 2003). There is evidence consistent with eutrophication intensifying the effect of *Ribeiroia* on prevalence of limb deformities in frogs (Chase 2003a,b, Johnson & Chase, 2004, Johnson *et al.*, 2007). The relationship between amphibian deformities and population declines is unknown, but high numbers of deformities could potentially contribute to these declines (Johnson & Lunde 2005).

1.8 Stressor-Pathogen Synergisms

Many studies show that amphibians are affected by synergisms between environmental factors (e.g. Pahkala *et al.*, 2002, Hatch & Blaustein, 2003, Boone *et al.*, 2005), including synergisms between infectious agents and environmental stressors (e.g. Kiesecker & Blaustein, 1995, Taylor *et al.*, 1999, Kiesecker, 2002, Christin et al. 2003, Forson & Storfer, 2006a). Nitrate is an abiotic environmental factor that may be contributing to amphibian population declines (Rouse *et al*., 1999). Exposure to toxic levels of nitrate causes a variety of effects in amphibian larvae, including mortality, impaired feeding ability, decreased growth, slowed development, altered behavior, and methemoglobinemia (Huey & Beitinger 1980, Baker & Waights 1993, Watt & Oldham

1995, Marco et al. 1999, Hatch & Blaustein 2000, Hatch & Blaustein 2003). Nitrate can also interact synergistically with UV-B radiation to decrease growth or survival in amphibian larvae (Hatch & Blaustein 2003). Thus, exposure of amphibians to nitrate may result in sublethal effects that lead to an increase in susceptibility to pathogens such as water molds.

Predators are a biotic environmental factor with strong direct influence on amphibian populations (Knapp et al. 2007, Walston & Mullin, 2007) that may also interact with pathogens to affect amphibians. Lefcort & Eiger (1993) demonstrated that *R. catesbeiana* (bullfrog) larvae injected with dead bacteria displayed behavioral fever, reduced anti-predator behavior, and increased susceptibility to predation by newts. Lefcort & Blaustein (1995) found similar effects for *R. aurora* (red-legged frog) larvae exposed to the pathogenic yeast *Cryptococcus humicolis* (=*Candida humicola*). Predatory *Notophthalamus viridescens* (red-spotted newt) interacted with the fungal pathogen *Batrachochytrium dendrobatidis* to slow development in *Hyla chrysoscelis* (gray treefrog) larvae (Parris & Beaudoin, 2004). However, there is evidence that predators and pathogens can interact in a less-than-additive fashion. BD caused decreased activity, enhanced antipredator behavior, and increased survival in the presence of predators in *Rana pipiens* (northern leopard frog) larvae (Parris *et al.*, 2006).

Chemical, visual, and/or mechanical stimuli from predators (predator cues) can act as stressors that interact with environmental contaminants to affect amphibian larvae (Relyea & Mills, 2001, Relyea, 2003, 2004, 2005). Predator cues also appear to be stressors potent enough to directly kill amphibian larvae (Relyea, 2003). Therefore, it

seems possible that predator cues may stress amphibians enough to increase their susceptibility to pathogens. Because exposure of amphibians to predator cues may in many cases occur with high frequency and/or occur for extended periods, predator cues deserve investigation as an environmental factor that may strongly influence the impact of pathogens on amphibian populations.

1.9 Pathogen-Pathogen Interactions

Although much research demonstrates that environmental factors and pathogens may interact synergistically to affect amphibians, possible synergisms between pathogens have received little attention (but see Cunningham *et al.*, 1996, Johnson *et al.*, 1999). At the level of the individual host, two or more pathogens can interact in a synergistic, additive, or less-than-additive fashion (e.g. Read & Phifer 1959, Holmes 1960, Sousa 1993, 1994, Briggs, 1993, Chang *et al.*, 1994, Briggs & Latto 2001, Thacker *et al.*, 2001, Safi *et al.*, 2003, Hassan *et al.*, 2006, Traina-Dorge *et al.*, 2007). Synergistic effects of two pathogens on individual hosts may lead to synergism between the two pathogens at the level of the host population (e.g. Tristão-Sá *et al.*, 2002, Elias *et al.*, 2006, Pion *et al.*, 2006). Alternatively, competition between pathogens at the level of the host individual may reduce infection prevalence (Icenhour *et al.*, 2006).

Zoosporic pathogens have the potential for interspecific competition at the level of the individual host because zoospores may compete for colonization sites on the host. Alternatively, damage to the epidermis of the host by one zoosporic pathogen may facilitate invasion by a second zoosporic pathogen. If a co-infection with two species has become established, one pathogen may outcompete the other for host resources. Water

molds are possible candidates for outcompeting other pathogens, due to the wide range of substrates they can utilize and the possibility that they will digest and absorb a second pathogen along with host tissue. Thus, water molds have the potential to interact with the zoosporic fungus BD. In addition, infection by water molds may be aided by other kinds of pathogens that damage the epidermis of the host, such as trematodes. Other pathogens such as water molds and BD may enhance the effects of *Ribeiroia* on limb development by damaging the skin or weakening the host such that the shedding of metacercarial cysts is inhibited, thus leading to higher prevalence of deformities. Alternatively, water molds may attack encysting cercaria and thereby prevent them from causing deformities.

1.10 Organization of thesis

Each chapter addresses possible effects of water molds on post-embryonic amphibians. In Chapters 2 and 4, I investigated whether *Saprolegnia* can kill amphibian larvae. In Chapter 3, I tested whether *Saprolegnia* can kill newly-metamorphosed juvenile frogs. In Chapter 5, I tested for effects of *A. flagellata* on survival, oomycete infection status, BD infection load, time to metamorphosis, mass at metamorphosis, and prevalence of limb deformities in a single amphibian species.

In addition, Chapters 2 and 4 include tests for possible interactive effects of water molds and environmental stressors on amphibian larvae. Possible synergistic interactions between *Saprolegnia* and nitrate, an environmental contaminant, on larval amphibians were investigated in Chapter 2. A possible synergism between cues from predators and *Saprolegnia* was examined in Chapter 4. The primary focus of Chapter 5 was to test for between-pathogen interactions.

Chapter 2

Effects of nitrate and the pathogenic water mold *Saprolegnia* on survival of amphibian larvae

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2.1 Abstract

We tested for a synergism between nitrate and *Saprolegnia*, a pathogenic water mold, using larvae of 3 amphibian species: *Ambystoma gracile* (northwestern salamander), *Hyla regilla* (Pacific treefrog) and *Rana aurora* (red-legged frog). Each species was tested separately, using a 3 × 2 fully factorial experiment with 3 nitrate treatments (none, low and high) and 2 *Saprolegnia* treatments (*Saprolegnia* and control). Survival of *H. regilla* was not affected significantly by either experimental factor. In contrast, survival of *R. aurora* was affected by a less-than-additive interaction between *Saprolegnia* and nitrate. Survival of *R. aurora* was significantly lower in the *Saprolegnia* compared to the control treatment when nitrate was not added, but there was no significant difference in survival between *Saprolegnia* and control treatments in the low and high nitrate treatments, consistent with increased nitrate preventing *Saprolegnia* from causing mortality of *R. aurora*. Survival of *A. gracile* followed a similar pattern, but the difference between *Saprolegnia* and control treatments when nitrate was not added was not significant, nor was the nitrate × *Saprolegnia* interaction. Our study suggests that *Saprolegnia* can cause mortality in amphibian larvae, that there are interspecific differences in susceptibility and that the effects of *Saprolegnia* on amphibians are context-dependent.

2.2 Introduction

Infectious diseases can bring about population declines and local host extinctions, contributing significantly to the global "biodiversity crisis". Emerging infections involving novel pathogens or bringing established pathogens in contact with naïve host

populations often have particularly drastic effects on their hosts (Daszak *et al.*, 2000, Cleaveland *et al.*, 2002, Harvell *et al.*, 2002, Altizer *et al.*, 2003). In addition, pathogens capable of infecting multiple species may cause extinctions, as more resistant host species act as reservoirs of infection, preventing the demise of the pathogen along with its susceptible hosts (e.g. Gog *et al.*, 2002, McCallum & Dobson, 2002). Furthermore, the effects of pathogens on hosts are often context-dependent (e.g. Grossholz, 1992, Kiesecker & Skelly, 2001, Mitchell *et al.*, 2002). For example, both biotic and abiotic stressors may act synergistically with pathogens to increase the adverse effects on hosts. Thus, if both stressors and pathogens are present, the effects on hosts may be greater than if either factor acted alone (Guth *et al.*, 1977, Brown & Pascoe, 1989, Khan, 1990).

In several locations, amphibians may exemplify systems in which stressors and pathogens interact, thereby influencing population dynamics. The documentation of amphibian population declines (Alford & Richards, 1999, Houlahan *et al.*, 2000, Stuart *et al.*, 2004) describes a number of factors as contributing to these declines, including abiotic and biotic stressors and pathogens (Alford & Richards, 1999, Collins & Storfer, 2003, Semlitsch, 2003, Blaustein *et al.*, 2004). Clearly, causes for amphibian population declines appear to be context-dependent and complex, often with more than one factor involved (Blaustein & Kiesecker, 2002). It is also becoming clear that some amphibian populations have been severely impacted by 1 or more pathogens (Daszak *et al.*, 2003). It is likely that the effects of these pathogens are influenced by co-factors

(Taylor *et al.*, 1999, Blaustein & Kiesecker, 2002, Kiesecker, 2002, Christin *et al.*, 2003, Gendron *et al.*, 2003).

Saprolegnia, a water mold, is one important pathogen found in many amphibian populations (e.g. Strijbosch, 1979, Banks & Beebee, 1988, Blaustein *et al.*, 1994b). Furthermore, *Saprolegnia*-associated mortality appears to increase in the presence of abiotic stressors (Strijbosch, 1979, Banks & Beebee, 1988, Kiesecker & Blaustein, 1995, Kiesecker *et al.*, 2001c).

Saprolegnia (family Saprolegniaceae) is both saprobic and parasitic, obtaining nutrition from decaying organic matter or living hosts (Seymour, 1970). *Saprolegnia* infects a wide variety of organisms, including insects, turtles, fishes, and amphibians (MacGregor, 1921, Seymour, 1970). In amphibians, embryos and larvae can become infected (Bragg & Bragg, 1958, Walls & Jaeger, 1987, Blaustein *et al.*, 1994b). *Saprolegnia*-infected embryos of fishes and amphibians become covered with visible white hyphal filaments and usually do not hatch (Blaustein *et al.*, 1994b). Infection can spread via contact from growing hyphae (in the case of immobile hosts such as amphibian egg masses) or through colonization by free-swimming zoospores (Wood & Willoughby, 1986). Transmission can occur between species, for example, between fishes and amphibians (Kiesecker *et al.*, 2001b). Fishes and amphibians may also be infected by *Saprolegnia* via contact with infected soil (Kiesecker *et al.*, 2001b). Host species show strong interspecific variation in their susceptibility to infection (Richards & Pickering, 1978, Wood & Willoughby, 1986, Kiesecker & Blaustein, 1995, 1997). Factors such as water temperature, pH, pollution, exposure to UV-B radiation, injury

from biting, silt, and host behavior may modify the effects of *Saprolegnia* on its hosts (MacGregor, 1921, Strijbosch, 1979, Walls & Jaeger, 1987, Banks & Beebee, 1988, Carballo & Muñoz, 1991, Bly *et al.*, 1993, Pickering, 1994, Carballo *et al.*, 1995, Kiesecker & Blaustein, 1995, 1997, Lefcort *et al.*, 1997). *Saprolegnia* may influence community structure by altering competition between hosts (Kiesecker & Blaustein, 1999). *Saprolegnia* has a world-wide distribution (Wood & Willoughby, 1986, Blaustein *et al.*, 1994b, Kiesecker & Blaustein, 1997). This ubiquitous distribution is influenced by the widespread introduction of hatchery-raised fishes that carry and transmit *Saprolegnia* to other species (Blaustein *et al.*, 1994b, Kiesecker *et al.*, 2001b).

The purpose of this study was to examine amphibian larvae for susceptibility to mortality from *Saprolegnia* and to test for a possible synergism between *Saprolegnia* and nitrate, an important stressor on amphibian populations. Nitrate contamination is of global importance. In 2000, 87×10^6 metric tons of nitrogen were used as fertilizer in agriculture (Tilman *et al.*, 2001). Nitrogen fertilization and sewage from humans and livestock are major sources of nitrate (Steinheimer *et al.*, 1998, Tilman *et al.*, 2001). In the 1990s in the Willamette Basin of Oregon about 63,000 t nitrate were applied (Rinella & Janet, 1998). Ninety-eight percent of stream samples in the Willamette Basin contained detectable nitrate concentrations (0.054 to 22 mg l^{-1}) (Wentz *et al.*, 1998). Anthropogenic nitrate enters aquatic ecosystems via runoff, groundwater, and sewage discharge (e.g. Giblin & Gaines, 1990, Steinheimer *et al.*, 1998, van Lanen & Dijksma 1999, Zhilang *et al.*, 2003). There is a direct correlation between nitrate concentration and the proportion of the drainage area in agriculture. Nitrate concentrations vary

seasonally, with highest concentrations coinciding with the beginning of rainfall induced run-off in the autumn and early winter (Wentz *et al.*, 1998).

Anthropogenic nitrate contributes to eutrophication and can cause nitrate concentrations to reach toxic levels (OECD, 1982, Rouse *et al.*, 1999). Nitrate is toxic to a variety of organisms, including humans (Comly, 1945, Lee, 1970, Muir *et al.*, 1991, Camargo & Ward, 1995). There are well-documented effects on the susceptibility of amphibian species to nitrogenous fertilizers (e.g. Hecnar, 1995, Marco *et al.*, 1999, Rouse *et al.*, 1999, Hatch *et al.*, 2001). Effects of nitrate on amphibians include mortality, impaired feeding ability, reduced growth, slowed development, altered behavior, and methemoglobinemia (Huey & Beitinger, 1980, Baker & Waights, 1993, Watt & Oldham, 1995, Marco *et al.*, 1999, Hatch & Blaustein, 2000, Hatch & Blaustein 2003). Nitrate can also interact with UV-B radiation to decrease growth or survival (Hatch & Blaustein, 2003). Furthermore, nutrient enrichment from nitrogen-based fertilizers may alter community dynamics by increasing the abundance of herbivores, such as snails that are hosts for parasites linked to amphibian deformities (Johnson *et al.*, 2002, Chase, 2003a,b, Johnson & Chase, 2004).

2.3 Materials and Methods

Embryos of the northwestern salamander *Ambystoma gracile*, Pacific treefrog *Hyla regilla* and red-legged frog *Rana aurora* (hereafter, *Ambystoma*, *Hyla*, and *Rana*) were collected in 2002. *Ambystoma* and *Rana* were collected on 10 February and 17 March, respectively, at Coast Pond (approximately 20 km south of Waldport, Lincoln County, Oregon, USA). *Hyla* were collected on 10 March from a pond at Baker Beach

(approximately 10.5 km north of Florence, Lane County, Oregon, elevation about 12 m). Whole clutches of *Ambystoma* and *Hyla* were collected, while portions of *Rana* clutches were collected.

Amphibians were maintained and hatched in the laboratory at 13 to 17.5°C in tanks filled with dechlorinated tapwater treated with Novaqua® and Amquel® water conditioners. These conditioners do not remove nitrate or nitrite. Each species was kept separately. *Hyla* and *Rana* were reared in tanks containing about 30 l of water and *Ambystoma* were reared in tanks containing about 8 l of water. Approximate densities of larvae were 6.3, 2.1 to 3.1, and 12.5 individuals l^{-1} for *Hyla, Rana*, and *Ambystoma,* respectively. Frog larvae were fed a ground mixture of alfalfa pellets and Tetramin fish flakes. *Ambystoma* were given *Artemia* (brine shrimp). A natural photoperiod was provided during the maintenance and experimental periods in the form of artificial light combined with natural daily sunlight through unshaded windows.

Each species was tested in a separate experiment. Each experiment used a 3 × 2 fully factorial design to manipulate nitrate and *Saprolegnia*. There were three nitrate treatments (no nitrate, low nitrate, and high nitrate) and two *Saprolegnia* treatments (*Saprolegnia* and control). Treatments were assigned to units randomly, and larvae were added to units haphazardly with respect to treatment. For each species, there were five replicates of each treatment combination. Thus, there were 30 units per species, for a total of 90 units.

Experimental units consisted of plastic boxes (dimensions: 31 × 18 × 8 cm) containing 2 l of water from one of three stock solutions (no nitrate, low nitrate, and high

nitrate stock solutions). We added 0.29 ml l^{-1} of Novaqua® and 0.29 ml l^{-1} of Amquel® to each stock solution. We added sodium nitrate to the low nitrate and high nitrate stock solutions to achieve nominal nitrate concentrations of 0, 5, and 20 mg l^{-1} in the no nitrate, low nitrate, and high nitrate treatments, respectively.

Ten larvae were added per unit for *Hyla* and *Ambystoma*, while seven larvae were added per unit for *Rana*. Larvae were added to units, and treatments were applied on 14 April 2002. *Hyla* and *Rana* ranged in age from about 2 to 5 and 7 to 9 weeks post-hatching, respectively. Gosner developmental stages (Gosner 1960) were 25 to 28 in *Hyla* and 25 to 30 in *Rana*. The oldest *Ambystoma* larvae used in experiments were 25 days post-hatch.

Larvae were chosen for experimentation haphazardly. For *Ambystoma*, one larva was chosen haphazardly from each of 10 clutches, and individuals that appeared to be small and recently hatched were excluded. *Hyla* were chosen from two tanks, one containing larvae from 12 clutches and one containing larvae from 13 clutches. *Rana* were chosen from 4 tanks and from 4 to 8 clutches.

Saprolegnia was isolated from a water sample taken next to a *Rana aurora* embryo mass in Coast Pond on 10 February 2002. Isolation of *Saprolegnia* was achieved using sterile hemp seeds and YpG (yeast-glucose) agar media (Fuller & Jaworski, 1987). *Saprolegnia* was prepared for experiments by placing a hemp seed laden with *Saprolegnia* into a Petri dish (diameter = 85 mm, height = 12 mm) filled approximately half full with ultrapure water and containing seven sterile hemp seeds. Dishes were incubated at ~13 to 15°C for six days. These hemp seeds, laden with *Saprolegnia,* were

used in *Saprolegnia* treatments. Three seeds were added to each unit in the *Saprolegnia* treatment. Immediately prior to the experiment, seeds were connected by *Saprolegnia* hyphae. Seeds were disconnected and added to units haphazardly. Units in the control treatment received three sterile hemp seeds. Addition of sterile seeds was haphazard with respect to nitrate treatment.

Leftover seeds laden with *Saprolegnia* were placed in a refrigerator (~4 to 5°C) at the start of the experiments. Non-spherical zoosporangia were counted four days later using a dissecting microscope on five seeds with heavy growth, selected haphazardly. Number of zoospores per seed (mean ± SE) was 16.6 ± 4.0. Spherical zoosporangia may have been present (Seymour 1970); however, they would have been indistinguishable from oogonia under the dissecting microscope. Since the number of spherical zoosporangia was not quantified, our counts of zoosporangia may be underestimates.

Within 5.5 hours of addition of larvae to units and application of *Saprolegnia* treatments, units from one of each treatment combination were selected haphazardly for each species and a water sample was taken to obtain measurements of initial water quality. Conductivity, total alkalinity, and calcium hardness were measured in subsets of water samples. We measured pH for every water sample.

To minimize handling stress of animals in the experiments, initial measurements of their total length and mass were not taken. Instead, on 15 April, unused larvae from the same stocks were selected (in the same manner that larvae were selected for experimentation) and measured to obtain estimates of total length and mass of the larvae in the experiments (Table 2.1).

During the experiment, anurans were fed ad libitum a ground mixture of alfalfa pellets and Tetramin fish flakes. On 20 April, *Ambystoma* were given *Artemia* (brine shrimp). The experiment was checked at least once per day. Live larvae were monitored visually for hyphal structures consistent with descriptions of *Saprolegnia* growth on amphibian larvae (Bragg & Bragg 1958, Bragg 1962). Dead larvae were removed and examined for hyphal structures with a dissecting microscope. Each experiment lasted for seven days. Surviving larvae were anesthetized with MS-222 and sacrificed. During the experiment, laboratory temperature was maintained at approximately 13 to 15°C.

2.4 Results

In *Hyla*, no significant effects of nitrate or *Saprolegnia* on survival were found (Fig. 2.1, Table 2.2). In contrast, for *Rana,* significant effects of *Saprolegnia* and the interaction between nitrate and *Saprolegnia* were detected, although the nitrate term was not significant (Table 2.2). Survival of *Rana* is presented in Figure 2.2. For *Rana*, in the no nitrate treatment, survival was significantly lower in the *Saprolegnia* treatment compared to the control treatment ($0.005 < p < 0.01$). However, in the low nitrate and in the high nitrate treatments, survival was not significantly different between *Saprolegnia* and control treatments. Nitrate alone did not significantly affect survival (none of the differences between treatment groups were significant). However, survival was always lower when nitrate was added, compared to the no nitrate, control treatment group (no nitrate or pathogen added). One *Rana* individual with severe tail damage was observed being preyed upon by two conspecifics. It was removed and scored as dead. Scoring this

individual as dead or eliminating it from the analysis did not alter the qualitative interpretations regarding *Rana* survival.

In *Ambystoma,* survival when nitrate was not added was lower in *Saprolegnia* compared to control treatments (Fig. 2.3), but all larvae, in both *Saprolegnia* and control treatments, survived when nitrate was added. Only two larvae died; both were in the no nitrate, *Saprolegnia* treatment. ANOVA revealed no significant effects of the treatments or their interaction; however, there were non-significant trends toward a main effect of nitrate treatment and a nitrate × *Saprolegnia* interaction (Table 2.2). Thus, there was a non-significant trend of the same less-than-additive interaction between the two factors that we detected in the experiment using *Rana*.

In *Rana*, four carcasses were observed being eaten by conspecifics, and signs of scavenging (i.e. holes in the skin or missing structures obviously due to scavenging) were observed on 25 of the 31 remaining carcasses. For an additional two carcasses, it could not be determined whether cannibalism was involved in causing the missing structures (this may have resulted from parasitism, e.g. from *Saprolegnia,* or decomposition without the involvement of cannibalism). Two larvae (one in the no nitrate, control treatment combination and one in the low nitrate, *Saprolegnia* treatment) were observed being chewed upon by one or more conspecifics while still alive.

No signs of hyphal growth were observed in *Hyla* (Table 2.3). *Rana* carcasses had hyphal growths more often in *Saprolegnia* compared to control treatments in the no nitrate and low nitrate treatments, but the reverse was true in the high nitrate treatment. In contrast, hyphae were noted in live *Rana* more frequently in *Saprolegnia* than in

control treatments in each of the nitrate treatments. At the end of the experiment, hyphal growths were not observed on any live *Rana*. It is possible that in *Rana*, shredded tadpole structures (e.g. muscle and connective tissue) due to scavenging by conspecifics may have been misidentified as hyphae in some cases. Both of the *Ambystoma* that died, which were in the no nitrate, *Saprolegnia* treatment combination, were covered with hyphae, but hyphae were not noted on any live *Ambystoma*.

Conductivity ranged from 153 to 195 μS cm^{-1} (n = 6, not all treatment combinations included), total alkalinity ranged from 33 to 41 mg $CaCO_3$ l^{-1} (n = 5, not all treatment combinations included), and calcium hardness ranged from 34 to 40 mg $CaCO_3$ l^{-1} (n = 5, not all treatment combinations included). pH varied from 6.5 to 7.2 (n = 18).

2.5 Discussion

Our results suggest that *Saprolegnia* caused mortality of *Rana,* but only in the no nitrate treatment. This is consistent with increased nitrate preventing *Saprolegnia* from causing mortality of *Rana.* In *Rana*, the combined effects of nitrate and *Saprolegnia* were less than additive, rather than synergistic. Although determining the mechanism behind such an interaction was not a goal of this study, some possible mechanisms deserve note. Nitrate may decrease zoospore production or kill zoospores in *Saprolegnia*. Nitrate may also have induced a physiological response in *Rana* that increased their resistance to *Saprolegnia*.

Less-than-additive stressor–pathogen interactions have been reported previously in different organisms, including amphibians. For example, Parris & Baud (2004) demonstrated a negative interaction between copper and the amphibian pathogen

Batrachochytrium dendrobatidis. Both copper and the pathogen increased the length of the larval period in gray treefrogs *Hyla chrysoscelis* larvae, but the magnitude of the effect of the pathogen on larval period was lower when the copper concentration was high than it was in regimes in which there was a lower copper concentration. Parris & Baud (2004) hypothesized that copper may have decreased the growth of the fungus on *H. chrysoscelis*. Poleo *et al.* (2004) found that aluminum and zinc decreased the number of parasites in Atlantic salmon *Salmo salar*. Parasite diversity and intensity in two snail species (*Physella columbiana* and *Lymnaea palustris*) were lower in lakes polluted by heavy metals than in reference lakes, which may have influenced competitive interactions between the 2 snail species (Lefcort *et al.*, 2002). Riggs & Esch (1987) and Riggs *et al.* (1987) studied the tapeworm *Bothriocephalus acheilognathi* in *Gambusia affinis* (mosquitofish), *Notropis lutrensis* (red shiners), and *Pimophales promelus* (fathead minnows) in a cooling pond receiving thermally and selenium-enriched fly ash from a coal-fired power plant in North Carolina, USA. Three sites were characterized as polluted, interface or unpolluted, based upon proximity to the fly ash input and selenium concentration. A complex pattern of mean number of worms per fish, mean number of gravid worms per fish, growth and biomass of worms, ratio of gravid proglottids per gravid worm, and number of eggs shed per gravid proglottid emerged (Riggs & Esch, 1987, Riggs *et al.*, 1987). In several cases, the pattern was consistent with increased pollution causing decreased worm population size or decreased performance of individual worms (Riggs & Esch, 1987, Riggs *et al.*, 1987).

Other stressors may also affect host–pathogen interactions. For example, high temperatures may also reduce the effects of infectious disease or even eliminate infection. Woodhams et al. (2003) found evidence consistent with high temperatures eliminating *Batrachochytrium dendrobatidis* infection in juvenile *Litoria chloris* (red-eyed treefrogs). However, the influence of temperature on amphibian diseases may not be straightforward. Aspects of diseases such as mortality from infection, time to death after exposure, and production of pathogenic propagules may show conflicting patterns with respect to temperature (Berger *et al.*, 2004, Rojas *et al.*, 2005).

Importantly, effects of stressor–pathogen interactions on parameters of individual hosts do not necessarily translate into corresponding effects at the level of the host population. Indeed, modeling of the influence of environmental stress on the impact of infectious disease caused by a parasite that is host specific and has a population closed to recruitment from outside the population, suggests that environmental stress that increases the susceptibility of individual hosts to the parasite is most likely to decrease the impact of the disease on the host population (Lafferty & Holt, 2003). However, if stress that increases the susceptibility of individual hosts to parasites occurs in an infectious disease system in which the parasite population is open to outside recruitment, or is not host-specific and the other host(s) are not affected by the stress, then the stress will most likely cause an increase in the impact of the disease on the host population (Lafferty & Holt, 2003). In this scheme, the ability to grow as a saprobe (as *Saprolegnia* can) should have the same qualitative effect on a host population as open recruitment or ability to use 1 or more alternate hosts unaffected by the stressor. Lafferty & Holt (2003) did not model

situations in which the effects of stress and an infectious disease on individual hosts were less-than-additive. However, it seems logical that if nitrate prevents *Saprolegnia* from affecting survival of amphibian larvae, then it will also prevent *Saprolegnia* from having effects on larvae that influence population-level parameters. However, effects on other life stages besides larvae may factor in to determine the combined effects of nitrate and *Saprolegnia* on an amphibian population, and the effects on survival of other life stages may be different from the effect on survival of larvae.

Our study concentrated on testing the separate and combined effects of nitrate and *Saprolegnia* on survival of amphibian larvae. Thus, we did not focus on possible sublethal effects. Nitrate and *Saprolegnia* may interact to influence sublethal parameters in amphibian larvae.

The nominal nitrate concentrations we used were realistic for amphibian larvae in habitats receiving fertilizer runoff. In the Willamette Valley of Oregon, average nitrate concentrations of 17.8 and 21.9 mg N l^{-1} were reported in water samples from some crop soils receiving recommended rates of nitrogen fertilization (Brandi-Dohrn *et al.*, 1997, Marco et al., 1999). These average values are highly toxic to some amphibians (Marco *et al.*, 1999). Flow-weighted mean nitrate concentrations ranging from zero to 14.8 mg l^{-1} have been recorded in streams draining the English Lake District, Cumbria, England (Thornton & Dise, 1998). Average nitrate concentrations in 30 mg l^{-1} have been recorded in relatively large streams in the Great Lakes region of North America (US EPA, 1998, Rouse *et al.*, 1999). Rouse et al. (1999) suggest that such streams will typically have average nitrate concentrations lower than small ponds and ditches close to

point sources of nitrate. Thus, amphibian larvae in agricultural landscapes may be exposed to nitrate levels well in excess of 30 mg l^{-1}. Exposure to these levels may occur for extended periods of time (Rouse *et al.*, 1999).

Observations of *Rana* carcasses suggest that the *Saprolegnia* treatment caused infection in the low and no nitrate treatments, but not in the high nitrate treatment. Furthermore, our results suggest *Saprolegnia* can kill larvae of *R. aurora* in water lacking nitrate pollution. Because most of the studies of the effects of *Saprolegnia* on amphibians have been examined in embryos, our study of larvae is an important step in determining the effects of this pathogen on postembryonic life history stages. The effects of *Saprolegnia* on amphibian populations may be far different, and possibly more severe, if the pathogen causes mortality in both embryos and larvae rather than in just 1 life stage. Recent models have shown differential effects on populations when mortality occurs in different life stages in amphibians (Biek *et al.*, 2002, Vonesh & De la Cruz, 2002a,b).

Under the conditions of this study, *Rana* was susceptible to mortality from *Saprolegnia* when nitrate was not added, while *Hyla* and *Ambystoma* were not susceptible to mortality from *Saprolegnia* in any of the nitrate treatments. These interspecific differences may indicate that *R. aurora* larvae are more susceptible to *Saprolegnia* than larvae of the other two species. However, the relative susceptibilities of these species may depend on the dose of the pathogen.

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Table 2.1. *Hyla regilla, Rana aurora* and *Ambystoma gracile.* Measurements of total length (mean ± 1SE) and mass (mean ± 1 SE) from laboratory stocks of amphibian larvae

Table. 2.1

Species	Total length (mm)	N	Mass (g)
Hyla regilla	12.7±0.3	32 (8 batches of 4 larvae each)	0.03[a]
Rana aurora	23.5±0.7	23 (23 larvae)	0.14±0.02
Ambystoma gracile	16.6±0.4	30 (10 batches of 3 larvae each)	0.03[a]

[a]SE could not be calculated because larvae were massed in batches.

Table 2.2. *Hyla regilla, Rana aurora* and *Ambystoma gracile.* ANOVA results for the survival of larvae of the 3 amphibian species

Source of variation	MS	df	*F*	*p*
Hyla regilla				
Nitrate	10.000	2	1.000	0.383
Saprolegnia	3.333	1	0.333	0.569
Nitrate × *Saprolegnia*	3.333	2	0.333	0.720
Error	10.000	24		
Rana aurora				
Nitrate	20.408	2	0.128	0.881
Saprolegnia	1149.660	1	7.192	0.013
Nitrate × *Saprolegnia*	700.680	2	4.383	0.024
Error	159.864	24		
Ambystoma gracile				
Nitrate	13.333	2	2.667	0.090
Saprolegnia	13.333	1	2.667	0.116
Nitrate × *Saprolegnia*	13.333	2	2.667	0.090
Error	5.000	24		

Table 2.3. *Hyla regilla, Rana aurora* and *Ambystoma gracile.* Examination of larvae for hyphal growth. We checked for hyphal growth on living larvae on 10 occasions for *Hyla* and *Ambystoma* and 12 times for *Rana.* At the end of the experiment, hyphal growths were not found on any of the live larvae. NA: no mortality; ND: microscopy data not recorded for the one individual that died in this treatment combination. No hyphae were noted during visual inspection of this individual

Treatment	Dead individuals with hyphae	Total # of instances live individuals were counted as having hyphal growth[a]
Hyla regilla		
No nitrate addition/control	ND	0
No nitrate addition/*Saprolegnia*	0/1 (0%)	0
Low nitrate addition/control	NA	0
Low nitrate addition/*Saprolegnia*	NA	0
High nitrate addition/control	0/1 (0%)	0
High nitrate addition/*Saprolegnia*	NA	0
Rana aurora		
No nitrate addition/control	0/2 (0%)[a]	0
No nitrate addition/*Saprolegnia*	6/13 (46%)[b]	2[d]
Low nitrate addition/control	2/7 (25%)	0
Low nitrate addition/*Saprolegnia*	3/7 (43%)[c]	4
High nitrate addition/control	1/7 (14%)	1
High nitrate addition/*Saprolegnia*	0/5 (0%)[c]	2
Ambystoma gracile		
No nitrate addition/control	NA	0
No nitrate addition/*Saprolegnia*	2/2 (100%)	0
Low nitrate addition/control	NA	0
Low nitrate addition/*Saprolegnia*	NA	0
High nitrate addition/control	NA	0
High nitrate addition/*Saprolegnia*	NA	0

[a]Including 1 individual that was removed prior to death. Hyphae were not noted on this individual

[b]Not including 1 carcass that may have had hyphal growth

[c]Not including 2 carcasses that were not discovered

[d]Not including 1 count in which an individual may have had hyphal growth on its tail

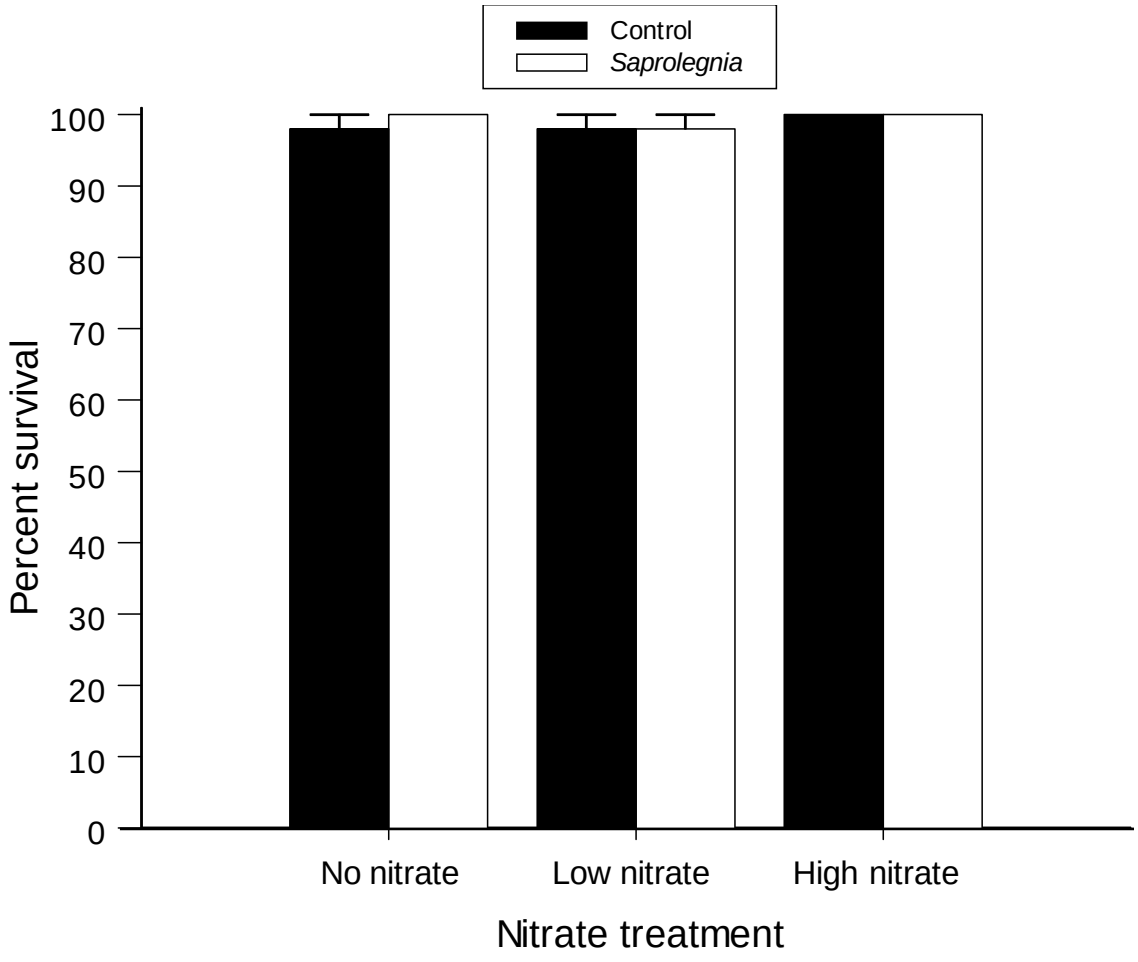

Figure 2.1. *Hyla regilla.* Survival of larvae. Error bars are + 1 SE.

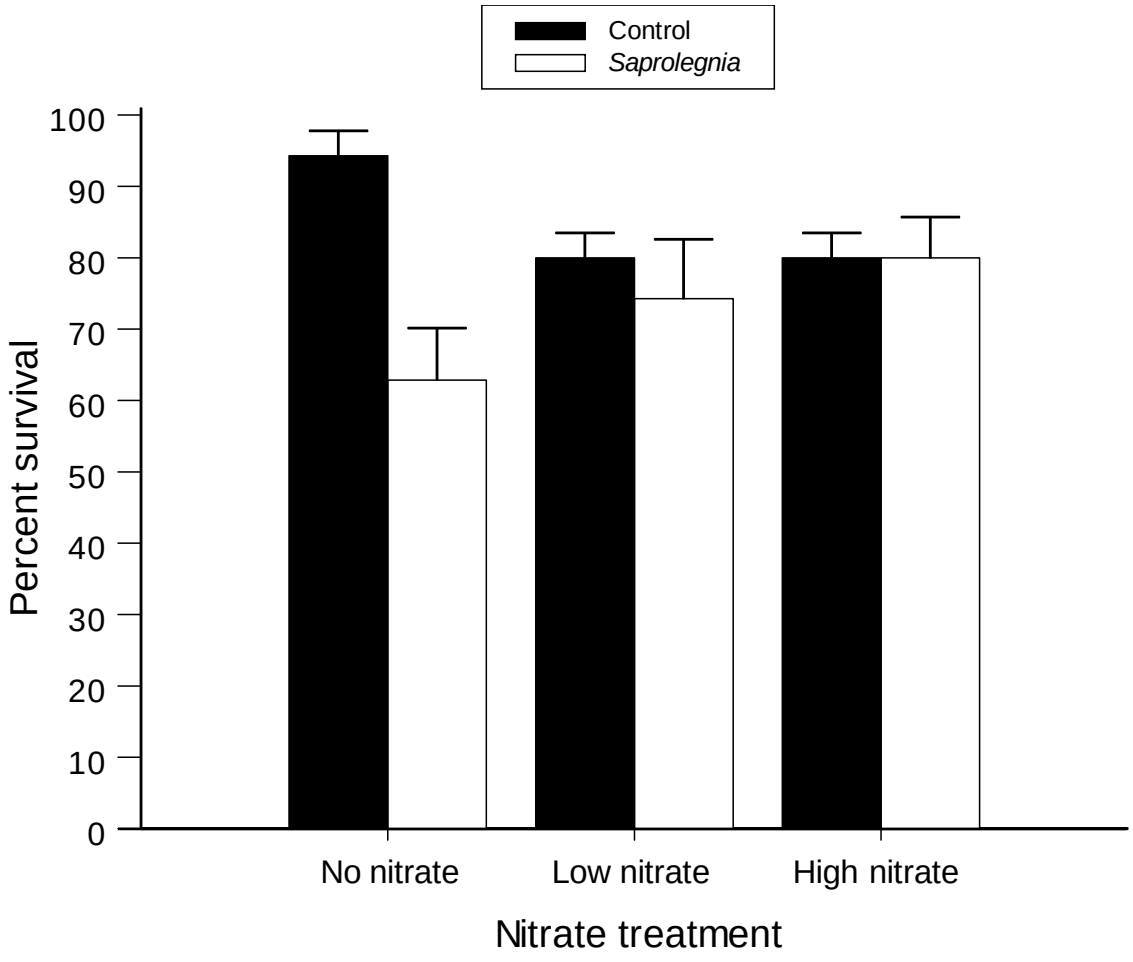

Figure 2.2. *Rana aurora*. Survival of larvae. Error bars are +1 SE.

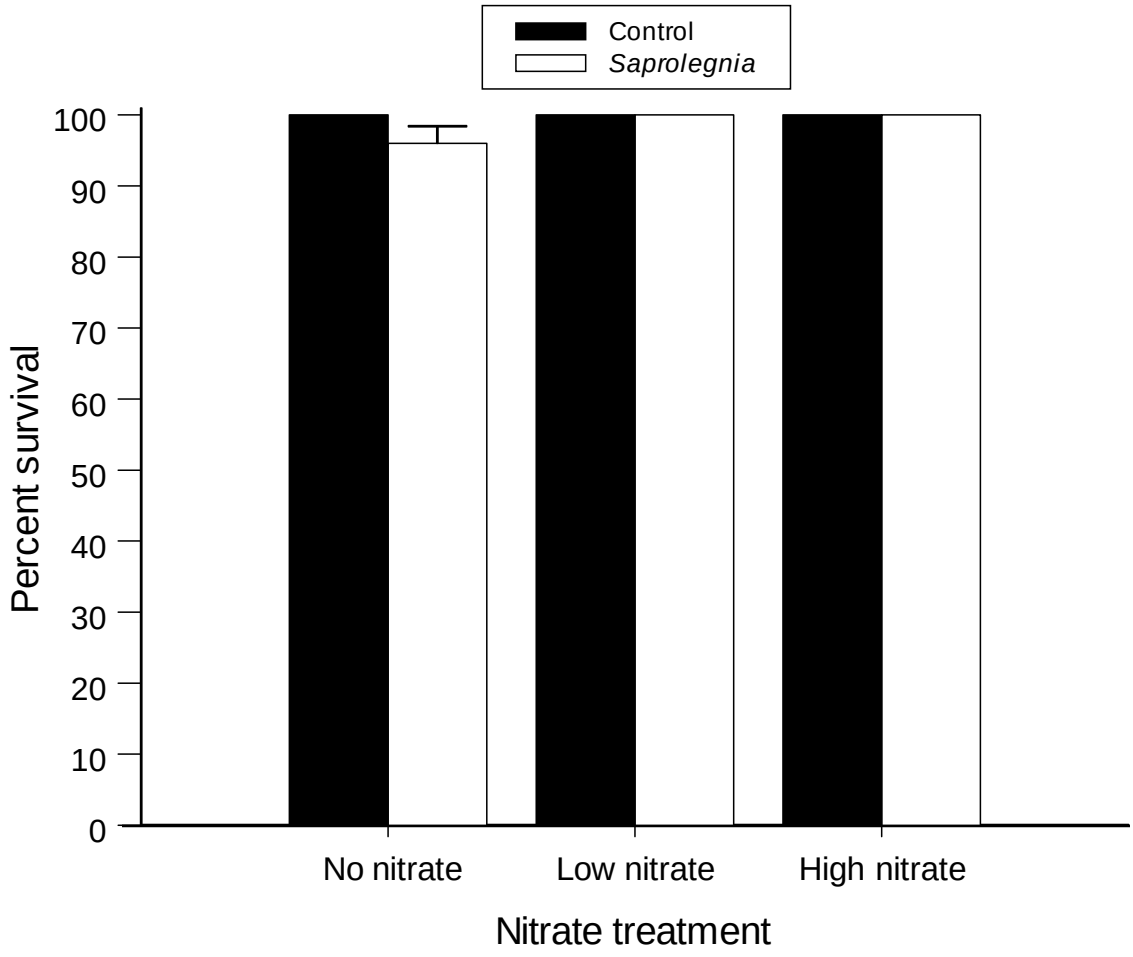

Figure 2.3. *Ambystoma gracile.* Survival of larvae. Error bars are +1 SE.

Chapter 3

Susceptibility of Newly-Metamorphosed Frogs to a Pathogenic Water Mold (*Saprolegnia sp.*)

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3.1 Abstract

Recent losses of worldwide biodiversity include population declines and extinctions in many amphibian populations. Many factors, including pathogens, are contributing to amphibian population declines. One pathogen, a water mold of the genus *Saprolegnia,* causes mortality in early life stages of amphibians and may contribute to the declines of specific amphibian populations. Most of our knowledge of how *Saprolegnia* affects amphibians comes from studies of embryos. The effects of *Saprolegnia* on post-metamorphic amphibians are poorly known. Therefore, in the laboratory, we investigated the susceptibility of newly-metamorphosed juvenile amphibians to *Saprolegnia* in four frog species: *Bufo boreas* (western toad), *Pseudacris regilla* (Pacific treefrog), *Rana aurora* (red-legged frog), and *R. cascadae* (Cascades frog). In this study, we found that juvenile *R. cascadae* exposed to *Saprolegnia* had greater rates of mortality than unexposed controls. In the other species, survival was also lower in the *Saprolegnia* treatments compared with controls but these differences were not significant statistically. Combined effects of *Saprolegnia* in both embryonic and juvenile stages may make the populations of *R. cascadae* especially vulnerable.

3.2 Introduction

As part of worldwide losses in biodiversity, many amphibian populations are declining and disappearing (Houlahan *et al.,* 2000, Stuart *et al.,* 2004). Numerous factors appear to be contributing to these declines. They include habitat destruction (Dodd & Smith, 2003), introduced predators (Knapp & Matthews, 2000, Kats & Ferrer, 2003, Vredenburg, 2004) global climate change (Pounds & Crump, 1994, Pounds *et al.,* 1999),

pollution (Beebee *et al.,* 1990) and ultraviolet-B (UV-B) radiation (Blaustein *et al.,* 1998).

Pathogens are causing mortality in various amphibian life stages (e.g. Blaustein *et al.,* 1994b) and are implicated in the declines of several amphibian populations (Berger *et al.,* 1998, Daszak *et al.,* 2003, Lips *et al.,* 2006, Pounds *et al.,* 2006). Pathogens of amphibians are diverse. They include parasitic worms (e.g. Platt *et al.,* 1993, Aisien *et al.,* 2003, Blaustein & Johnson, 2003), oomycetes (e.g. Blaustein *et al.,* 1994b), fungi (e.g. Berger *et al.,* 1998), viruses (e.g. Jancovich *et al.,* 1997), bacteria (e.g. Worthylake & Hovingh, 1989), and myxozoans (e.g. Delvinquier *et al.,* 1992). The oomycete *Saprolegnia* is a genus of water mold that is commonly present in soil and freshwater (Johnson *et al.,* 2002a). *Saprolegnia* species are capable of both saprotrophism and parasitism (Johnson *et al.,* 2002a). *Saprolegnia* infects a variety of organisms, including mosquitoes (MacGregor, 1921), turtles (Tiffney, 1936), fish (e.g. Schaefer *et al.*, 1981), and amphibians (e.g. Blaustein *et al.,* 1994b). *Saprolegnia* is associated with mass mortality in fish populations (Schaefer *et al.,* 1981, Johansson *et al.,* 1982).

Saprolegnia infects amphibian embryos (e.g. Blaustein *et al.,* 1994b, Kiesecker *et al.,* 2001b), larvae (e.g. Schnetzler, 1888, Walls & Jaeger, 1987), and adults (Ford *et al.,* 2004). Banks and Beebee (1988) found that *Saprolegnia*-associated mortality of *Bufo calamita* (Natterjack toad) in England was associated with low temperature and acid conditions. In the Oregon Cascade Range, a synergism between UV-B radiation and *Saprolegnia ferax* contributes to massive mortality of *B. boreas* and *Rana cascad*ae embryos (Blaustein *et al.,* 1994b, Kiesecker & Blaustein, 1995). Thus, there is the

potential for *Saprolegnia* to greatly affect amphibian populations. For *B. boreas* and *R. cascadae*, laying in communal egg masses increases embryo mortality, apparently because of the high density of susceptible hosts when eggs are laid in close proximity to one another (Kiesecker & Blaustein, 1997). For *B. boreas, S. ferax*-associated embryo mortality becomes greater when El Niño Southern Oscillation events cause a decrease in water levels and exposure to UV-B radiation increases (Kiesecker *et al.,* 2001c). Koch's postulates (Pelczar & Reid, 1965) have been fulfilled for *B. boreas* embryos, illustrating that *Saprolegnia* is indeed one pathogen causing mortality in amphibians in the Pacific Northwest (U.S.A.) (Kiesecker *et al.,* 2001c).

There are only limited data on the effects of *Saprolegnia* on post-embryonic amphibians. In the Oregon Cascades Range, there is experimental evidence that *Saprolegnia* influences competitive interactions between *R. cascadae* and *Pseudacris regilla* (Pacific treefrog). In an experiment performed in outdoor ponds, *Saprolegnia* prevented *R. cascadae* larvae from reducing survival, growth, and development of *P. regilla* larvae, apparently because of decreased density of *R. cascadae* larvae due to *Saprolegnia*-induced mortality of their embryos (Kiesecker & Blaustein, 1999). Bragg & Bragg (1958) and Bragg (1962) reported *Saprolegnia* on dead amphibian larvae after mass mortality events. Walls & Jaegar (1987) noticed that mortality of *Ambystoma maculatum* (spotted salamander) larvae caused by exposure to *A. talpoideum* (mole salamander) larvae was associated with *Saprolegnia* infection of bite wounds from aggressive *A. talpoideum*. Lefcort *et al.* (1997) observed that mortality of *A. tigrinum* (tiger salamander) caused by exposure to silt was associated with *S. parasitica* infection.

Romansic *et al.* (2006) showed that *Saprolegnia* can kill *R. aurora* (red-legged frog) larvae, and *Saprolegnia* infection was associated with red leg syndrome and mortality in captive *Xenopus laevis* (African clawed frog) adults that had undergone oocyte-harvesting surgery (Ford *et al.,* 2004).

One step in examining the potential population effects of *Saprolegnia* is to test their susceptibility to the pathogen at various life stages. This seems especially important in light of models suggesting that mortality involving post-embryonic individuals may contribute significantly to population declines (Vonesh & de la Cruz, 2002, Biek *et al.,* 2002).

The purpose of this study was to test experimentally the susceptibility of newly-metamorphosed amphibians (metamorphs) to mortality from *Saprolegnia*. We used four anuran species: *B. boreas, P. regilla, R. cascadae,* and *R. aurora* (red-legged frog). *B. boreas, P. regilla* and *R.cascadae,* are susceptible to mortality from *Saprolegnia* at the embryonic stage (Kiesecker & Blaustein, 1995, Kiesecker *et al.,* 2001b). There have been no tests for the possible effect of *Saprolegnia* on *R. aurora* embryos. Romansic *et al.* (2006) found that larvae of *R. aurora* are susceptible to mortality from this pathogen, but found no evidence that larvae of *P. regilla* were. There have been no studies examining the effects of *Saprolegnia* on post-metamorphic frogs.

3.3 Materials and Methods

We tested a set of three frog species (*B. boreas, P. regilla,* and *R. cascadae*) collected from the Oregon Cascade Range because *Saprolegnia* causes massive mortality in embryos of *B. boreas* and *R. cascadae* in this region (Blaustein *et al.,* 1994b,

Kiesecker & Blaustein, 1995). We also tested *R. aurora* to gain a broader understanding of the effects of *Saprolegnia* on frogs. Testing frogs from the Oregon Cascades and *R. aurora* simultaneously was impossible due to differences in timing of oviposition and larval period between frog populations in the Oregon Cascades and the populations of *R. aurora* known to the authors. Therefore, *B. boreas, P. regilla,* and *R. cascadae* were tested simultaneously in experiment 1 and *R. aurora* alone was tested in a separate experiment (experiment 2).

Amphibians were collected as larvae. *B. boreas* were collected on 22 and 27 August 2002 from Todd Lake Deschutes, County, Oregon, USA (elevation about 1864 m). *P. regilla* were collected on 23 August from a subalpine meadow about 0.6 km NW of Todd Lake, Deschutes County (elevation about 1982 m) and *R. cascadae* and were collected from this meadow on 23 August and 18 September 2002. *R. aurora* were collected from one pond (elevation about 12 m) at Baker Beach, Lane County, Oregon on 31 May 2003 and a second pond (elevation about 6 m) at Baker Beach in June, 2003. Larvae were brought to the laboratory and maintained in aquaria filled with tapwater conditioned with NovAqua and Amquel water conditioners (hereafter, dechlorinated tapwater). *B. boreas* tanks were aerated. Larvae were fed a ground mixture of alfalfa pellets and Tetramin fish flakes. Metamorphs were transferred to tanks containing dechlorinated tapwater and tilted (part of the bottom of the tank was above water and part was below water), with the exception that *B. boreas* metamorphs were put in a plastic shoebox with paper towels moistened with dechlorinated tapwater placed on the bottom.

Prior to use in experimentation, metamorphs were fed crickets, except for *P. regilla* metamorphs, which were fed a combination of *Drosophila* and crickets.

Saprolegnia was isolated from a water sample that was collected on 10 September 2002 at the shore of Lost Lake in the Oregon Cascade Range (Linn County, elevation 1220 m), a site where *Saprolegnia* has contributed to massive mortality of *B. boreas* embryos (Blaustein *et al.,* 1994b, Kiesecker & Blaustein, 1995, Kiesecker *et al.,* 2001c). The isolate was grown in pure culture in Petri dishes using YpG agar media (Fuller & Jaworski, 1987). To obtain *Saprolegnia* for use in experiments, sterile hemp seeds were added directly to Petri dishes containing *Saprolegnia* cultures to allow seeds to become inoculated with *Saprolegnia*. Seeds were then removed and added to standardized Petri dishes (diameter = 85 mm, height = 12 mm) filled approximately half full with ultrapure water. Thirty seeds were added to each dish. After seeds were added to water, dishes were incubated for seven days at approximately 20-23º C and then transferred to a refrigerator (~4º C) for two days prior to use. *Saprolegnia* hyphae grew between seeds in dishes, producing clumps of seeds connected by a mycelium of *Saprolegnia* containing hyphae and zoosporangia. These clumps of seeds were used to apply *Saprolegnia* treatments in laboratory experiments.

Experimental units consisted of plastic cups (diameter: 9.5 cm, height = 7.5 cm) with covers of 1.5-mm fiberglass mesh. Unbleached paper towels were placed at the bottom of each cup and flooded initially with 15 ml of ultrapure water. In experiment 1, (*B. boreas, P. regilla,* and *R. cascadae* tested simultaneously), we manipulated treatment and frog species in a 2 × 3 fully factorial design. Units received, at random, either a

Saprolegnia treatment (a clump of 30 hemp seeds overgrown with *Saprolegnia* hyphae and zoosporangia) or a control treatment (30 sterile hemp seeds) and five metamorph individuals of either *B. boreas, P. regilla*, or *R. cascadae*. There were five replicates of each species-treatment combination, for a total of 30 units and 150 metamorphs. Experiment 2 used the same methods, except that it used *R. aurora* metamorphs only (five metamorphs per unit). Here, there were five replicates of each treatment (*Saprolegnia* and control), for a total of 10 units and 50 metamorphs. Metamorphs were visually inspected for hyphal growths consistent with *Saprolegnia* infection (Ford et al. 2004) immediately prior to addition to units. No hyphal growths were found during these inspections. Addition of all metamorphs to units was random with respect to treatment. Each experiment lasted for two weeks. Experiment 1 began on 27 October 2002. Experiment 2 began on 6 August 2003.

The initial number of *Saprolegnia* zoospores and zoospore cysts (a combined total) applied in the *Saprolegnia* treatment was estimated at the start of each experiment using leftover clumps of *Saprolegnia*-laden hemp seeds from the same stock used in the experiment but not added to cups. These clumps were lifted out of their water, placed in a sterile container, and washed with ultrapure water. The number of zoospores and cysts in a sample of the resulting solution was counted using a hemacytometer and these counts provide an estimate of the initial number of zoospores and cysts per cup in the *Saprolegnia* treatment, assuming that the number of zoospores and cysts present from sources besides the *Saprolegnia*-laden hemp seeds was zero. Estimates of initial number

of *Saprolegnia* zoospores and cysts were 1.1×10^8 (standard error = 2.4×10^7, n = 2) and 2.4×10^7 (standard error = 3.3×10^6, n = 2) in experiments 1 and 2, respectively.

Throughout each experiment, dechlorinated tapwater was added as necessary to maintain a thin film of water over towels, thus allowing hydration of amphibians and preventing *Saprolegnia* from desiccating. Experiments were checked at least once per day. Frogs were not fed during the experiments. Dead individuals were removed and preserved in 70% ethanol. At the end of each experiment, percent survival was recorded for each cup and surviving individuals were anaesthetized with MS-222 and preserved in 70% ethanol.

After the experiments, preserved specimens were measured for length (Table 3.1). Snout-vent length was measured, unless there was some tail present, in which case the snout-base of tail length was measured. Specimens that were damaged or distorted due to desiccation or decomposition prior to preservation or handling after preservation were not measured. Approximate range of ages at the start of experimentation was 2-6 weeks post-metamorphosis for *B. boreas* and *P. regilla* and 2-8 weeks post-metamorphosis for *R. aurora* and *R. cascadae*. We defined metamorphosis as having about 50% or more of the tail resorbed.

Preserved specimens were examined for oomycotic growth consistent with *Saprolegnia* infection (Ford et al. 2004) using a dissecting microscope (25× magnification). For each metamorph, a sample of or the entirety of any hyphae, shedding skin, unusual-looking skin, unusual-looking structures, or unidentified debris present on the exterior of the specimen was removed using a pair of forceps, and examined using a

compound microscope (100-400× magnification). Metamorphs with coenocytic hyphae were scored as having oomycotic growth consistent with *Saprolegnia* infection.

Each experiment was analyzed separately. In experiment 1, percent survival data and prevalence of oomycotic growth data were heteroscedastic, violating the parametric assumption of homoscedasticity. In the survival data there was no variation in the *B. boreas*, control treatment combination, while in the prevalence of oomycotic growth data there was no variation in the control treatment. Therefore, all analyses were nonparametric. For Experiment 1, survival and prevalence of oomycotic growth were analyzed separately, using multiple-comparisons procedures. For each dependent variable, an analog to the Student-Newman-Keuls test (Zar, 1999) to test for pairwise differences between treatments for each of the three frog species in the experiment (*B. boreas, P. regilla*, and *R. cascadae*). For experiment 2, we tested for a difference in survival between the two treatments in *R. aurora* using a rank-sum test. No oomycotic growth was observed on *R. aurora.*

3.4 Results

Mean percent survival of *B. boreas, P. regilla,* and *R. cascadae* in experiment 1 is displayed in Figure 3.1. Survival was not significantly different between *Saprolegnia* and control treatments in *B. boreas* ($q_{0.05,\infty,3,}$= 2.85, $0.10 < p < 0.20$) and *P. regilla* ($q_{0.05,\infty,2,}$= 2.6588, $0.05 < p < 0.10$). In *R. cascadae*, survival was significantly lower in the *Saprolegnia* treatment compared to the control treatment ($q_{0.05,\infty,3,}$= 5.0292, $0.005 < p < 0.01$). Mean percent and proportion of specimens showing oomycotic growth is displayed in Table 3.2. In *R. cascadae, B. boreas*, and *P. regilla*, prevalence of

oomycotic growth was higher in the *Saprolegnia* compared to the control treatment ($p >$ 0.50 for *R. cascadae*, $p >0.05$ for *B. boreas* and *P. regilla*). In these three species, oomycotic growth was not observed on any of the individuals in the control treatment.

Mean percent survival of *R. aurora* in experiment 2 is also displayed in Figure 3.1. Survival was lower in the *Saprolegnia* treatment compared to the control treatment, but this difference was not significant statistically ($p = 0.2828$). Oomycotic growth was not observed on any *R. aurora.*

3.5 Discussion

Our results suggest that newly metamorphosed *R. cascadae* are susceptible to mortality from *Saprolegnia*. In each of the other three species, mortality was higher in the exposed compared to the control group, but none of these differences were statistically significant. However, longer exposures and/or higher doses of the pathogen may cause significant mortality in these three species. Also, differences in zoospore concentrations and differences in age may have caused some of the differences in results across the four frog species. For example, despite the use of the same culturing methods in both experiments, estimated initial number of zoospores and cysts applied in the *Saprolegnia* treatment for experiment 2 (*R. aurora*) was only about one-fifth that of experiment 1 (*B. boreas, P. regilla* and *R. cascadae*). Furthermore, approximate age post-metamorphosis was 2-6 weeks in *B. boreas* and *P. regilla*, and 2-8 weeks in *R. cascadae* and *R. aurora*.

Prevalence of oomycotic growth consistent with *Saprolegnia* infection was higher in the *Saprolegnia* compared to the control treatment for *B. boreas, P. regilla*, and *R.*

cascadae, but none of these differences were statistically significant. Oomycotic growth was not observed in *R. aurora.* It is possible that some metamorphs had oomycotic growth that was not detected and that failure to detect cases of oomycotic growth prevented detection of a statistically significant effect of the *Saprolegnia* treatment on prevalence of oomycotic growth in *R. cascadae*, the species in which we found a statistically significant negative effect of the *Saprolegnia* treatment on survival.

Our study suggests that *Saprolegnia* kills newly-metamorphosed individuals of at least one anuran species (*R. cascadae*). Our study constitutes an important step in determining the effects of this pathogen on post-embryonic life history stages in nature. Taken together, the results of this study and those of previous studies (Romansic *et al.,* 2006, Walls & Jaegar, 1987, Lefcort *et al.,* 1997) suggest that *Saprolegnia*-induced mortality of amphibians in nature is not restricted to embryos and may greatly influence mortality rates in larvae and newly-metamorphosed individuals. Efforts to evaluate whether or not a pathogen contributes to amphibian population declines should not be restricted to a single life history stage.

3.6 Acknowledgements

A SEED grant from the Declining Amphibian Population Task Force, grants from the NSF IRCEB Program (DEB0213851 and IBN9977063), D. Olson of the USDA Forest Service Pacific Northwest Research Station, and an award from the Zoology Research Fund of the Oregon St. University Department of Zoology provided funding. J.M.R. was supported by an Oregon Sports Lottery Scholarship and an EPA STAR Fellowship (FP-91640201-0). We would like to thank J. Spatafora for instruction on

isolation of *Saprolegnia* and use of his laboratory. S. Andrew, J. Gonzales, Mona Jones-Romansic, J. Martin, L. Payton, E. Richmond, and A. Waggener provided assistance.

Table 3.1. Mean length (± 1 standard error) of preserved specimens.

Species	Mean length (mm)
Experiment 1	
Bufo boreas	14.0 ± 0.1, n = 50
Pseudacris regilla	12.5 ± 0.1, n = 39
Rana cascadae	13.6 ± 0.2, n = 47
Experiment 2	
Rana aurora	15.8 ± 0.2, n = 27

Table 3.2. Mean percent and proportion (in parentheses) of preserved specimens showing oomycotic growth consistent with *Saprolegnia* infection in control and *Saprolegnia* treatments. If variation in mean percent is present, ± 1 standard error is given. Mean percent data are for means across all relevant experimental units. Proportions are for all individuals in that category lumped together.

Table 3.2

Species	Treatment	Died[a]	Survived[b]	Total
Experiment 1				
Bufo boreas	Control	NA	0.0 (0/25)	0.0 (0/25)
	Saprolegnia	100.0 (3/3)	0.0 (0/22)	12.0 ± 4.9 (3/25)
Pseudacris regilla	Control	0.0 (0/12)	0.0 (0/13)	0.0 (0/25)
	Saprolegnia	16.7 ± 10.5 (2/15)	0.0 (0/10)	8.0 ± 4.9 (2/25)
Rana cascadae	Control	0.0 (0/2)	0.0 (0/23)	0.0 (0/25)
	Saprolegnia	66.7 ± 28.9 (3/5)[c]	0.0 (0/19)	13.0 ± 5.4 (3/24)[c]
Experiment 2				
Rana aurora	Control	0.0 (0/10)	0.0 (0/15)	0.0 (0/25)
	Saprolegnia	0.0 (0/14)	0.0 (0/11)	0.0 (0/25)

[a]Individuals that died during the experiment
[b]Individuals that survived the experiment
[c]Mean percent and proportion do not include one individual with possible oomycete growth.

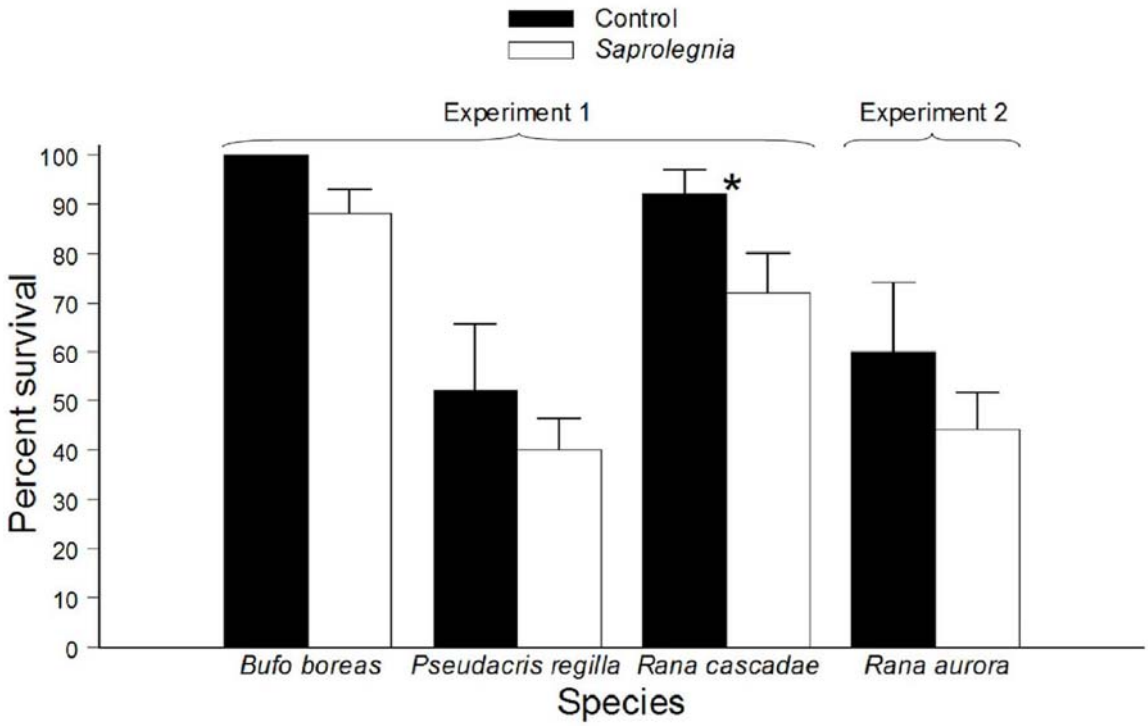

Figure 3.1. Percent survival of metamorphs of *Bufo boreas, Pseudacris regilla,* and *Rana cascadae* in experiment 1 and *Rana aurora* in experiment 2. Error bars are + 1 standard error. * Denotes the statistically significant difference between treatments for *Rana cascadae*.

Chapter 4

Effects of the Pathogenic Water Mold *Saprolegnia ferax* and Predator Cues on Survival of Amphibian Larvae

John M. Romansic, Kristin A. Diez, Elise M. Higashi and Andrew R. Blaustein

4.1 Abstract

Infectious diseases are a significant threat to worldwide biodiversity. Amphibian declines, a significant part of current biodiversity losses, are in many cases associated with infectious disease. The effects of pathogenic water molds on amphibian embryos have been studied extensively. However, little information is available about how water molds affect post-embryonic amphibians. We tested the effects of a species of water mold, *Saprolegnia ferax,* on larvae of four amphibian species; *Pseudacris regilla* (Pacific treefrogs), *Rana cascadae* (Cascades frogs), *Ambystoma macrodactylum* (long-toed salamanders), and *Rana aurora* (red-legged frogs). We also tested for a possible synergism between cues from predatory adult *Taricha granulosa* (roughskin newts) and *S. ferax*. *Saprolegnia ferax*, depending upon the species, can kill eggs and juvenile amphibians. A one-week exposure to *S. ferax* caused mortality in *P. regilla* larvae and a two-week exposure to *S. ferax* caused mortality in *R. aurora* larvae. Predator cues did not interact with *S. ferax* to affect survival of early stage *R. aurora* larvae. Our results suggest that *S. ferax* can kill amphibians at the larval life history stage and further suggests that evaluation of how water molds affect amphibian populations requires investigation of possible effects on various life stages.

4.2 Introduction

Biodiversity losses characterized by population declines, range reductions, and species extinctions are occurring at alarming rates (Raven, 1987, Myers, 1990, Wilson, 1992, Singh, 2002). One contributing factor is infectious disease (Daszak et *al.*, 2000, Harvell *et al.*, 2002, Altizer *et al.*, 2003). As part of these worldwide losses in

biodiversity, many amphibian populations are declining and disappearing (Houlahan *et al.,* 2000; Stuart *et al.,* 2004). Although many factors probably contribute to amphibian population declines (Alford & Richards, 1999, Blaustein & Kiesecker, 2002, Semlitsch, 2003, Collins & Storfer, 2003), infectious disease can be a major causal factor (Daszak *et al.*, 1999, 2003). The pathogenic fungus *Batrachochytrium dendrobatidis* (BD) is associated with many amphibian population declines (e.g. Berger *et al.*, 1998, Bosch & Martinez-Solano, 2001, Lips *et al.,* 2006, Pounds *et al.,* 2006, Rachowicz *et al.*, 2006, Schloegel *et al.*, 2006). However, other pathogens, including viruses, bacteria, trematodes, mesomycetozoans, and water molds may also contribute to increased mortality and population declines in amphibians (e.g. Worthylake & Hovingh, 1989, Blaustein *et al.*, 1994b, Cunningham *et al.*, 1996, Green et al., 2002, Greer, 2005, Johnson & Lunde, 2005).

Water molds (Stramenopila: Oomycota: Oomycetes: Saprolegniales) are fungus-like protists that inhabit aquatic habitats and moist soils (Dick, 1990). Most aquatic water molds are saprobes that grow on dead organic matter (Dick, 1990). Some water molds, including aquatic species, are capable of parasitism (Dick 1990, Johnson *et al.*, 2002a). Water molds that infect amphibians include *Achlya flagellata*, *Aphanomyces sp.*, *Saprolegnia ferax, S. parasitica,* and probably a number of other species (Tiffney & Wolf, 1939, Blaustein *et al.*, 1994b, Berger *et al.*, 2001, Lefcort *et al.*, 1997). *Saprolegnia ferax* contributes to massive mortality of frog embryos (Blaustein *et al.*, 1994b, Kiesecker & Blaustein, 1995). A mixed-species culture containing multiple species of both *Achlya* and *Saprolegnia* caused mortality and sublethal effects in embryos

of *R. sylvatica* (wood frog) and *B. americanus* (American toad) (Touchon *et al.*, 2006, Gomez-Mestre *et al.*, 2006). The effects of water molds on frog embryos appear to be influenced by several environmental factors, including temperature, pH, ultraviolet-B radiation, precipitation, plant cover, and oviposition behavior (Banks & Beebee, 1988, Kiesecker & Blaustein, 1995, Kiesecker & Blaustein, 1997, Green, 1999, Kiesecker *et al.*, 2001c, Ruthig, 2006).

In contrast to the extensive research on the effects of water molds on amphibian embryos, there is relatively little information available about how water molds affect post-embryonic amphibians. There is experimental evidence that *Saprolegnia* influences competitive interactions between larvae of *R. cascadae* and *P. regilla*, apparently by mediating densities of the competing species through differential effects on embryos (Kiesecker & Blaustein, 1999). Larvae with water mold infections occur occasionally in North America (Converse & Green, 2005). Berger *et al.* (2001) reported massive mortality of *Bufo marinus* (cane toad) larvae associated with *Aphanomyces* infection, and *Saprolegnia* has been found on dead amphibian larvae after mass mortality events (Bragg & Bragg, 1958, Bragg, 1962). Romansic *et al.* (2006) demonstrated that *Saprolegnia* can kill larvae of *R. aurora* (northern red-legged frog). Romansic *et al*. (*in press*) found that *Saprolegnia* is capable of killing newly metamorphosed *R. cascadae*.

Models suggest that mortality involving post-embryonic individuals may contribute significantly to population declines in amphibians (Vonesh & de la Cruz, 2002a,b, Biek *et al.,* 2002). Therefore, a full investigation of the potential effects of water molds on amphibian populations requires determining how these pathogens affect

various amphibian life history stages. We tested the effects of *S. ferax* on three kinds of amphibian larvae: 1) *P. regilla* in mid-late larval stages, 2) *R. cascadae* in mid-late larval stages 3) *Ambystoma macrodactylum* (long-toed salamander) larvae, and 4) *R. aurora* in an early larval stage. In addition, because the effects of *Saprolegnia* species on amphibians appear to be influenced by ecological conditions (Walls & Jaeger, 1987, Banks & Beebee, 1988, Kiesecker & Blaustein, 1995, Lefcort *et al.*, 1997, Kiesecker *et al.*, 2001c, Romansic *et al.*, 2006, Ruthig, 2006), we tested for a possible synergism between *S. ferax* and predator cues.

Many studies show that amphibians are affected by synergisms between environmental stressors, including pH-UV-B synergism (Pahkala *et al.*, 2002) nitrate-UV-B synergism (Hatch & Blaustein, 2003), and synergism between environmental contaminants (Boone *et al.*, 2005). Amphibians are also susceptible to synergisms between environmental contaminants and infectious agents (e.g. Taylor *et al.*, 1999, Kiesecker *et al.*, 2002, Christin et al. 2003, Forson & Storfer, 2006a). Predator cues are an environmental stressor that, due to their widespread occurrence, may be important in the disease ecology of amphibians. Amphibian larvae exposed to predator cues display a variety of responses, including behavioral avoidance, increased refuge use, reduced activity, reduced feeding, and hastened metamorphosis (e.g. Petranka *et al.*, 1987, Kats *et al.*, 1988, Feminella & Hawkins, 1992, Wilson & Lefcort, 1993, Kiesecker *et al.*, 2002, reviewed in Kats & Dill, 1998). Although these responses may reduce risk of predation in nature, they may lead to negative effects. Over the long term, reduced intake of food or the physiological demands of hastened development may prevent amphibians from

mounting an effective immune response. In addition, predator cues appear to be capable of direct negative effects on stressing amphibians. There is evidence consistent with predator cues acting alone to directly kill amphibian larvae (Relyea, 2003). Also, predator cues can interact with environmental contaminants to increase mortality in amphibian larvae (Relyea & Mills, 2001, Relyea, 2003, 2004, 2005). Therefore it seems possible that long-term exposure to predator cues may increase the susceptibility of amphibians to pathogens.

We tested for a synergism between predator cues and *S. ferax*, using *R. aurora* larvae as prey/hosts, and *T. granulosa* (roughskin newts) as predators. *Taricha granulosa* and *R. aurora* are sympatric in much of the Pacific northwest of North America (McAllister & Leonard, 2005, Kuchta, 2005) and adult *T. granulosa* (hereafter; newts) prey on *R. aurora* larvae (Nussbaum *et al.*, 1983). *Rana aurora* larvae respond to cues from adult newts fed *R. aurora* larvae with decreased activity, decreased time to metamorphosis, and reduced mass at metamorphosis (Wilson & Lefcort, 1993, Lefcort & Blaustein, 1995, Kiesecker *et al.*, 2002).

4.3 Materials and Methods

Collection and maintenance of amphibians

Pseudacris regilla larvae (Gosner (1960) developmental stages 25-35) were collected on 2 September 2002 from the Potholes subalpine meadow about 0.6 km NW of Todd Lake, Deschutes County, Oregon, USA (elevation about 1980 m). *Pseudacris regilla* were kept at a density of approximately 5 larvae per l of water for four days,

transferred to into new tanks with new water at a density of approximately 2.3 larvae/l, and transferred to new tanks with new water every seven days thereafter.

Rana cascadae larvae were collected from the Potholes meadow on 23 August and 18 September 2002. *R. cascadae* collected on 23 August ranged from stages 25-35. *R. cascadae* collected on 18 September ranged from stages 36 to 40. Prior to experimentation, *R. cascadae* were maintained in aquaria filled with about 35 l of water and not aerated. *Rana cascadae* were housed at a density of approximately 1.6 larvae/l and transferred to new tanks with new water every 6-9 days. *Rana cascadae* were kept at a lower density than *P. regilla* because they were larger in size than *P. regilla.*

Ambystoma macrodactylum larvae were collected on 28 August and 2 September from the Potholes meadow. Cannibalism sometimes occurs among *Ambystoma macrodactylum* larvae (Anderson, 1967, Walls *et al.*, 1993a,b). To prevent cannibalism, larvae were maintained in separate plastic cups (diameter at bottom = 8 cm, diameter at top = 11 cm, height = 11 cm) filled with 0.6 l of water. Prior to their use in experiment 2, *A. macrodacylum* were transferred to new cups with new water every 1-9 days and fed worms (*Tubifex sp.*).

Rana aurora embryo masses (approximately 290-350 embyros/mass) were collected from Coast Pond (~ 20 km south of Waldport, Lincoln County, Oregon, USA). Six masses (stages 18-21) were collected on 16 January 2003 (group A) and five masses (stages 19-22) were collected on 5 February 2003 (group B). Embryos were placed in aquaria (as above) filled with about 30 L of water and aerated. *Rana aurora* were transferred to new aquaria with new water every 7-8 days when not being tested in an

experiment. In group A, hatchlings (group A1) were separated from unhatched individuals (group A2) fifteen days after hatching began. Larvae in group A1 were used in experiment 2. During the time between separation and experiment 1, larvae in group A1 ranged in density from 2.5-9.5 larvae/l of water. Larvae in group B were used as focal larvae for experiment 3. Larvae in group B ranged in density from 2.8-10.5 larvae/l water at completion of hatching and from 2.8-8.8 larvae/l immediately prior to experiment 2. Density of larvae dropped in two aquaria due to removal of larvae for use as food for newts.

Fifteen adult male newts were collected from Coast Pond on 13 and 16 January, 2003. Newts were placed in individual 38-l aquaria (dimensions as above) filled with approximately 3 l of water to a depth of 2.5 cm. Each aquarium contained three square ceramic tiles (length = 11 cm) stacked to a height of 2 cm and placed against one end of the aquarium. Newts were transferred to new aquaria with new water every 7-9 days until the start of experiment 2.

Amphibians were kept in a laboratory maintained at approximately 13-15˚ C in 2002 and 10.5-14˚ C in 2003. All water used in the study, unless otherwise noted, was tapwater conditioned with NovAqua® and Amquel® water conditioners. Amphibians were kept under a natural photoperiod, except that *P. regilla* and *R. cascadae* in experiment 1 were transferred from a natural photoperiod to a 12 light:12 dark photoperiod at the start of experiment 1, as were *A. macrodactylum* larvae at the start of experiment 2. Prior to experimentation frog embryos and larvae were kept in 38-L aquaria (length × width × height = 50 × 25 × 31 cm). Frog larvae were fed a mixture (3:1

by volume) of rabbit chow and Tetramin fish flakes (hereafter; tadpole food). Metamorphosed frogs (≥ 50% tail resorption) were removed. Newts were fed *Tubifex sp.* 1-2 days after collection, *R. aurora* larvae from group A from January 20 to 13 February, and *R. aurora* larvae from group B from 17 to 25 February.

Collection, isolation, and culturing of Saprolegnia ferax

Saprolegnia ferax was isolated from a water sample that was collected on 10 September 2002 at the shore of Lost Lake in the Oregon Cascade Range (Linn County; elevation 1220 m), a site where *S. ferax* has contributed to massive mortality of *B. boreas* embryos (Blaustein *et al.,* 1994b, Kiesecker & Blaustein, 1995, Kiesecker *et al.,* 2001c). This isolate can also kill juvenile frogs (Romansic *et al.*, *in press*). *Saprolegnia ferax* was isolated using hemp seeds and YpG (yeast-glucose) agar media (Fuller & Jaworski, 1987). The isolate was identified using available keys and standard methods (Johnson *et al.* 2002, Seymour, 1970). Identification was confirmed using a DNA barcoding procedure and comparison to available DNA sequences in Genbank (J. E. J. *et al.*, unpublished).To obtain *S. ferax* for use in experiments, sterile hemp seeds were added to Petri dishes containing *S. ferax* cultures on YpG agar media to allow seeds to become inoculated with *Saprolegnia*. Seeds were then removed and added to standardized Petri dishes (diameter = 85 mm, height = 12 mm) filled approximately half full with ultrapure water and incubated for seven days at approximately 20-23º C. In experiments 1, 2, and 3, these dishes each contained fifty seeds. In experiment 4, these dishes each contained thirty seeds. *S. ferax* hyphae grew between seeds in dishes, producing clumps of seeds

connected by a mycelium of *S. ferax* containing hyphae and zoosporangia. These clumps of seeds were used to apply *S. ferax* treatments in experiments.

Experiment 1

Pseudacris regilla and *R. cascadae* larvae were exposed to *S. ferax* in a 2 × 2 factorial experiment that manipulated frog species and *S. ferax*. Experimental units consisted of plastic boxes (dimensions: 31 ×18 ×8 cm) filled with 2 l of water to a depth of 4 cm and stocked with six larvae. Larvae were added to units haphazardly, except that each unit received either 1) *P. regilla* (*P. regilla* treatment) or 2) *R. cascadae* (*R. cascadae* treatment). On the day following the start of the experiment, ten larvae of each species were haphazardly selected from the unused larvae. These larvae ranged in Gosner (1960) stage from 31 to 39 and in total length from 24.5 to 33.2 mm (*P. regilla*) and from 33 to 41 and in total length from 31.5 to 53.0 mm (*R. cascadae*). There were two pathogen treatments: 1) *S. ferax,* and 2) control. There were five replicates of each treatment combination, for a total of 20 experimental units. Treatment combinations were assigned to units randomly. The *S. ferax* treatment consisted of a clump of 50 hemp seeds overgrown with *S. ferax* hyphae and zoosporangia. The control treatment consisted of 50 sterile hemp seeds. A cage consisting of a small plastic box (length × width × height = 9.5 × 9.5 × 6.5 cm) with sides of 1-mm fiberglass mesh fiberglass mesh and no top was placed in the center of each unit. Seeds were placed inside the cage to prevent larvae from feeding on *S. ferax* and thus reducing the production of zoospores, but allowed passage of *S. ferax* zoospores. The experiment began on 30 September and lasted for seven days.

Experiment 2

Ambystoma macrodactylum larvae were exposed to *S. ferax* and control treatments. Experimental units consisted of plastic boxes and cages filled with water (as above). To prevent cannibalism, each unit was stocked with only one larva. Larvae were selected haphazardly and assigned to units randomly. Units received larvae in random sequence. The four remaining larvae not used in the experiment ranged from 40.8-58.2 mm in total length (mean ± 1 SE = 51.6 ± 3.9). There were two treatments: 1) *S. ferax,* and 2) control. Treatments were assigned to units randomly. There were 17 replicates of each treatment, for a total of 34 units. The *S. ferax* treatment consisted of a clump of 50 hemp seeds overgrown with *S. ferax* hyphae and zoosporangia. The control treatment consisted of 50 sterile hemp seeds. Six days after the experiment began, *A. macrodactylum* were fed *Tubifex* sp. The experiment lasted for seven days.

Experiment 3

Rana aurora larvae were exposed to *S. ferax* and control treatments. Experimental units consisted of plastic boxes and cages filled with water (as above) and stocked with 5 *R. aurora* larvae from group 1A. Five of the six embryo masses (masses 1 through 5) in group A were chosen at random and each unit received one haphazardly selected Gosner stage 25 larva (2-3 weeks post-hatching) from each of these masses. Units received larvae in random sequence. At the start of the experiment, two Gosner stage 25 group A1 larvae not used in the experiment were haphazardly selected from each of masses 1 through 5. These larvae ranged in total length from 15.7 to 25.1 mm (mean + 1 SE = 20.1 ± 0.80).

There were two treatments: 1) *S. ferax,* and 2) control. Treatments were assigned to units randomly. There were five replicates of each treatment, for a total of ten units. The *S. ferax* treatment consisted of a clump of 50 hemp seeds overgrown with *S. ferax* hyphae and zoosporangia. The control treatment consisted of 50 sterile hemp seeds. After seven days, larvae were transferred to new boxes with new water and new cages and treatments were re-applied. Each unit received 0.03 g of food at the start of the experiment and immediately after transfer to new boxes. The experiment began on 7 February and lasted for fourteen days.

Experiment 4

Rana aurora larvae were exposed to newt cues and *S. ferax* in a 2 × 2 factorial experiment. There were two cue newt cue treatments: 1) present and 2) absent. There were two pathogen treatments: 1) *S. ferax,* and 2) control. There were five replicates of each treatment combination, for a total of 20 experimental units. Treatment combinations were assigned to units randomly. The experiment consisted of two stages: 1) the newt cue treatment stage (stage 1) and 2) the pathogen treatment stage (stage 2).

At the start of stage 1, larvae were placed in aquaria (dimensions as above) filled with 5 l of water to a depth of 4 cm and containing a wooden cage placed in the center. Brown cardboard was placed on the sides of each aquarium to a height of 9 cm to block visual cues from neighboring aquaria. Cages (length × width × height = 20 × 12 × 5.5 cm) had 1-mm fiberglass mesh on all sides. Aquaria in the newt cues present treatment had one newt placed inside the cage, while aquaria in the newt cues absent treatment lacked newts. Ten newts were selected at random for use in the experiment from the

newts in the laboratory. Newts were assigned to aquaria in the newt cues present treatment at random, and these aquaria received their newts in random sequence. Each aquarium was stocked with ten larvae (focal larvae) from group 2. There were more larvae per unit in experiment 3 than in experiments 1 and 2 to increase precision of the results. Each aquarium received two haphazardly selected Gosner stage 25 larvae (2-3 weeks post-hatching) from each of the five masses in group 2. At the start of the experiment, four Gosner stage 25 larvae were haphazardly selected from the unused larvae of each of the five masses in group 2. These larvae ranged in total length from 17.8 to 23.9 mm (mean + 1 SE = 20.9 ± 0.4) and ranged in mass from 48 to 184 mg (mean + 1 SE = 89 ± 7). The newts used in the experiment were massed three days after the end of stage 1. They ranged in mass from 11.068 to 13.501 g (mean ± SE = 12.215 ± 0.303).

At the start of the experiment, 0.03 g of tadpole food was added to each aquarium. Every three days, each aquarium had waste and 75% of the water removed and replaced with fresh water and 0.03 g of tadpole food. Every two days, newts were fed by adding five larvae group 2 larvae to each cage containing a newt. The center of the top of each cage had a 1 cm slit that allowed addition of larvae, but prevented newt escape. Stage 1 lasted for a total of 16 days, after which larvae were transferred to stage 2. In stage 2, larvae were housed in boxes with cages as above and no newts were present. At the start of stage 2, *S. ferax* and control treatments were applied as in experiment 1, except that cages in the *S. ferax* treatment received clumps of thirty seeds and cages in the control treatment received thirty sterile seeds. After seven days, larvae were transferred to new

boxes with new water and new cages, the treatments were re-applied. During stage 2, all units received 0.03 g of tadpole food on the day of and four days following each application of the pathogen treatments. Stage 2 lasted a total of fourteen days. The experiment began on 27 February and lasted a total of thirty days.

All experiments were checked once per day. Larvae were examined visually for hyphal growths consistent with water mold infection in amphibian larvae (Converse & Green, 2005). Dead larvae were removed and preserved in 70% ethanol. Larvae surviving to the end of their experiment were euthanized using MS-222 and preserved in 70% ethanol.

Densities of zoospores and zoospore cysts

For every application of an *S. ferax* treatment, the combined number of zoospores and zoospore cysts of *S. ferax* were estimated using a cytometer for seed clumps randomly selected from the unused clumps in the batch used in the application (Table 4.1). These estimates were extrapolated to an estimated density of zoospores and cysts in the *S. ferax* treatment at application using the volume of water in units (Table 4.1).

Statistical Analyses

No *A. macrodactylum* larvae died in experiment 2. In all other experiments, survival data had heteroscedasticity that could not be removed by transformation. Therefore, for these experiments, we analyzed survival using nonparametric methods.

Survival in experiment 3 was analyzed using a rank-sum test. For experiment 1, we tested for differences among treatments and among treatment groups using a multiple comparisons procedure. We used a modified Bonferroni adjustment (Keppel, 1982) to

set $\alpha = 0.05$ for this procedure. There were k = 4 treatment combinations and 7 pairwise comparisons of interest *a priori*, so the *p*-value for rejection of null hypotheses in this procedure was set at 0.0286. Pairwise comparisons were made using rank-sum tests. To test for interactive effects of the frog species and *S. ferax* treatments, we used nonparametric multiple contrasts (Zar, 1999). One contrast was used to test whether the effect of the *S. ferax* treatment was different in *P. regilla* compared to *R. cascadae*. A second contrast was used to test whether the effect of frog species was different in the *S. ferax* treatment compared to the control treatment.

To analyze experiment 4, we used the same multiple comparisons and multiple contrast procedures used for experiment 1. One contrast was used to test whether the effect of the *S. ferax* treatment was different in the newt cues present compared to the newt cues absent treatment. A second contrast was used to test whether the effect of the newt cues present treatment was different in the *S. ferax* treatment compared to the control treatment.

In experiment 4, some larvae died during stage 1. To focus on testing the hypothesis that mortality from *S. ferax* was influenced by the presence of newt cues, for this experiment we analyzed only survival after the *S. ferax* treatment began.

4.4 Results

Experiment 1 (mid-late stage Psuedacris regilla *and* Rana cascadae *larvae)*

Survival was lower in the *S. ferax* treatment compared to the control treatment and lower in the *P. regilla* treatment compared to the *R. cascadae* treatment (Fig. 4.1, both comparisons: $Z = -2.4918$, $p = 0.0127$). Survival was lower in the *S. ferax* treatment

compared to the control treatment in the *P. regilla* treatment (Z = -2.8347, p = 0.0127). In the *R. cascadae* treatment, survival was 100% in both the *S. ferax* and control treatments. Also, survival was lower in the *P. regilla* treatment compared to the *R. cascadae* treatment in the *S. ferax* treatment (Z = -2.8347, p = 0.0127). In the control treatment, survival was 100% in both the *P. regilla* and *R. cascadae* treatments. The effect of the *S. ferax* treatment was not different in the *P. regilla* treatment compared to the *R. cascadae* treatment, and the effect of the *P. regilla* treatment was not different in the *S. ferax* treatment compared to the control treatment (both contrasts: S = -4.8941, $0.10 < p < 0.25$). Thus, there was no evidence that the effects of the *S. ferax* treatment on survival were greater in *P. regilla* compared to *R. cascadae*.

In the *S. ferax* treatment, there were thirteen observations of hyphae consistent with *S. ferax* infection on live *P. regilla* (Table 4.2). All larvae that died were in the *P. regilla*/*S. ferax* treatment combination and were partially or completely eaten by conspecifics before inspection for hyphae. Hyphae were not observed on either of the three partially eaten carcasses. Hyphae were not observed on *P. regilla* in the control treatment or on *R. cascadae*.

*Experiment 2 (*Ambystoma macrodactylum *larvae)*

Survival was 100% in both the control treatment and the *S. ferax* treatment. Hyphae were never observed on any *A. macrodactylum*.

Experiment 3 (stage 25 Rana aurora *larvae)*

Survival was lower in the *S. ferax* treatment compared to the control treatment (Fig. 4.2, Z = 2.8247, p = 0.0047). In the *S. ferax* treatment hyphae consistent with water

mold infection were observed on live individuals twelve times and on sixteen out of seventeen dead individuals found. One individual in the *S. ferax* treatment was completely eaten before inspection. No hyphae were observed any larvae in the control treatment.

Experiment 4 (Effects of Saprolegnia ferax *and predator cues on* Rana aurora *larvae)*

Survival was lower in the *S. ferax* treatment compared to the control treatment (Figure 4.3, $Z = -2.8120$, $p = 0.0049$). Survival was not different between the two newt cue treatments ($Z = -0.4687$, $p = 0.6393$). In addition, there were trends of lower survival in the newt cues present, *S. ferax* treatment combination compared to: 1), the newt cues absent, control treatment combination and 2), the newt cues present, control treatment combination (both comparisons: $Z = -1.9276$, $p = 0.0539$). There were also trends of lower survival in the newt cues absent, *S. ferax* treatment combination compared to: 1), the newt cues absent, control treatment combination and 2), the newt cues present, control treatment combination (both comparisons: $Z = -1.9640$, $p = 0.0495$). The other two pairwise differences between treatment combinations were not significant both $p \geq 0.5038$). The effect of the *S. ferax* treatment on survival was not different in the newt cues present treatment compared to the newt cues absent treatment ($S = -0.5624$, $0.90 < p < 0.95$). Also, the effect of the newt cues present treatment on survival was not different in the *S. ferax* treatment compared to the control treatment ($S = -0.5624$, $0.90 < p < 0.95$). Thus there was no evidence of an interaction between newt cues and *S. ferax* affecting survival.

In the newt cues present/*S. ferax* treatment combination, there were thirteen observations of hyphae consistent with *S. ferax* infection on live individuals (Table 4.2). Hyphae were not found on any of the three dead individuals found in this treatment combination, nor were hyphae observed on larvae in any of the other treatment combinations. One dead individual in the newt cues present/*S. ferax* treatment combination and one dead individual in the newt cues absent/*S. ferax* treatment combination were completely eaten by conspecifics before inspection.

During stage 1, survival was 92% in the newt cues absent/control treatment combination, 100% in the newt cues absent/*S. ferax* treatment combination, 96% in the newt cues present/control treatment combination, and 96% in the newt cues present/*S. ferax* treatment combination.

4.5 Discussion

Experiment 1 suggests that one week of exposure to *S. ferax* killed mid-late stage *P. regilla* larvae. In contrast, one week of exposure to *S. ferax* did not kill mid-late stage *R. cascadae*, although there was insufficient statistical evidence to demonstrate that *P. regilla* was more susceptible to mortality from *S. ferax* than *R. cascadae*. In Experiment 2, one week of exposure to *S. ferax* did not kill *A. macrodactylum* larvae. Experiments 3 and 4 suggest that two weeks of exposure to *S. ferax* killed early stage *R. aurora* larvae. In addition, hyphal growths consistent with *S. ferax* infection were observed on live *P. regilla* and live and dead *R. aurora* exposed to *S. ferax* treatments. Taken together, the results of our study demonstrate that *S. ferax* is capable of killing amphibian larvae.

Thus, evaluation of the possible effects of this pathogen on amphibian populations should take into account potential effects on larvae.

Although previous studies have found that predator cues and environmental contaminants can interact synergistically to kill amphibian larvae (Relyea & Mills, 2001, Relyea, 2003, 2004, 2005), we found no evidence that cues from predatory newts interacted synergistically with *S. ferax* to affect survival of *R. aurora* larvae. However, whether predator cues interact with pathogens may be context-specific. Synergisms between predator cues and environmental contaminants appear to be context-specific, and whether or not they occur may depend on the species of predator, the type of biocide, and the species of prey (Relyea 2003, 2004, 2005). It is possible that predator cues may influence susceptibility to infectious disease in other host-parasite systems. Also, in our study, the predator cue and pathogen treatments were not applied simultaneously, and this may have influenced the occurrence of a synergism between these factors.

Although we found no evidence that predator cues interacted with *S. ferax* to affect survival in our study, predators may interact with water molds in other ways. For example, water molds may colonize wounds on amphibians that are injured but not killed by predators. Skin wounds appear to make fish more susceptible to *Saprolegnia* infection (Richards & Pickering, 1978, Pickering, 1994, Hatai & Hoshai, 1994). Walls and Jaeger (1987) found that mortality of *A. maculatum* (spotted salamander) larvae caused by exposure to *A. talpoideum* (mole salamander) larvae in a laboratory experiment was associated with *Saprolegnia* infection of bite wounds inflicted by aggressive *A. talpoideum*.

Many of the pathogenic water molds have a wide host range and can persist and proliferate as a saprobe on dead organic matter (Tiffney, 1936, Dick, 1990, Johnson *et al.*, 2002). These characteristics give water molds considerable potential to affect amphibian populations. Water molds are likely to persist at fairly high densities even if the density of one or more amphibian species it utilizes drops. Thus, if mortality from a pathogenic water mold leads to a decline in an amphibian population, such mortality could continue as the population dwindles, thereby driving it to extinction or making it more susceptible to other factors.

Our results, together with previous research on amphibians and water molds (Kiesecker & Blaustein, 1995, Berger *et al.*, 2001, Kiesecker et al., 2001b, Romansic *et al.*, 2006, Romansic *et al.*, *in press*) suggest that multiple life history stages of amphibians are susceptible to these pathogens. However, this may depend upon the species of amphibian. For different species, the life stage most susceptible to water molds may differ. Additionally, patterns of mortality may depend on the species of water molds present. Our results highlight the need to investigate possible effects on multiple life stages of the host species when evaluating the potential effects of pathogens on host populations. If only one life history stage of the host species is considered, the effect of a pathogen on the host population may be underestimated.

Although infectious diseases are a significant threat to global biodiversity (Daszak et *al.*, 2000, Harvell *et al.*, 2002, Altizer *et al.*, 2003), the degree to which infectious diseases have contributed to the current biodiversity crisis is highly uncertain (Smith *et al.*, 2006). Thus there is an urgent need for better understanding of disease ecology so

that the risk of disease-related biodiversity losses may be more accurately measured and species and communities at risk of disease-related losses may be identified (Smith *et al.*, 2006). Evidence that anthropogenic environmental changes, pathogen pollution, and introduction of non-native pathogens by humans have already increased the impact of infectious disease some species (e.g. Anagnostakis, 1987, Conrad *et al.*, 2005, Johnson *et al.*, 2007), combined with the prediction that climate change will cause an overall increase in the impact of infectious diseases on natural communities (Harvell *et al.*, 2002), underscores the importance of making meaningful progress in our understanding of the ecology of infectious disease.

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Table 4.1. Mean estimates (± 1 SE) of the combined number of zoospores and zoospore cysts and mean estimates of density of the combined number of zoospores and zoospore cysts in each application of an *S. ferax* treatment.

Experiment	Application	Mean estimate ± 1 SE	n	Mean estimate of density (zoospores and zoospore cysts/l of water) at application
1	Sole application	1.3×10^8	1	6.6×10^7
2	Sole application	$1.3 \times 10^8 \pm 6.5 \times 10^6$	3	6.5×10^7
3	Initial application	$4.4 \times 10^7 \pm 7.8 \times 10^6$	2	2.2×10^7
	Re-application	3.3×10^7	1	1.6×10^7
4	Initial application	2.9×10^7	1	1.5×10^7
	Re-application	4.6×10^7	1	2.3×10^7

Table 4.2. Results of examinations for hyphal growths. For experiment 3, only data for stage 2 are shown. Larvae that were completely eaten before inspection are not included.

Experiment	Treatment/treatment combination	# (percentage) of dead individuals with hyphae	# of instances hyphal growth was observed on a live individual
1	*P. regilla*/control	NA	0[a]
	P. regilla/*S. ferax*	0/3[c]	13[b]
	R. cascadae/control	NA	0[a]
	R. cascadae/*S. ferax*	NA	0[a]
2	Control	NA	0[a]
	S. ferax	NA	0[a]
3	Control	NA	0[a]
	S. ferax	16/17[d]	12[a]
4	Newt cues absent/control	NA	0[a]
	Newt cues absent/*S. ferax*	0/2[e]	0[a]
	Newt cues present/control	NA	0[a]
	Newt cues present/*S. ferax*	0/3[e]	8[b]

NA denotes that they were no carcasses in that category.
[a]At the end of the experiment, hyphal growths were not found on any of the live larvae.
[b]At the end of the experiment, one live individual had hyphal growth.
[c]All three carcasses were partially eaten.
[d]One carcass with hyphae was partially eaten.
[e]Two carcasses were partially eaten.

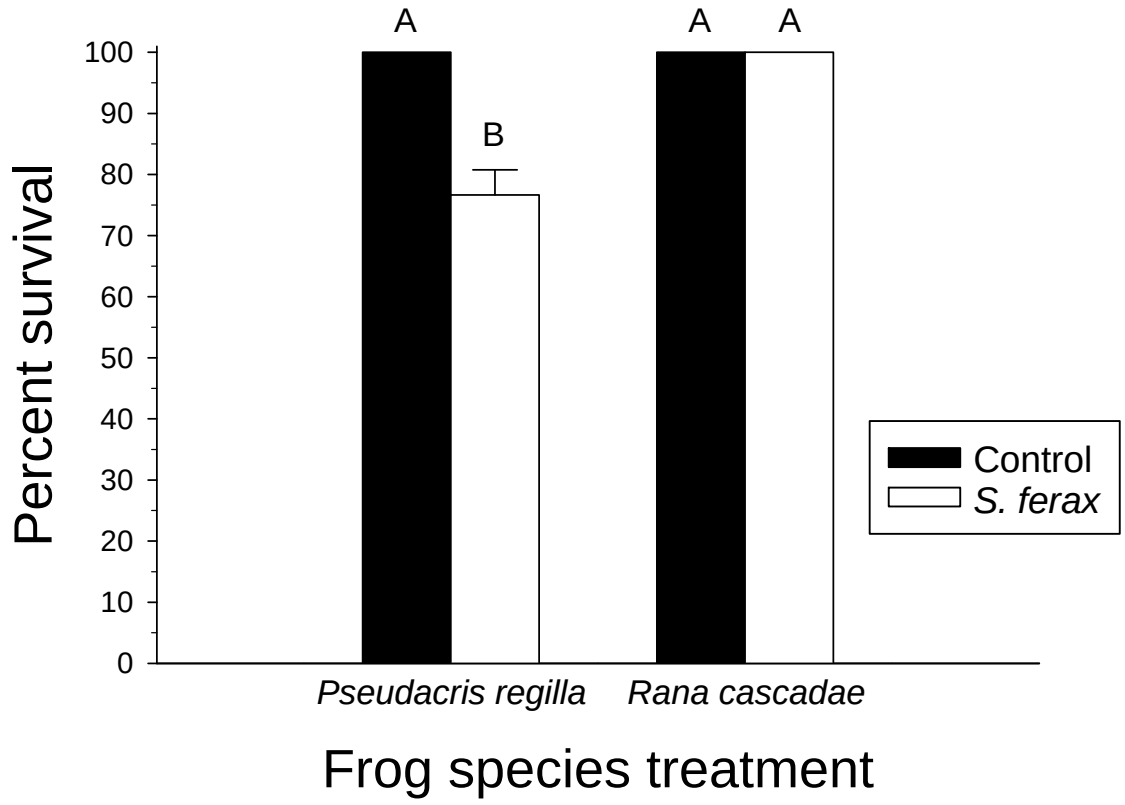

Figure 4.1. Survival (+ 1 SE) of *Pseudacris regilla* and *Rana cascadae* larvae exposed to *Saprolegnia ferax* and control treatments in experiment 1. Treatments that share a letter are not different statistically ($\alpha = 0.05$).

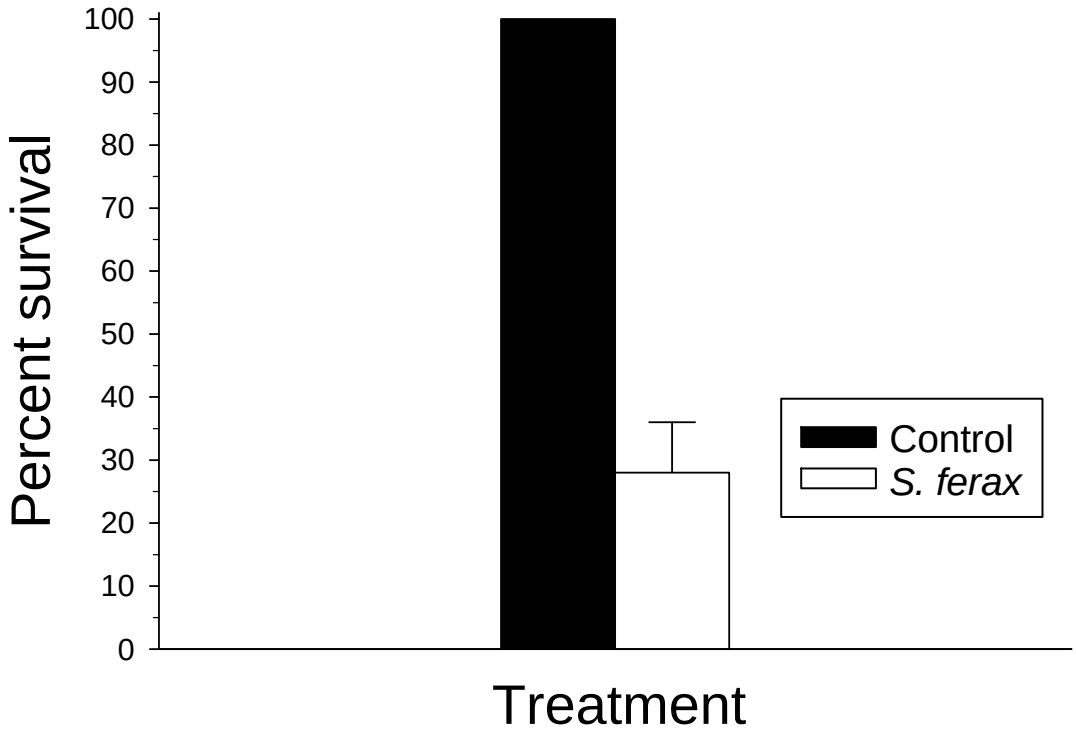

Figure 4.2. Survival (+1 SE) of *Rana aurora* larvae exposed to *Saprolegnia ferax* and control treatments in experiment 3.

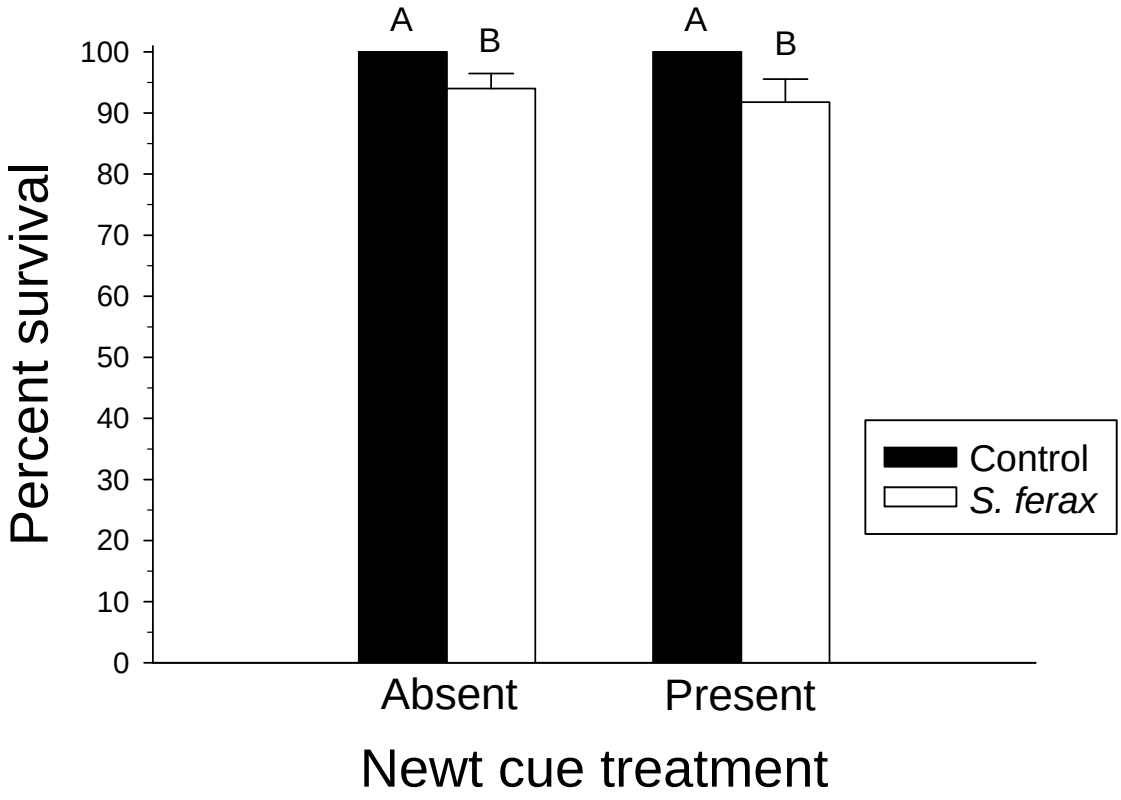

Figure 4.3. Survival (+ 1 SE) during stage 2 (exposure to pathogen treatments) of *Rana aurora* larvae exposed to the presence and absence of newt cues in experiment 4. Treatments that share a letter are not different statistically ($\alpha = 0.05$).

Chapter 5

Individual and Combined Effects of Multiple Pathogens (*Achlya flagellata, Ribeiroia*, and *Batrachochytrium dendrobatidis*) on Pacific Treefrogs (*Pseudacris regilla*)

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5.1 Abstract

In nature, individual hosts encounter multiple pathogens simultaneously. Two or more pathogens may have synergistic, additive, or antagonistic effects on hosts. If their effects on hosts are severe, they may affect whole host populations. Although several pathogens are implicated in amphibian population declines, interactions between pathogens have received very little attention. Using *Pseudacris regilla* (Pacific treefrog) larvae as hosts, we investigated interactions among three amphibian pathogens: the trematode *Ribeiroia*, the fungus *Batrachochytrium dendrobatidis* (BD), and the oomycete *Achlya flagellata* using a 2 × 2 × 2 factorial experimental design. We measured survival, prevalence of limb deformities, mass at metamorphosis, rate of development, prevalence of oomycete infection, and BD infection load. Exposure to *Ribeiroia* cercariae caused elevated mortality, increased frequency of limb deformities, and delayed development in *P. regilla*. The composition of deformity types produced by *Ribeiroia* differed from that in a previous study of *P. regilla*, suggesting that this composition is context-dependent. Extra limbs and limb elements accounted for few of the *Ribeiroia*-induced deformities. Also, prevalence of extra limbs or limb elements in *Ribeiroia*-exposed individuals was low. Missing limbs and limb elements were approximately four times as common as extra limbs and limb elements among *Ribeiroia*-exposed individuals, suggesting that extra limbs and limb elements are not always the most common class of deformities this pathogen produces. Exposure to BD accelerated development in larval treefrogs, despite the fact that no BD infections were found. Thus, in our study, exposure to BD caused sublethal but biologically significant effects, even in the apparent absence of infection.

Larvae exposed to BD may have responded to its presence by increasing their rate of development. Thus, in our study, *Ribeiroia* and BD both affected rate of development in their amphibian host, but in opposite directions. We found no effects of *Achlya* treatment or strong evidence of interactions between pathogens. However, the occurrence of such interactions may be context-dependent. Our study is one step in testing whether between-pathogen interactions play a role in amphibian population declines. Due to the context dependency in the disease ecology of amphibians, tests for possible between-pathogen interactions in different pathogen and host species and different ecological scenarios are warranted.

5.2 Introduction

Infectious diseases cause a multitude of negative effects on human health, agriculture, and livestock and biodiversity (Daszak *et al.*, 2000, Harvell *et al.*, 2002, Altizer *et al.*, 2003, Scholthof, 2003). The social and economic costs of infectious diseases to human societies are far-reaching and severe (for reviews see Marra *et al.*, 1998, O'Lorcain & Holland, 2000, Sachs & Malaney, 2002, Korsak *et al.*, 2004, Russell, 2004). Strategies to lessen the unwanted effects of infectious diseases rely on conceptual and mathematical models of disease ecology. Most models in disease ecology include only one pathogen (we include microparasites and macroparasites under the term "pathogen") (e.g., Anderson and May, 1991, Lafferty & Holt, 2003, Schauber & Ostfeld, 2002). However, in real environments, individual hosts are exposed to multiple pathogens simultaneously throughout their lives. Multiple pathogens can have synergistic, additive, or less-than-additive effects on hosts (Briggs, 1993, Chang *et al.*,

1994, Briggs & Latto 2001, Thacker *et al.*, 2001, Safi *et al.*, 2003, Hassan *et al.*, 2006, Traina-Dorge *et al.*, 2007). How multiple infectious agents influence host populations, however, is less clear, owing to the difficulties inherent in experimentally manipulating host populations. Despite this, a pattern consistent with "ecological interference", in which one pathogen negatively affects a second pathogen by removing individuals from the pool of susceptible hosts, has been found in records of measles and pertussis in humans (Rohani *et al.*, 2003). Similar between-pathogen interactions at the level of the host population are included in several mathematical models (e.g. Briggs *et al.*, 1993, Kirschner, 1999, Allen *et al.*, 2003, Pedersen & Mills, 2004). Importantly, in some host-pathogen systems, two pathogens appear to interact synergistically to influence population-level host parameters. These phenomena may arise from the two pathogens interacting synergistically at the level of the host individual (e.g. Tristão-Sá *et al.*, 2002, Elias *et al.*, 2006, Pion *et al.*, 2006). However, antagonistic interactions can also occur between pathogens (Read & Phifer 1959, Holmes 1960, 1961, Lim & Heyneman, 1972, Kuris, 1990, Sousa, 1990, 1993). There is evidence consistent with competition between pathogens reducing infection prevalence (Icenhour *et al.*, 2006).

We tested for possible interactions among three amphibian pathogens acting at the level of individual hosts, using larvae of *Pseudacris regilla* (Pacific tree frog). The pathogens are the trematode *Ribeiroia,* the oomycete *Achlya flagellata* and the fungus *Batrachochytrium dendrobatidis* (BD). All three of these pathogens are implicated in amphibian mortality or pathology (Johnson *et al.*, 2002b, Tiffney & Wolf 1939, Berger *et al.*, 1998). Our study has important implications for conservation, especially

conservation of amphibians, due to numerous pathogen-associated population declines in amphibians (e.g. Worthylake & Hovingh, 1989, Cunningham *et al.*, 1996, Berger *et al.*, 1998, Greer, 2005, Rachowicz *et al.*, 2006, Schloegel *et al.*, 2006).

Ribeiroia uses snails of the family Planorbidae as first intermediate hosts, fish, frogs and salamanders as second intermediate hosts, and birds and mammals as definitive hosts (e.g. Marin, 1928, Price, 1931, Beaver 1939, Yamaguti, 1971, Molnar *et al.*, 1974, Bowerman & Johnson, 2003, Johnson *et al.*, 2004). Infected snails shed free-swimming *Ribeiroia* cercariae, which infect frog and salamander larvae and encyst as metacercariae (Johnson *et al.*, 1999). *Ribeiroia* infection causes a variety of limb deformities in amphibians (Blaustein and Johnson 2003; Johnson *et al.*, 1999, 2001a, 2006, Schotthoefer *et al.*, 2003). It can also cause elevated mortality, delayed metamorphosis, reduced mass at metamorphosis, and delayed regeneration following limb injury (Johnson *et al.*, 1999, 2001a, 2006, Kiesecker, 2002, Schotthoefer *et al.*, 2003). *Ribeiroia*-associated limb deformities are widespread in the United States (Johnson *et al.*, 2002b, 2003, Kiescecker 2002), and there is qualitative evidence supporting the hypothesis that the prevalence of *Ribeiroia*-induced limb deformities in amphibians in the western USA has recently increased (Johnson *et al.*, 2003; Johnson and Lunde 2005).

Achlya flagellata, which is closely related to *Saprolegnia*, is capable of saprobism and parasitism (Johnson *et al.,* 2002a). It can contribute to mortality in amphibians and fish (e.g. Srivastava & Srivastava, 1975, 1976, Khulbe 1992, Khulbe *et al.* 1995, Bisht *et. al.*, 1996; see Johnson *et al.* (2002a) for additional references), and

has been linked to large die-offs of fish in natural and hatchery environments (Khulbe, 1992, Bisht *et al.*, 1996; Tiffney and Wolf, 1939).

Tiffney and Wolf (1939) found diseased red-spotted newts (*Notopthalamus viridescens*) infected with hyphal growths in Massachusetts, USA. Examination of infected individuals revealed *A. flagellata/Saprolegnia parasitica* co-infections (Tiffney & Wolf, 1939). In addition, other species of *Achlya* are associated with mortality of amphibian embryos. *Achlya glomerata* has been isolated from dead embryos of *Rana luteiventris* (Columbia spotted frog) in Wyoming, USA and *Ambystoma maculatum* (spotted salamander) in Maryland, USA (Green & Converse 2005). *Achlya racemosa* has been found on embryos of *A. macrodactylum* (long-toed salamander) in northwest North America (Pilliod & Fronzuto, 2005). Multiple species of *Achlya* have been found on dead embyros of *R. sylvatica* (wood frog) and *B. americanus* (American toad) in Massachusetts, USA (Touchon *et al.*, 2006, Gomez-Mestre *et al.*, 2006). Water mold cultures containing multiple species of both *Achlya* and the closely related genus *Saprolegnia* caused mortality and early hatching in embryos of *R. sylvatica* and *B. americanus* and in de-jellied embryos of *A. maculatum* (spotted salamander) (Touchon *et al.*, 2006, Gomez-Mestre *et al.*, 2006).

The recently described chytridiomycte fungus *Batrachochytrium dendrobatidis* (BD) has been linked to amphibian mortality in several populations (Berger *et al.*, 1998, Longcore *et al.*, 1999, Bosch & Martinez-Solano, 2001, Bradley *et al.*, 2002, Lips *et al.*, 2006, Schloegel *et al.*, 2006, Rachowicz *et al.*, 2006, Bosch *et al.*, 2007). BD infects the keratinized mouthparts of frog tadpoles and the keratinized skin of adult and newly

metamorphosed frogs and salamanders (Berger *et al.*, 1998, Davidson *et al.*, 2003). BD can kill both larval and post-metamorphic frogs (Berger *et al.*, 1998, Woodhams et *al.*, 2003, Rachowicz & Vredenburg, 2004, Blaustein *et al.*, 2005). Moreover, BD can directly and indirectly (through interactions with predators and competitors) cause a variety of sublethal effects in frog larvae. These effects include partial or complete loss of the keratinized mouthpart structures, reduced mass at metamorphosis, increased time to metamorphosis, and increased asymmetry (Berger *et al.*, 1998, Parris, 2004, Parris and Baud, 2004, Parris and Beaudoin, 2004, Parris and Cornelius, 2004, Blaustein *et al.*, 2005). In addition, Parris et al. (2006) found that BD caused decreased activity, enhanced antipredator behavior, and increased survival in the presence of predators in *Rana pipiens* (northern leopard frog) larvae.

Ribeiroia, A. flagellata, and BD have high potential to interact on amphibian hosts, since all three infect the epidermis. *Achlya flagellata* may be a wound parasite (Tiffney and Wolf, 1939). Pickering and Willoughby (1997) found that the early lesions in diseased *Perca fluviatilis* L. (perch) that developed water mold infection were similar to bites from the fish louse *Argulus foliaceous*, suggesting that the initial lesions were caused by these bites. Thus, it is possible that *A. flagellata* and *Ribeiroia* interact synergistically, with *A. flagellata* colonizing wounds in the skin of amphibian larvae caused by entry of *Ribeiroia* cercariae. Alternatively, *A. flagellata* zoospores may attack *Ribeiroia* cercaria, leading to less-than-additive effects of these two pathogens on amphibian hosts. In addition, zoospores of *A. flagellata* and BD may compete for colonization sites, possibly leading to less-than-additive effects on amphibians.

Our study tested for interactive effects of the pathogens on survival, frequency of limb deformities, rate of development, mass at metamorphosis, BD infection, and hyphal infection consistent with *A. flagellata*. *Pseudacris regilla* is an ideal host species in which to measure these parameters because it can be kept at warm temperatures that allow rapid development to metamorphosis, at which time limb deformities may be easily assessed. Also, *P. regilla* is susceptible to infection, mortality, and limb deformities from *Ribeiroia* (Johnson *et al.*, 1999, 2002b, 2003) and infection with BD (Blaustein *et al.*, 2004, T. T., *unpublished data*). In addition, mid to late stage *P. regilla* larvae are susceptible to mortality from *S. ferax*, a water mold closely related to *A. flagellata* (Romansic *et al.*, Chapter 4, this volume).

5.3 Materials and Methods

Collection and maintenance of Pseudacris regilla

Thirty masses of *P. regilla* embryos (Gosner (1960) developmental stages: 12-16) still in their egg case were collected on 26 June 2006 at Little Three Creek Lake (Deschutes County, Oregon, USA, about 21 km southwest of Sisters; elevation: about 2,040 m). Prior to the start of the experiment, *P. regilla* were kept in 38 L glass aquarium (length × width × height = 50 × 25 × 31 cm, 15 embryo clutches/aquarium) filled with approximately 35 L of tap water and aerated. Tap water was conditioned with NovAqua® and Amquel® (~0.14 ml of each conditioner per l of water). *P. regilla* were kept in a laboratory maintained at 13.5-16.5˚ C under a natural photoperiod and transferred to new aquaria 7-8 days until 27 days after collection, when they were transferred to new aquaria and moved to a separate laboratory maintained at 18.5-22.5˚

C. The light cycle in this laboratory was set to last from ten minutes before sunrise to ten minutes after sunset to approximate a natural photoperiod and was reset every 15-22 days. Three days after transfer to the warmer laboratory, larvae were transferred to new aquaria. *P. regilla* spent the seven days prior to the start of the experiment in the warmer room. Throughout the study, larvae were fed a mixture (3:1 by volume) of rabbit chow and Tetramin fish flakes. Prior to the start of the experiment, larvae were kept at a density of 12 larvae/l of water.

Experimental protocol

We used a 2 × 2 × 2 factorial design with two treatments (exposure and control) for each of the three pathogens (*Achlya flagellata* (hereafter: *Achlya*), *Ribeiroia*, and BD). There were 20 replicates of each treatment combination, except for *Achlya* control, *Ribeiroia* exposure, BD control combination, which had 21 replicates. There was one larva per experimental unit and a total of 161 experimental units. Treatments were applied to experimental units randomly.

Ribeirioa *treatments*

Snails of the genus *Planorbella* were collected on 26 July from a pond in the E. E. Wilson Wildlife Refuge (18 km north of Corvallis (Benton County, Oregon; elevation 60 m) and were maintained in the laboratory at 19-20˚ C for five days without food. This site has a history of high *Ribeiroia* infection and severe limb deformities in Pacific treefrogs (Johnson *et al.*, 2002b, Romansic *et al.*, unpublished). Six snails that were shedding *Ribeiroia* cercariae were placed in individual plastic beakers (diameter at bottom = 5 cm, diameter at top = 6 cm, height = 8 cm) filled with 100 ml of water

(conditioned as above) and placed in darkness for six hours, after which the snails were removed. Five of the six beakers were chosen haphazardly and their contents were combined in a single large plastic beaker (diameter at bottom = 9 cm, diameter at top = 11 cm, height = 14 cm). The resulting water with cercaria was mixed by gently pouring back and forth between two beakers. The water was then divided equally between two large beakers and 500 ml of water (conditioned as above) was added to each beaker. The contents of each beaker were mixed by gently pouring back and forth using a second large beaker and used as sources of the doses for the *Ribeiroia* exposure treatment. Each dose consisted of five ml of water and 15 cercaria from one of the two sources were placed in small plastic beakers (diameter at bottom = 2 cm, diameter at top = 3 cm, height = 2 cm). The *Ribeiroia* exposure treatment was applied by using stainless steel forceps to tilt the small beaker sideways, submerge it in the water of the experimental unit, and place it on its side against the side of the unit such that it opened toward the center of the unit. The *Ribeiroia* control treatment was applied the same way, except that five snails not shedding cercaria were used, and no cercaria were added to the small beakers.

Achlya *treatments*

Achlya flagellata (hereafter: *Achlya*) was isolated from water taken on 2 July 2006 at Scott Lake (about 14 km northeast of Belknap Springs, Lane County, Oregon; elevation: ~ 1,460 m) using hemp seeds and YpG (yeast-glucose) agar media (Fuller & Jaworski, 1987). The isolate was identified using available keys and standard methods (Johnson, 1956, Johnson *et al.* 2002a). Identification was confirmed using a DNA

barcoding procedure and comparison to available DNA sequences in Genbank (Johnson *et al.*, *unpublished data*). Sterile hemp seeds were inoculated by addition to Petri dishes (diameter = 85 mm, height = 12 mm) containing YpG agar media and a YpG agar plug containing *Achlya*. Thirty-five inoculated seeds were added to each of four dishes filled with about 46 ml of sterile ultrapure water. Dishes were incubated at 22-22.5˚ C for three days. One dish was selected haphazardly for a count of zoospores and zoospore cysts. Its contents were gently stirred using a pipette, a sample of its water was taken, and a total count of zoospores and zoospore cysts was obtained using a cytometer. Estimated number of zoospores and zoospore cysts in the dish was 1.6×10^8. The contents of the other three dishes were gently stirred using a pipette and the water (containing zoospores and zoospore cysts) was removed. The water from each of these three dishes was combined and 1 ml of the combined solution was added to each unit in the *Achlya* exposure treatment. Multiples species of *Achlya* and *Saprolegnia*, including *A. flagellata*, have each been measured at zoospore concentrations in the range of 5-25 $\times$ 10^3 zoospores per l in a reservoir during a three-year period in which massive mortality of fish occurred in association with water molds (Bisht *et al.*, 1996). We used a level of *Achlya* exposure that approximated higher concentrations of *A. flagellata* zoospores that may occur, for example, in the vicinity of carcasses of fish or amphibians being decomposed by *A. flagellata*. Units in the *Achlya* control received water in the same way as units in the *Achlya* exposure treatment, except that the hemp seeds in the dishes were sterile and had been sham-inoculated by adding them to sterile YpG agar dishes containing sterile YpG agar plugs. Extrapolation using the zoospore and zoospore cyst

count and the volume of water in each unit yields an estimated concentration of a total of 3.4×10^6 zoospores and zoospore cysts per l in the *Achlya* exposure treatment.

BD treatments

BD isolate JEL 274 was grown on thirteen Petri dishes containing 1% tryptone agar media and incubated for 13 days at 22-22.5˚ C. Each BD treatment was applied in two stages to minimize handling time of zoospores and possible settling of zoospores prior to addition to experimental units. The eleven dishes that had an average level of growth (determined by visual inspection) were selected for the experiment. Five of these eleven dishes were selected haphazardly and used for the units in the *Achlya* control/BD exposure treatment combination. Each of the five dishes was flooded with 3.0 ml of ultrapure water and 2.5 ml of resulting BD zoospore solution was removed from each dish. The solutions from the five dishes were combined. Each unit in the *Achlya* control/BD exposure treatment combination received 0.25 ml of this combined zoospore solution. This treatment level was chosen to approximate the zoopore concentration that resulted in high prevalence of BD infection in *P. regilla* larvae in a previous study (Blaustein *et al.*, 2005). Five of the remaining six selected BD dishes were chosen haphazardly and used in the above manner to treat the units in the *Achlya* exposure/BD exposure treatment combination. Units in the *Achlya* control/BD control and *Achlya* exposure/BD control treatment combination were treated the same way, except that each received solution from five haphazardly selected dishes from a batch of ten sterile dishes containing 1% tryptone agar media. Zoospores were counted for the remaining BD dish using a hemacytometer. Estimated number of zoospores on this dish was 6.5×10^6.

Extrapolation using this number and the volume of water in each unit yields an estimate zoospore concentration in the BD treatment of 1.1×10^6 zoospores per l, which was close to the estimated 1.4×10^6 zoospores per l to which *P. regilla* larvae were exposed in Blaustein *et al.*, 2005).

Experimental procedure

At the start of the experiment, *P. regilla* larvae were selected haphazardy and examined with a hand lens (10× magnification) to determine Gosner (1960) developmental stages. After staging, larvae were placed in plastic bins (length × width × height = 27 × 16 × 12 cm) filled with 4 l of water (1 bin/stage for stages 26-28). Once a sufficient number of stage 26-28 individuals were obtained, individuals of other stages were returned to their aquaria. Stage 26-28 individuals were placed in individual, plastic beakers (dimensions as above) filled with 100 ml of water and no food. Larvae were added to beakers haphazardly.

The *Ribeiroia* treatments were applied between 2000 and 2230 hours, after which larvae were kept in darkness until dawn the following morning. Approximately ten hours after the *Ribeiroia* treatments were applied, the small beakers were removed and the contents (including the larva) of each medium sized beaker was poured into a large beaker (dimensions as above) containing 500 ml of water. Immediately prior to this transfer, six haphazardly chosen beakers in the *Ribeiroia* treatment were inspected visually and with a dissecting microscope for cercariae. No cercariae were found, consistent with all cercariae entering the larvae. Approximately six and 12 hours later, the *Achlya* and BD treatments were applied, respectively.

Ten minutes after application of the BD treatments, each larva received food. Thereafter, food was applied daily such that each larva always had food present. Three days after the application of treatments was completed, each larva was transferred to a new beaker with 600 ml of fresh water, with water and beaker changes occurring every 4-5 days thereafter.

Dead individuals were removed, swabbed for BD, and preserved in 70% ethanol. Individuals with both forelimbs emerged (stage 42) were considered metamorphosed and were removed, inspected on the dorsal side visually and with a dissecting microscope for hyphae consistent with oomycte infection, checked for abnormalities in range of motion of the joints of the limbs, swabbed, euthanized with MS-222, inspected on the ventral side visually and with a dissecting microscope for hyphae, and preserved in 70% ethanol. The experiment ran for 54 days after application of the treatments was completed, at which time 87% of individuals were dead and/or metamorphosed. At the end of the experiment, all surviving individuals were inspected, swabbed, euthanized, and preserved the same way metamorphosed individuals were.

Preserved individuals were inspected visually and with a hand lens (10× magnification) and dissecting microscope for deformities and preserved stage 42 individuals were massed (wet mass) to the nearest 0.1 mg. Hyphae were examined under a compound microscope (400× magnification) for the presence of coenocytic hyphae and reproductive structures consistent with *Achlya*.

Each swab was analyzed by real-time PCR, following the protocol of (Boyle *et al.*, 2004), which provided a quantitative estimate of BD DNA on each swab. Standards

of 100, 10, 1, 0.1, and zero BD zoospore genome equivalents were included in triplicate on each of the four reaction plates run.

Leftover larvae not used in the experiment were used to estimate the initial lengths and masses of the larvae used in the experiment. Unused larvae were transferred to the colder laboratory room at the start of the experiment to minimize their growth and development before measurement. Two days after the start of the *Ribeiroia* treatments, 21 unused larvae were selected haphazardly, with the constraint that they did not appear to fall outside the stage 26-28 range. The seventeen individuals in this sample that were between stages 26 and 28 ranged from 14.24 to 22.18 mm in total length (mean ± 1 SE = 18.94 ± 4.59) and from 31 to 97 mg in mass (mean ± 1 SE = 64 ± 4). At the start of the experiment, all larvae ranged in age from 18 to 27 days post-hatching.

Statistical Analyses

Frequency of limb deformities (frequency of individuals with ≥ 1 limb deformity) among stage 42 individuals and survival were analyzed using multiple contrasts of proportions (Zar, 1999), an adaptation of the Scheffé procedure for multiple contrasts among means, to test for main effects of each treatment and to test for interactions between treatments. We tested for interactions between treatments by testing whether the effect of a particular treatment or treatment combination was different in different treatments or treatment combinations. We also tested for pairwise differences in survival and frequency of limb deformities between treatment combinations using Tukey-type multiple comparisons of proportions (Zar, 1999).

Rate of development was analyzed by adapting using Cox Proportional Hazards models, which are commonly used in survival analysis (Parmar & Machin, 1996). Our Cox Proportional Hazards models quantified the hazard of metamorphosis not occurring. Differences in the hazard of an event occurring (or not occurring) serve as an overall measurement of the differences between survival curves (Parmar & Machin, 1996). Thus, differences in hazard of metamorphosis not occurring described differences in the production of metamorphs among different treatments. Three separate models were used to analyze rate of development. Model 1 included all individuals and tested for effects of the experimental treatments. Model 2 tested for treatment effects among individuals that did not die or display limb deformities following *Ribeiroia* exposure. Model 2 excluded individuals in the *Ribeiroia* exposure treatment that died or displayed limb deformities to test whether *Ribeiroia* affected development in individuals that did not die or display limb deformities following *Ribeiroia* exposure. From this data set, individuals not reaching stage 37 (separation of toes on hindlimbs) were excluded. Model 3 used only individuals in the *Ribeiroia* exposure treatment that reached stage 37 and tested for effects of treatment and limb deformity status. Mass at metamorphosis was analyzed using ANOVA. In the developmental rate and mass at metamorphosis analyses, initial stage was included in the model as a continuous variable. Mass data met parametric assumptions of normality and homoscedasticity and thus were not transformed.

5.4 Results

Survival

Survival was lower in the *Ribeiroia* exposure treatment (83%) compared to the *Ribeiroia* control treatment (99%) (Fig. 5.1, Table 5.1, S = -4.5358, $0.0005 < p < 0.001$). Neither *Achlya* (S = 1.2180, $p > 0.25$) nor BD treatment (2.0852, $p > 0.25$) influenced survival. None of the multiple contrasts testing for interactions were significant (all $p > 0.25$), indicating that the effect of *Ribeiroia* on survival did not depend on the other treatments. Survival was 75% in the *Achlya* control/*Ribeiroia* exposure/BD exposure treatment and the *Achlya* exposure/*Ribeiroia* exposure/BD exposure treatment combination. In each of these two treatment combinations, survival was lower than in the *Achlya* control/*Ribeiroia* control/BD control treatment combination ($q_{\infty, 8}$ = 4.7414, $0.01 < p < 0.025$, Table 5.2), the *Achlya* exposure/*Ribeiroia* control/BD control treatment combination ($q_{\infty, 8}$ = 4.7414, $0.01 < p < 0.025$), and the *Achlya* exposure/*Ribeiroia* control/BD exposure treatment combination ($q_{\infty, 8}$ = 4.6818, $0.01 < p < 0.025$). No other pairwise comparisons between treatment combinations were significant (all $p > 0.05$).

Deformities

Frequency of limb deformities (frequency of individuals with ≥ 1 limb deformity) in stage 42 individuals was greater in the *Ribeiroia* exposure treatment (59%) compared to the *Ribeiroia* control treatment (1%) (S = 9.6680, $p < 0.0005$, Fig. 5.2, Table 5.3). There was no evidence that frequency of deformities was influenced by *Achlya* treatment (S = 2.2696, $p > 0.25$) or BD treatment (S = -0.7235, $p > 0.25$). None of the multiple contrasts testing for interactions were significant (all $p > 0.25$), indicating that the effect

of *Ribeiroia* on frequency of deformities did not depend on the other treatments. All the treatment combinations in the *Ribeiroia* exposure treatment had higher frequency of deformities than all the treatment combinations in the *Ribeiroia* control treatment (all $p < 0.025$, Table 5.4), except that there was no difference in frequency of deformities between the *Achlya* control/*Ribeiroia* exposure/BD exposure treatment combination and the *Achlya* exposure/*Ribeiroia* control/BD control treatment combination ($q_{\infty, 8} = 3.2372$, $0.2 < p < 0.05$). In both *Ribeiroia* treatments (exposure and control) there were some deformities that did not directly involve a limb, but all deformed individuals had at least one limb deformity.

Deformities in the Ribeiroia *exposure treatment*

Limb deformities in the *Ribeiroia* exposure treatment consisted mostly of missing, partially missing, reduced, and extra limbs and digits, abnormally small limbs, abnormal joints (permanent flexion and permanent extension), rotated feet, finger-like projections, small bumps (~1 mm diameter), and skin webbings (Table 5.5). Most (70 out of 76 (92.1%)) deformities were limb deformities, and most (65 out of 70 (92.9%)) limb deformities involved forelimbs rather than hindlimbs. Extra limbs and limb elements observed included extra limbs (polymely), extra toes (polydactyly), one case of mirror-image duplication of toes. However, these types of deformities were not common. Only five out of 54 (9.3%) of *Ribeiroia*-exposed individuals reaching metamorphosis had one or more extra limbs or limb elements, with prevalence of extra limbs or limb elements ranging from 7.1 to 14.3% across the different *Achlya* and BD treatments. Frequency of deformities involving extra limbs or limb elements among deformity types

ranged from 5.3 to 14.3% across these treatments. Notably, frequency of deformities involving missing or reduced limbs or limb elements constituted a substantial proportion of deformities, ranging in frequency from 40.0 to 47.7% across treatments. Also notable were low frequencies of cutaneous fusion, which ranged in frequency among deformities from 0.0 to 6.3%.

Deformities in the Ribeioia *control treatment*

Only one individual in the *Ribeiroia* control treatment displayed any deformities. This individual had a missing forelimb, an abnormally small hindlimb, and two abnormal hindlimb joints (permanent flexion).

Rate of development

Hazard of metamorphosis not occurring was higher in the *Ribeiroia* exposure treatment compared to the *Ribeiroia* control treatment (Model 1, $X^2 = 16.7755$, $p < 0.001$, Fig. 5.3, Table 5.6), indicating that the *Ribeiroa* exposure treatment decreased rate of development. Risk of metamorphosis not occuring was 1.46 times higher for *Ribeiroia*-exposed individuals compared to individuals not exposed to *Ribeiroia*. Also, hazard of metamorphosis not occurring was higher in the BD control treatment compared to the BD exposure treatment ($X^2 = 7.1022$, $p = 0.0077$), indicating that the BD exposure treatment increased rate of development. Risk of metamorphosis not occurring was 1.28 times higher for individuals not exposed to BD compared to BD-exposed individuals. There was no evidence that risk of metamorphosis occurring was influenced by *Achlya* treatment ($X^2 = 0.2755$, $p = 0.5997$) or initial stage ($X^2 = 2.8239$, $p = 0.0929$). None of the interaction terms were significant (all $p \geq 0.1025$), indicating that there was no

evidence that rate of development was influenced by interactions involving multiple pathogens and/or initial stage.

Hazard of of metamorphosis not occurring was higher among individuals in the *Ribeiroia* exposure treatment that did not die or display limb deformities compared to individuals in the *Ribeiroia* control treatment (Model 2, $X^2 = 16.3306$, $p = 0.0001$, Table 5.7, data not shown). Risk of metamorphosis not occurring was 1.58 times higher for *Ribeiroia*-exposed individuals that did not die or display limb deformities compared to individuals not exposed to *Ribeiroia.* In addition, Model 2 shows an interaction between *Ribeiroia* and initial stage ($X^2 = 4.5714$, $p = 0.0325$). The effect of *Ribeiroia* on development was greater for individuals with higher initial stages. None of the other terms in Model 2 were significant (all $p \geq 0.1245$). Among individuals in the *Ribeiroia* exposure treatment, there was a trend of faster development in individuals displaying one or more limb deformities compared to individuals not displaying any limb deformities (Model 3, $X^2 = 2.7839$, $p = 0.0952$, Table 5.8, data not shown) and also a trend of interaction between limb deformity status and *Achlya* treatment ($X^2 = 3.1703$, $p = 0.0750$). Individuals displaying limb deformities tended to develop faster than those not displaying any limb deformities in the *Achlya* control treatment, but not in the *Achlya* exposure treatment. None of the other terms in Model 3 were significant (all $p \geq 0.1031$).

One individual in the *Achlya* exposure/*Ribeiroia* control/BD control treatment combination was excluded from the Model 2 data set on the basis of not reaching stage 37. In this data set, fourteen individuals (five in the *Ribeiroia* control treatment and nine

in the *Ribeiroia* exposure treatment) were between stages 37 and 41 and thus did not complete limb development. Nine individuals, all of which died, were excluded from the Model 3 data sets on the basis of not reaching stage 37. In this data set, there were eighteen individuals (nine displaying limb deformities and nine not displaying limb deformities) between stages 37 and 41. It is possible that some of these individuals between stages 37 and 41 that did not display deformities would have done so had their development continued. However, deformities such as abnormally small or bent toes or abnormal range of motion in foot joints that might have developed in these individuals had their development continued were seen on only three stage 42 individuals. Also, these kinds of deformities appeared minor and appeared unlikely to have substantial biological significance for frogs in nature. In addition, forelimb deformities, which would not be visible until forelimb emergence at stage 42, were observed in only one stage 42 individual in the *Ribeiroia* control treatment and only two stage 42 individuals in the *Ribeiroia* exposure treatment.

One individual in the *Achlya* exposure/*Ribeiroia* control/BD control treatment combination (initial stage: 27) had only one forelimb and was removed at Gosner (1960) stage 43. This individual was excluded from all analyses of rate of development. Alternate models were constructed for all Models 1 and 2 with this individual included and scored as reaching metamorphosis on a range days (day 33-37) on which it may have reached stage 42. The qualitative interpretations of the results of the models did not differ than those of the models that excluded this individual.

Mass at metamorphosis

There was no evidence that mass at metamorphosis was influenced by initial stage or any of the treatments (Fig. 5.4, Table 5.9). Mass was lower in the BD exposure treatment compared to the BD control treatment, but this difference was not statistically significant ($t_{1, 111}$ = -0.3167, p = 0.7521). The individual that reached stage 43 was excluded from the analysis of mass at metamorphosis.

Infection

A hyphal infection was observed on only one live individual. This individual was in the *Ribeiroia* exposure/*Achlya* exposure/BD exposure treatment combination and had whitish hyphal growth on the foot on its left forelimb. No clearly identifiable oomycete reproductive structures were seen during microscopic examination of these hyphae. However, coenocytic hyphae consistent with *Achlya* infection were abundant in this sample. No BD DNA was detected by real-time PCR on any of the swabs. Thus the assays revealed no evidence of sustained BD infection in any individuals. All standards in the assays amplified normally (Tables 5.10, 5.11).

5.5 Discussion

We found main effects of *Ribeiroia* and BD on *P. regilla* that may contribute to the population-level effects of these pathogens on amphibians, but we did not find evidence of interactions among different pathogens. Within this study, one-time exposure to small numbers of *Ribeiroia* cercariae (n=15) caused elevated mortality and increased frequency of limb malformations. Consistent with previous experimental exposures of frog larvae, including larvae of *P. regilla,* to *Ribeiroia* (Johnson *et al.,* 1999,

2001a, Schotthoefer *et al.*, 2003, Skelly *et al.*, 2007), most limb malformations among *Ribeiroia*-exposed individuals involved the hindlimbs. In addition, the range of limb deformity types produced by *Ribeiroia* in our study and these previous studies were all similar. However, relative frequencies of types of deformities produced were different among all these studies. The differences between studies that used different species suggest that these relative frequencies are species-specific. Also, comparison of our study with another study in *P. regilla* (Johnson *et al.*, 1999) suggests that within a single species, the relative frequencies of different deformities caused by *Ribeiroia* depend on ecological context. Most notable among the differences between these two studies were that frequencies of extra limbs and limb elements and cutaneous fusion among deformity types were lower in our study. These differences may be due to differences in exposure regime, host populations, *Ribeiroia* populations, and/or experimental protocols. Skelly *et al.* (2007) noted that the difference in prevalence of limb deformities in sensitive stages of *R. pipiens* (northern leopard frog) following exposure to *Ribeiroia* in their study compared to the study of Schotthoefer *et al.* (2003) may have been due to differences in ecological context. Moreover, Schotthoefer *et al.* (2003) found that prevalence of deformities following exposure to *Ribeiroia* depended on developmental stage of the host at exposure. Thus *Ribeiroia* appears to cause a different prevalence and composition of deformities in different ecological situations.

Skelly *et al.* (2007) observed a preponderance of extra limbs and limb elements among the deformities in *R. pipiens* (northern leopard frog) they exposed to *Ribeiroia* and state that this preponderance is consistent with the results of previous experimental

exposures of amphibians to this pathogen (Johnson *et al.*, 1999, 2001a, Schotthoefer *et al.*, 2003), which Skelly *et al.* describe as having produced primarily extra limbs and limb elements. However, extra limbs and limb elements have not always been the majority or even the most common class of deformities produced by a particular *Ribeiroia* treatment in a particular amphibian species. Among the deformities in *Ribeiroia*-exposed individuals, and looking across treatment levels and studies, frequency of extra limbs and limb elements among deformities is variable and sometimes low, ranging from 26.7 to 30.4 percent in *B. boreas*, from 20.0 to 50.0 percent in *A. macrodacylum* (long-toed salamander), from 48.2 to 100% in *R. pipiens*, and from 5.3 to 55.3% in *P. regilla*, a species that is highly susceptible to extra limbs and limb elements from *Ribeiroia* exposure (Johnson *et al.*, 1999, 2001a, 2002b, 2003, 2006, Schotthoefer *et al.*, 2003, Skelly *et al.*, 2007, this study). In addition, prevalence of extra limbs or limb elements of *Ribeiroia*-exposed *P. regilla* was low in our study, ranging from 7.1 to 14.3% across treatments. Furthermore, deformities involving missing or reduced limbs or limb elements were approximately four times more common than deformities involving extra limbs or limb elements in *P. regilla* we exposed to this pathogen. Thus extra limbs and limb elements are not the sole, nor necessarily the most common class of deformities produced by *Ribeiroia* under all ecological conditions. Moreover, the occurrence of extra limbs and limb elements at sites that harbor *Ribeiroia* varies among different amphibian species and different sites. Among these sites, frequency of extra limbs or limb elements among deformity types in species displaying *Ribeiroia* infection and high (>5%) prevalence of deformities has been measured at levels as low as 5.3% in frogs and 0.2%

in salamanders (Johnson *et al.*, 2001b, 2002b, 2003, 2006). These field data are consistent with *Ribeiroia* producing a different pattern of deformities under different ecological conditions.

Skelly *et al.* (2007) also observed a relatively low frequency of extra limbs and limb elements (5 out of 150 (3%)) among deformities found in Vermont amphibians and used this as evidence against the involvement of *Ribeiroia* in these deformities. However, in our study, extra limbs and limb elements constituted few (7.1 to 14.3%) of the deformities produced by *Ribeiroia*. This suggests that a low frequency of extra limbs and limb elements among deformities is not strong evidence against the involvement of *Ribeiroia*. Skelly *et al.* (2007) further state that the preponderance of missing limbs and limb elements they observed among the deformities found in Vermont amphibians is inconsistent with *Ribeiroia* as a cause. Missing limbs and limb elements have not accounted for the majority of deformities in any experimental *Ribeiroia* treatments reported so far, but due to the context-dependency in the pattern of deformities this pathogen produces, this possibility should not be ruled out at this point. Furthermore, multiple causative factors may be involved in causing deformities. Even if a single factor does not seem likely to be responsible for causing the full pattern of deformities considered, it may be responsible for some of the deformities, while one or more other causes factor in to produce the full pattern.

We also found evidence that *Ribeiroia* delayed development of *P. regilla* larvae. Similarly, Johnson *et al.* (2006) found that *Ribeiroia* exposure slowed development in long-toed salamander (*Ambystoma macrodactylum*) larvae. Such delayed development

may occur because amphibians shift resources away from development and toward repair of wounds caused by entry of *Ribeiroia* cercaria. We found no strong evidence that rate of development among *Ribeiroia*-exposed *P. regilla* was related to the presence or absence of limb deformities, BD treatment, *Achlya* treatment, initial stage, or their interactions. Thus, limb deformities resulting from exposure to *Ribeiroia* did not appear to alter rate of development. The lack of association between the presence of limb deformities and rate of development in *Ribeiroia*-exposed individuals further suggests that the mechanism by which *Ribeiroia* alters rate of development and the mechanism by which this pathogen alters the pattern of development appear to be somewhat independent.

Though we found no evidence that limb deformities altered developmental rate, there was an overall negative effect of *Ribeiroia* on rate of development in *P. regilla*. In addition, *Ribeiroia* delayed development among *P. regilla* that survived *Ribeiroia* exposure without obvious limb deformities. In nature, slowed development among individuals that do not die from *Ribeiroia* exposure or incur deformities likely to impair their survival may have particularly negative consequences for amphibian populations. Species that breed in temporary aquatic habitats may be particularly affected by delayed development, since their larvae will die if they fail to metamorphose before the habitat dries.

Ribeiroia-induced delayed development will lengthen the amount of time amphibian larvae are exposed to *Ribeiroia* cercaria, and thus is likely to increase *Ribeiroia* infection load and the severity of *Ribeiroia*-induced effects, including delayed

development, thus creating a positive feedback. Importantly, limb deformities may be particularly influenced by delayed development. Frog larvae are only susceptible to *Ribeirioa*-induced limb deformities if *Ribeiroia* infects during early hindlimb development (Schotthoefer *et al.*, 2003, Bowerman & Johnson, 2003). Extension of the amount of time larvae are in these sensitive stages of development is likely to increase the frequency, number, and severity of limb deformities in habitats where *Ribeiroia* is present. Limb deformities may greatly increase the susceptibility of amphibians to predation by other vertebrates that serve as the definitive hosts for *Ribeiroia,* thereby aiding transmission of the pathogen (Johnson *et al.*, 2005). Therefore, delayed development of amphibian hosts may provide an advantage to *Ribeiroia* genotypes that cause it or take advantage of other *Ribeiroia* genotypes that do.

Importantly, and unlike exposure to *Ribeiroia*, exposure to BD accelerated development. Intriguingly, however, none of the *P. regilla* exposed to BD tested positive for BD infection. All BD standards in our PCR assays amplified normally, indicating that the PCR assays were capable of detecting BD DNA. Previous work by our group (see Blaustein *et al.* 2005) found that *P. regilla* larvae were susceptible to infection with BD at when dosed with a concentration of BD zoospores (~1.4×10^6 zoospores per l) only slightly higher than the concentration we used (~1.1×10^6 zoospores per l) at temperatures (14 ± 1˚C) lower than the temperatures we used (~18.5-22.5˚ C). In addition, Blaustein *et al.* (2005) exposed *P. regilla* to BD by adding BD culture dishes to their water, while we added a BD zoospore solution to their water. Differences in BD infection between our study and that of Blaustein *et al.* (2005) could have been due to

methodological differences in dosage, exposure methods and/or temperatures. Also, we cannot eliminate the possibility that short-term BD infections occurred and then disappeared before individuals were swabbed. Most of our swabs were made on individuals that were stage 42 and thus had lost their larval mouthparts, in contrast to the study of Blaustein *et al.* (2005) in which *P. regilla* analyzed for BD infection all still had their larval mouthparts. During metamorphosis, the amount of keratin on a frog larva drops because resorption of the keratinized larval mouthparts starts before keratinization of the skin begins (Gosner 1960, Warburg *et al.*, 1994), which may result in elimination of BD infection. Some individuals in our study may have had BD infections that were eliminated during metamorphosis and thus not detected. Also, we cannot exclude the possibility that very light BD infections occurred in the BD treatment and were not detected by real-time PCR.

Even though we did not detect BD infection, our results suggest that the level of BD exposure used affected the biology of *P. regilla*, increasing their rate of development. In nature, such a response could be advantageous to the host. If an individual larva responds to the presence of BD by metamorphosing sooner, it may be able to leave its water body sooner and move to a terrestrial habitat or other water body where the risk of becoming infected with BD may be lower.

Many amphibian species exhibit phenotypic plasticity in behavior, morphology, and rate of development (e.g. Relyea and Werner 2000, Warkentin 2000, Relyea 2001). There is ample evidence that rate of development in amphibian larvae may increase from falling water level, low food resources, or the presence of predators or competitors (Sokol

1984, Werner 1986, Newman 1988, Crump 1989a,b). There is also evidence that adult and larval amphibians respond behaviorally to increased disease risk (Kiesecker & Skelly, 1999, Pfennig 2000, Han *et al.*, *in preparation*). Amphibians at the embryo stage can decrease time to hatching in response to the presence of pathogens (Warkentin *et al.*, 2001, Warkentin, 2005, Touchon *et al.*, 2006, Gomez-Mestre *et al,* 2006). Therefore it seems likely that amphibian larvae may alter their rate of development during the larval stage in response to increased disease risk.

Altering rate of development or the timing of developmental events in response to the presence of one or more pathogens may be costly. *Rana sylvatica* and *B. americanus* embryos and de-jellied *A. maculatum* (spotted salamander) embryos exposed to multiple species of each of the water mold genera *Achlya* and *Saprolegnia* had earlier hatching but also shorter length and earlier developmental stage as hatchings compared to controls (Gomez-Mestre *et al.*, 2006, Touchon *et al.*, 2006). Early hatching in *R. sylvatica* increased susceptibility to predators (Touchon *et al.*, 2006). Faster development during the larval stage could carry the cost of decreased mass at metamorphosis. There is some evidence that smaller size at metamorphosis is correlated with decreased numbers of lymphocytes during metamorphosis, reduced survival as a juvenile, delayed sexual maturity, smaller adult size, and smaller clutch size (Smith 1987, Semlitsch *et al.,* 1988, Berven, 1990, Scott, 1994). We did not detect such a reduction in mass at metamorphosis in our study. Mass at metamorphosis was lower in the BD exposure treatment compared to the BD control treatment, but this difference was not statistically significant. Nevertheless, our results raise the possibility that BD has negative effects

such as reduced size or mass at metamorphisis on individuals that are exposed to BD but do not become infected. Such effects on uninfected individuals may contribute to the effects this pathogen has on host populations and therefore deserve further investigation as a means by which this pathogen may contribute to population declines in amphibians.

The increase in rate of development we observed in *P. regilla* larvae may have been a manipulation of rate of development by the pathogen. BD appears to infect only the keratinized parts of amphibians, and recently metamorphosed juveniles (metamorphs) become covered in keratinized skin, while the only parts of larvae that contain keratin are their relatively small jaw sheaths and tooth rows. Thus, metamorphs with keratinized skin are probably better hosts for BD than larvae. If a particular BD clone causes an increase in rate of development among potential hosts, it may cause more metamorphs, hence more suitable hosts, to arise sooner.

The net effects on amphibians to the presence of BD may be a mixture of a response of the potential host and manipulation of the host by the pathogen. Interestingly, the net effect of BD on rate of development in amphibian larvae when prevalence of infection is high appears to be negative, not positive as in our study in which no infection was detected (Parris 2004, Parris & Baud 2004, Parris & Beaudoin 2004, Parris & Cornelius 2004). Further research should expose amphibians to cues from BD without the possibility of infection occurring so that the effects of BD presence per se on larval development may be measured.

We found no evidence that the parameters we investigated were influenced by intitial stage, the *Achlya* treatment, or interactions involving treatments or initial stage.

However, it is possible that different levels of exposure would result in interactions between the pathogens we investigated. Furthermore, it is possible that environmental stressors, which can influence susceptibility to disease in amphibians (e.g. Kiesecker & Blaustein, 1995, Forson & Storfer, 2006a), may influence whether interactions between pathogens occur. This study, which used three pathogens and one host species, is one step in evaluating the importance of interactions among pathogens in the disease ecology of amphibians. Other pathogens and hosts should be investigated to explore how multiple pathogens may be acting in combination to influence the population-level biology of amphibians.

5.6 Acknowledgements

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Table 5.1. Results of the multiple contrasts procedure for survival (numerator df = 7, denominator df = 153, $\alpha = 0.05$). Numerical values in the Contrast column are arcsine square root transformed proportions (proportions of individuals surviving). Abbreviations: A_e = *Achlya* exposure treatment, A_c = *Achlya* control treatment, R_e = *Ribeiroia* exposure treatment, R_c = *Ribeiroia* control treatment, BD_e = BD exposure treatment, BD_c = BD control treatment. One individual in the *Achlya*, *Ribeiroia* control, BD treatment combination (initial stage: 26) was accidentally sacrificed at stage 41 (one of its forelimbs was not finished emerging). This individual was excluded from analyses of survival.

Table. 5.1

Hypothesis	Difference	SE	*S*	*p*
A_e affected survival	5.5190	4.5310	1.2180	> 0.25
R_e affected survival	-20.5519	4.5310	-4.5358	$0.0005 < p < 0.001$
BD_e affected survival	-9.4481	4.5310	-2.0852	> 0.25
The effect of A_e was different in R_cBD_c vs. in R_cBD_e	-6.4605	6.3677	-1.015	> 0.25
The effect of A_e was different in R_cBD_c vs. in R_eBD_c	-4.6434	6.2904	-0.7382	> 0.25
The effect of A_e was different in R_cBD_c vs. in R_eBD_e	0	6.3273	0	did not test
The effect of A_e was different in R_eBD_c vs. in R_eBD_e	4.6434	6.2904	0.7382	> 0.25
The effect of A_e was different in R_eBD_c vs. in R_cBD_e	-1.8172	6.3310	-0.2870	> 0.25
The effect of A_e was different in R_cBD_e vs. in R_eBD_e	6.4605	6.3677	1.0146	> 0.25
The effect of R_e was different in A_cBD_c vs. in A_cBD_e	-2.5644	6.2900	-0.4077	> 0.25
The effect of *R*e was different in A_cBD_c vs. in A_eBD_c	-4.6434	6.2900	-0.7382	> 0.25
The effect of *R*e was different in A_cBD_c vs. in A_eBD_e	3.8962	6.3310	0.6154	> 0.25
The effect of R_e was different in A_eBD_c vs. in A_eBD_e	8.5395	6.3671	1.3412	> 0.25
The effect of R_e was different in A_eBD_c vs. in A_cBD_e	2.0790	6.3270	0.3286	> 0.25
The effect of R_e was different in A_cBD_e vs. in A_eBD_e	6.4605	6.3677	1.0146	> 0.25
The effect of BD_e was different in A_cR_c vs. in A_eR_c	-6.4605	6.3677	-1.0146	> 0.25
The effect of BD_e was different in A_cR_c vs. in A_cR_e	-2.5644	6.2904	-0.4077	> 0.25
The effect of BD_e was different in A_cR_c vs. in A_eR_e	2.0790	6.3273	0.3286	> 0.25
The effect of BD_e was different in A_cR_e vs. in A_eR_e	4.6434	6.2904	0.7382	> 0.25
The effect of BD_e was different in A_cR_e vs. in A_eR_c	-3.8962	6.3310	-0.6154	> 0.25
The effect of BD_e was different in A_eR_c vs. in A_eR_e	8.5395	6.3677	1.3411	> 0.25
The effect of A_e was different in R_e vs. in R_c	0.9086	4.5310	-0.6594	> 0.25
The effect of A_e was different in BD_e vs. in BD_c	-0.9086	4.5310	-0.2005	> 0.25
The effect of R_e was different in A_e vs. in A_c	0.9086	4.5310	0.2005	> 0.25
The effect of R_e was different in BD_e vs. in BD_c	2.9876	4.5310	0.6594	> 0.25
The effect of BD_e was different in A_e vs. in A_c	-0.9086	4.5310	-0.2005	> 0.25
The effect of BD_e was different in R_e vs. in R_c	-2.9876	4.5310	0.2005	> 0.25

Table 5.1 (Continued)

Hypothesis	Difference	SE	*S*	*p*
The effect of A_eR_e was different in BD_e vs. in BD_c	8.5395	6.3273	1.3496	> 0.25
The effect of R_eBD_e was different in A_e vs. in A_c	0	6.3273	0	did not test
The effect of A_eBD_e was different in R_e vs. in R_c	3.8962	6.3310	0.6158	> 0.25

Table 5.2. Results of the multiple comparison procedure comparing survival (proportion of individuals surviving) among treatment combinations ($\alpha = 0.05$). Numerical values in the Difference column are the differences between arcsine square root transformed proportions (proportions of individuals surviving). Abbreviations are as in Table 5.1. Differences between data points enclosed by data points that were not statistically different ($p < 0.05$) were not tested for significance (Zar, 1999). Differences not tested for significance all have $p > 0.05$.

Comparison	Difference	SE	$q_{\infty, 8}$	p
$A_cR_cBD_c$ - $A_eR_eBD_e$	30	6.3273	4.7414	$0.01 < p < 0.025$
$A_cR_cBD_c$ - $A_cR_eBD_e$	30	6.3273	4.7414	$0.01 < p < 0.025$
$A_cR_cBD_c$ - $A_cR_eBD_c$	22.22	6.2533	3.5533	$0.1 < p < 0.2$
$A_cR_cBD_c$ - $A_eR_eBD_c$	12.92	—	—	did not test
$A_cR_cBD_c$ - $A_cR_cBD_e$	12.92	—	—	did not test
$A_cR_cBD_c$ - $A_eR_cBD_e$	0	—	—	did not test
$A_cR_cBD_c$ - $A_eR_cBD_c$	0	—	—	did not test
$A_eR_cBD_c$ - $A_eR_eBD_e$	30	6.3273	4.7414	$0.01 < p < 0.025$
$A_eR_cBD_c$ - $A_cR_eBD_e$	30	6.3273	4.7414	$0.01 < p < 0.025$
$A_eR_cBD_c$ - $A_cR_eBD_c$	22.22	6.2533	3.5533	$0.1 < p < 0.2$
$A_eR_cBD_c$ - $A_eR_eBD_c$	12.92	—	—	did not test
$A_eR_cBD_c$ - $A_cR_cBD_e$	12.92	—	—	did not test
$A_eR_cBD_c$ - $A_eR_cBD_e$	0	—	—	did not test
$A_eR_cBD_e$ - $A_eR_eBD_e$	30	6.4079	4.6818	$0.01 < p < 0.025$
$A_eR_cBD_e$ - $A_cR_eBD_e$	30	6.4079	4.6818	$0.01 < p < 0.025$
$A_eR_cBD_e$ - $A_cR_eBD_c$	22.22	6.3348	3.5076	$0.2 < p < 0.5$
$A_eR_cBD_e$ - $A_eR_eBD_c$	12.92	—	—	did not test
$A_eR_cBD_e$ - $A_cR_cBD_e$	12.92	—	—	did not test
$A_cR_cBD_e$ - $A_eR_eBD_e$	17.08	6.3273	2.6994	$p > 0.5$
$A_cR_cBD_e$ - $A_cR_eBD_e$	17.08	—	—	did not test
$A_cR_cBD_e$ - $A_cR_eBD_c$	9.3	—	—	did not test
$A_cR_cBD_e$ - $A_eR_eBD_c$	0	—	—	did not test
$A_eR_eBD_c$ - $A_eR_eBD_e$	17.08	—	—	did not test
$A_eR_eBD_c$ - $A_cR_eBD_e$	17.08	—	—	did not test
$A_eR_eBD_c$ - $A_cR_eBD_c$	9.3	—	—	did not test
$A_cR_eBD_c$ - $A_eR_eBD_e$	7.78	—	—	did not test
$A_cR_eBD_c$ - $A_cR_eBD_e$	7.78	—	—	did not test
$A_cR_eBD_e$ - $A_eR_eBD_e$	0	—	—	did not test

Table 5.3. Results of the multiple contrasts procedure for frequency of limb deformities (frequency of individuals with ≥ 1 limb deformity) (numerator df = 7, denominator df = 153, $\alpha = 0.05$). Numerical values in the Contrast column are arcsine square root transformed proportions. Abbreviations are as in Table 5.1.

Table 5.3

Hypothesis	Difference	SE	*S*	*p*
A_e affected prevalence of limb deformities	11.4632	5.0508	2.2696	>0.25
R_e affected prevalence of limb deformities	48.8313	5.0508	9.6680	<0.0005
BD_e affected prevalence of limb deformities	-3.6541	5.0508	-0.7235	>0.25
The effect of A_e was different in R_cBD_c vs. in R_cBD_e	6.8165	6.5837	1.0354	>0.25
The effect of A_e was different in R_cBD_c vs. in R_eBD_c	6.0017	7.2224	0.8310	>0.25
The effect of A_e was different in R_cBD_c vs. in R_eBD_e	-8.4787	7.2224	-1.1739	>0.25
The effect of A_e waA different in R_eBD_c vA. in R_eBD_e	-14.4804	7.6614	-1.8900	>0.25
The effect of A_e was different in R_eBD_c vs. in R_cBD_e	0.8148	7.0625	0.1154	>0.25
The effect of A_e was different in R_cBD_e vs. in R_eBD_e	-15.2952	7.0625	-2.1657	>0.25
The effect of R_e was different in A_cBD_c vs. in A_cBD_e	7.4860	7.1471	1.0474	>0.25
The effect of *R*e was different in A_cBD_c vs. in A_eBD_c	6.0017	7.2224	0.8310	>0.25
The effect of *R*e was different in A_cBD_c vs. in A_eBD_e	-7.8092	7.2555	-1.0763	>0.25
The effect of R_e was different in A_eBD_c vs. in A_eBD_e	-13.8109	7.1387	-1.9347	>0.25
The effect of R_e was different in A_eBD_c vs. in A_cBD_e	1.4843	7.0284	0.2112	>0.25
The effect of R_e was different in A_cBD_e vs. in A_eBD_e	-15.2952	7.0625	-2.1657	>0.25
The effect of BD_e was different in A_cR_c vs. in A_eR_c	-6.8165	6.5837	-1.0354	>0.25
The effect of BD_e was different in A_cR_c vs. in A_cR_e	7.4860	7.1471	1.0474	>0.25
The effect of BD_e was different in A_cR_c vs. in A_eR_e	-6.9944	7.1471	-0.9786	>0.25
The effect of BD_e was different in A_cR_e vs. in A_eR_e	-14.4804	7.6614	-1.8900	>0.25
The effect of BD_e was different in A_cR_e vs. in A_eR_c	-14.3025	7.1387	-2.0035	>0.25
The effect of BD_e was different in A_eR_c vs. in A_eR_e	-0.1779	7.1387	-0.0249	>0.25
The effect of A_e was different in R_e vs. in R_c	-4.6467	5.0508	-0.9200	>0.25
The effect of A_e was different in BD_e vs. in BD_c	-3.8319	5.0508	-0.7587	>0.25
The effect of R_e was different in A_e vs. in A_c	-4.6467	5.0508	-0.9200	>0.25
The effect of R_e was different in BD_e vs. in BD_c	-3.1624	5.0508	-0.6261	>0.25
The effect of BD_e was different in A_e vs. in A_c	-3.8319	5.0508	-0.7587	>0.25
The effect of BD_e was different in R_e vs. in R_c	3.1624	5.0508	0.6261	>0.25

Table 5.3 (Continued)

Hypothesis	Difference	SE	*S*	*p*
The effect of A_eR_e was different in BD_e vs. in BD_c	-6.9944	7.1471	-0.9640	>0.25
The effect of R_eBD_e was different in A_e vs. in A_c	11.3553	7.2224	1.5722	>0.25
The effect of A_eBD_e was different in R_e vs. in R_c	-7.8092	7.2555	-1.0926	>0.25

Table 5.4. Results of the multiple comparison procedure comparing frequency of deformities (frequency of individuals with at least 1 limb deformity) among treatment combinations ($\alpha = 0.05$). Numerical values in the Difference column are the differences between arcsine square root transformed proportions (proportions of individuals surviving). Abbreviations are as in Table 5.1. Differences between data points enclosed by data points that were not statistically different ($p < 0.05$) were not tested for significance (Zar, 1999). Differences not tested for significance all have $p > 0.05$.

Comparison	Difference	SE	$q_{\infty, k}$	P
$A_cR_cBD_c$ - $A_eR_eBD_e$	61.27	7.1002	8.6293	<0.001
$A_cR_cBD_c$ - $A_cR_eBD_e$	61.27	7.1722	8.5427	<0.001
$A_cR_cBD_c$ - $A_cR_eBD_c$	61.27	7.3379	8.3498	<0.001
$A_cR_cBD_c$ - $A_eR_eBD_c$	47.58	7.2510	6.5619	<0.001
$A_cR_cBD_c$ - $A_cR_cBD_e$	24.58	7.6614	3.2083	0.2 < p <0.5
$A_cR_cBD_c$ - $A_eR_cBD_e$	9.62	—	—	did not test
$A_cR_cBD_c$ - $A_eR_cBD_c$	7.96	—	—	did not test
$A_eR_cBD_c$ - $A_eR_eBD_e$	47.24	6.9511	6.7960	<0.001
$A_eR_cBD_c$ - $A_cR_eBD_e$	47.24	7.0245	6.7250	<0.001
$A_eR_cBD_c$ - $A_cR_eBD_c$	47.24	7.1937	6.5669	<0.001
$A_eR_cBD_c$ - $A_eR_eBD_c$	39.62	7.1050	5.5764	<0.001
$A_eR_cBD_c$ - $A_cR_cBD_e$	16.62	—	—	did not test
$A_eR_cBD_c$ - $A_eR_cBD_e$	1.66	—	—	did not test
$A_eR_cBD_e$ - $A_eR_eBD_e$	47.24	7.1002	6.6533	<0.001
$A_eR_cBD_e$ - $A_cR_eBD_e$	47.24	7.1722	6.5865	<0.001
$A_eR_cBD_e$ - $A_cR_eBD_c$	47.24	7.3379	6.4378	<0.001
$A_eR_cBD_e$ - $A_eR_eBD_c$	33.55	7.2510	4.6269	0.01 <p < 0.025
$A_eR_cBD_e$ - $A_cR_cBD_e$	14.96	—	—	did not test
$A_cR_cBD_e$ - $A_eR_eBD_e$	36.69	6.9510	5.2783	0.005<p<0.01
$A_cR_cBD_e$ - $A_cR_eBD_e$	36.69	7.0245	5.2231	0.005<p<0.01
$A_cR_cBD_e$ - $A_cR_eBD_c$	36.69	7.1936	5.1003	0.005<p<0.01
$A_cR_cBD_e$ - $A_eR_eBD_c$	23	7.1050	3.2372	0.2<p<0.5
$A_eR_eBD_c$ - $A_eR_eBD_e$	13.69	6.4960	2.1075	p>0.5
$A_eR_eBD_c$ - $A_cR_eBD_e$	13.69	—	—	did not test
$A_eR_eBD_c$ - $A_cR_eBD_c$	13.69	—	—	did not test
$A_cR_eBD_c$ - $A_eR_eBD_e$	0	—	—	did not test
$A_cR_eBD_c$ - $A_cR_eBD_e$	0	—	—	did not test
$A_cR_eBD_e$ - $A_eR_eBD_e$	0	—	—	did not test

Table 5.5. Relative frequencies (in percentages) of deformity types among *Pseudacris regilla* metamorphs. The numbers in parentheses indicate the number of deformed (metamorphs/total number of metamorphs). Abbreviations are as in Table 4.1. Scoring methods were those of Johnson *et al.* (2001b). Deformities that were part of other deformities (e.g. a missing toe on an extra limb) were not counted. Multiple cases of the same deformity in the same individual (e.g. two missing hindlimbs on a single individual) were scored as one deformity. Deformity types in the table are those of Johnson *et al.* (2001b, Table 1), with the following exceptions: 1) "limb hyperextension" was not used as a category, 2) "permanent extension" describes a joint that does not fully flex, 3) "permanent flexion" describes a joint that does not full extend, and 4) "other" types of deformities, which fell outside the categories of Johnson *et al.* (2001b), were given short descriptions.

Table 5.5

Deformity type	$A_cR_cBD_c$ (0/17)	$A_cR_cBD_e$ (0/20)	$A_cR_eBD_c$ (8/13)	$A_cR_eBD_e$ (5/14)	$A_eR_cBD_c$ (1/18)	$A_eR_cBD_e$ (0/19)	$A_eR_eBD_c$ (9/14)	$A_eR_eBD_e$ (10/13)
Cephalic and axial								
Edema	—	—	0	0	0	—	0	5.0
Other deformities	—	—	5.3[c]	6.3[e]	0	—	9.5[h]	5.0[k]
Forelimb								
Ectromely (completely missing limb)	—	—	0	0	33.3	—	0	0
Permanent flexion in joint	—	—	0	0	0	—	0	0
Other forelimb deformities	—	—	0	0	0	—	9.5[i]	0
Hind limb								
Brachydactyly (abnormally short toe)	—	—	5.3	0	—	—	4.8	0
Ectrodactyly (completely missing toe)	—	—	5.3	6.3	0	—	4.8	5.0
Polydactyly (extra toe)	—	—	0	0	0	—	4.8	5.0
Syndactyly (fused toes)	—	—	10.5	—	—	—	—	—
Brachymelia (abnormally short limb)	—	—	5.3	12.5	—	—	0	0
Hemimelia (partially missing limb)	—	—	10.5	12.5	0	—	28.6	15.0
Ectromelia (completely missing limb)	—	—	15.8	12.5	0	—	9.5	20.0
Polymelia (extra limb or part of limb)	—	—	0	6.3	0	—	9.5	5.0
Mirror-image duplication (of toes)	—	—	5.3[d]	0	0	—	0	0
Femoral projection[a]	—	—	5.3	6.3	0	—	4.8	0
Cutaneous fusion (skin webbing)	—	—	0	6.3	0	—	4.8	0
Micromelia (abnormally small limb)	—	—	5.3	6.3	33.3	—	4.8	0
Permanent extension in joint	—	—	5.3	0[f]	0	—	0	5.0
Permanent flexion in joint	—	—	10.5	6.3	33.3	—	0	10.0
Other hindlimb deformities	—	—	0	18.8[g]	0	—	4.8[j]	20.0[l]
Total number of deformities	0	0	19	16	3	0	21	20
Number of deformities per deformed individual	—	—	2.4	3.2	3	—	2.3	2.0

Table 5.5 (Continued)

[a]Finger-like appendage (projection) on the dorsal skin of a hindlimb
[b]Finger-like projection on lower limb (2 cases), foot rotated with respect to lower limb (2 cases)
[c]Finger-like projection on ventrum near hindlimb
[d]Mirror-image duplication of the digit pattern 5-4-3-3-4-5, digits 1 and 2 present
[e]Bulge in abdomen near forelimb
[f]Possible partial permanent extension in joint that had partial permanent flexion (not scored as a deformity)
[g]abnormally shaped foot, bump on lower limb, finger-like projection on lower limb segment
[h]Bump on sacrum, finger-like projection on cloacal tail piece
[i]Toe abnormally shaped and abnormally bent at base
[j]Abnormal orientation of limb joints
[k]Ridge on ventrum near forelimb
[l]No range of motion in knee joint, bump on knee, bump on lower limb segment, finger-like projection on severely truncated limb

Table 5.6. Results of the Cox Proportional Hazards Model 1 (full data set) for risk of metamorphosis occuring.

Source of variation	df	X^2	p
Ribeiroia	1	16.7755	<0.0001
Achlya	1	0.2755	0.5997
Ribeiroia × *Achlya*	1	1.0093	0.3151
BD	1	7.1022	0.0077
Ribeiroia × BD	1	1.5015	0.2204
Achlya × BD	1	2.6663	0.1025
Ribeiroia × *Achlya* × BD	1	0.2114	0.6457
Initial stage	1	2.8239	0.0929
Ribeiroia × initial stage	1	3.0371	0.0814
Achlya × initial stage	1	0.5723	0.4493
Ribeiroia × *Achlya* × initial stage	1	0.7345	0.3914
BD × initial stage	1	0.3180	0.5728
Ribeiroia × BD × initial stage	1	0.0179	0.8935
Achlya ×BD × initial stage	1	1.6377	0.2006
Ribeiroia ×*Achlya* × BD × initial stage	1	16.7755	0.6149
Error	144		

Table 5.7. Results of the Cox Proportional Hazards Model 2 for risk of metamorphosis occuring. Model does not include individuals in the *Ribeiroia* exposure treatment that died or displayed limb deformities.

Source of variation	df	X^2	*p*
Ribeiroia	1	16.3306	0.0001
Achlya	1	2.2671	0.1322
Ribeiroia × *Achlya*	1	0.3894	0.5326
BD	1	2.3602	0.1245
Ribeiroia × BD	1	0.6674	0.4140
Achlya × BD	1	0.6525	0.4192
Ribeiroia × *Achlya* × BD	1	0.0079	0.9293
Initial stage	1	0.0015	0.9693
Ribeiroia × initial stage	1	4.5714	0.0325
Achlya × initial stage	1	1.3300	0.2488
Ribeiroia × *Achlya* × initial stage	1	0.2064	0.6496
BD × initial stage	1	0.1603	0.6889
Ribeiroia × BD × initial stage	1	0.0837	0.7724
Achlya ×BD × initial stage	1	0.8567	0.3547
Ribeiroia ×*Achlya* × BD × initial stage	1	0.5009	0.4791
Error	90		

Table 5.8 Results of the Cox Proportional Hazards Model 3 for risk of metamorphosis occuring. Model contains individuals in the *Ribeiroia* exposure treatment only.

Source of variation	df	X^2	P
Deformity status	1	2.7839	0.0952
Achlya	1	0.0041	0.9490
Deformity status × *Achlya*	1	3.1703	0.0750
BD	1	0.4078	0.5231
Deformity status × BD	1	0.0208	0.8853
Achlya × BD	1	1.9183	0.1660
Deformity status × *Achlya* × BD	1	0.0203	0.8868
Initial stage	1	0.0087	0.9257
Deformity status × initial stage	1	2.6564	0.1031
Achlya × initial stage	1	0.0769	0.7815
Deformity status × *Achlya* × initial stage	1	0.0647	0.7992
BD × initial stage	1	0.2037	0.6518
Deformity status × BD × initial stage	1	1.4831	0.2233
Achlya ×BD × initial stage	1	0.0646	0.7993
Deformity status ×*Achlya* × BD × initial stage	1	0.1966	0.6575
Error	56		

Table 5.9. Multiple linear regression results for mass at metamorphosis. Residual standard error: 22.63 (df: 111), multiple r^2: 0.1284.

Source of variation	Value	SE	*T*	*p*
Intercept	196.9535	71.5480	2.7527	0.0069
Ribeiroia	62.6190	71.5480	-0.8752	0.3834
Achlya	-82.8751	71.5480	-1.1583	0.2492
BD	-22.6587	71.5480	-0.3167	0.7521
Initial stage	-1.0169	2.6621	-0.3820	0.7032
Ribeiroia × *Achlya*	-87.8884	71.5480	-1.2284	0.2219
Ribeiroia × BD	-47.2804	71.5480	-0.6608	0.5101
Achlya × BD	-81.3896	71.5480	-1.1376	0.2578
Ribeiroia × initial stage	2.3151	2.6621	0.8696	0.3864
Achlya × initial stage	3.1941	2.6621	1.1998	0.2328
BD × initial stage	0.8124	2.6621	0.3052	0.7608
Ribeiroia × *Achlya* × BD	43.1025	71.5480	0.6024	0.5481
Ribeiroia × *Achlya* × initial stage	3.3676	2.6621	1.2650	0.2085
Ribeiroia × BD × initial stage	1.6406	2.6621	0.6163	0.5390
Achlya ×BD × initial stage	2.8893	2.6621	1.0853	0.2801
Ribeiroia ×*Achlya* × BD × initial stage	-1.7229	2.6621	-0.6472	0.5189

Table 5.10. Results of real-time PCR measurement of *Batrachochytrium dendrobatidis* (BD) DNA for standards with known numbers of BD genome equivalents. Ct serves as a measurement of the amount of BD DNA present and is defined as the cycle number at which the change in the fluorescence signal from the fluorescent labelled probe (ΔRn) crosses a threshold set at the midpoint of the log ΔRn versus cycle number (Boyle *et al.*, 2004). Ct value was zero for all swabs. Undetermined: no change in fluorescence was detected, consistent with a lack of DNA amplification.

Table 5.10

Reaction plate	Standard (number of BD genome equivalents)	Mean Ct ± 1 SD
1	100	26.226 ± 0.399
	10	30.707 ± 0.077
	1	34.060 ± 0.184
	0.1	37.513 ± 0.418
	0	undetermined[a]
2	100	26.688 ± 1.170
	10	30.432 ± 0.668
	1	36.208 ± 0.267
	0.1	39.707 ± 0.775[b]
	0	undetermined[a]
3	100	27.034 ± 0.067
	10	31.383 ± 0.090
	1	36.797 ± 1.050
	0.1	39.400 ± 0.997[b]
	0	undetermined[a]
4	100	27.467 ± 0.280
	10	31.444 ± 0.086
	1	36.358 ± 0.314
	0.1	39.317 ± 0.779
	0	undetermined[a]

[a]Undetermined for all three samples
[b]Undetermined for one sample; mean and SD are for the other two samples

Table 5.11. Regression parameters for real-time PCR-derived standard curves of log number of *Batrachochytrium dendrobatidis* (BD) genome equivalents plotted against Ct value.

Reaction plate	Slope	Intercept	r^2
1	-3.770	33.860	0.992
2	-4.520	35.549	0.973
3	-4.333	35.889	0.970
4	-4.046	35.670	0.986

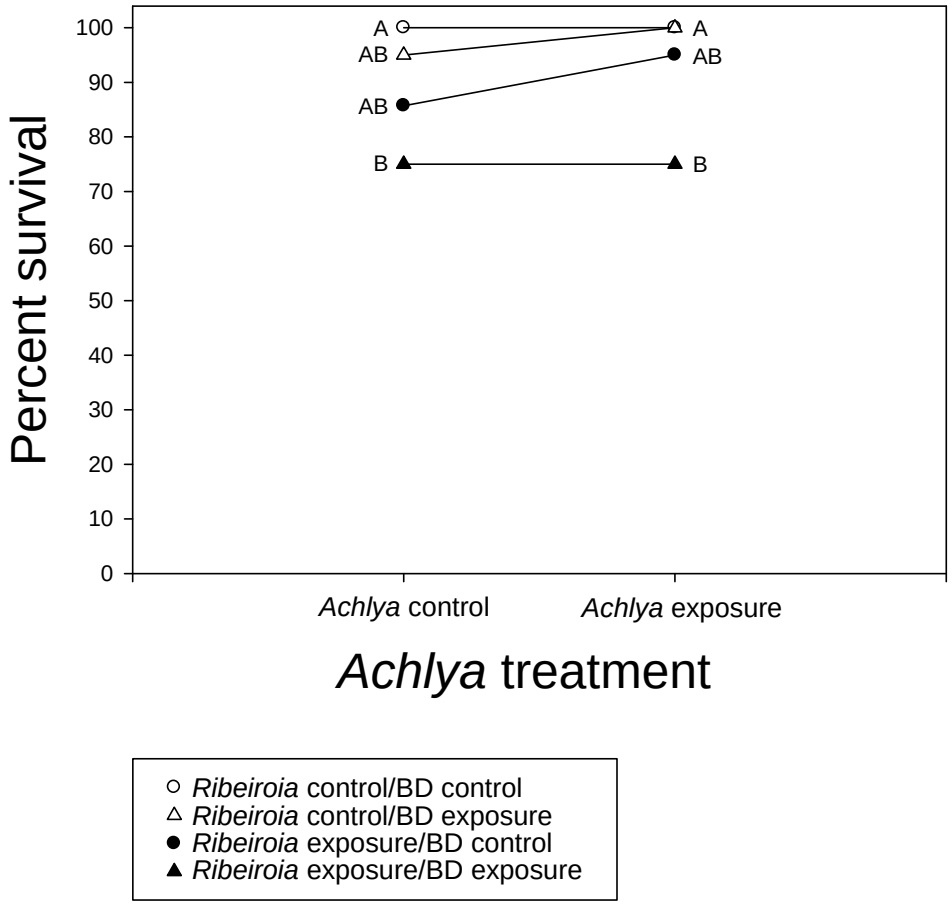

Figure 5.1. Survival of *Pseudacris regilla.* Treatment combinations that do not share a letter are significantly different from each other (multiple comparisons of proportions, $\alpha = 0.05$).

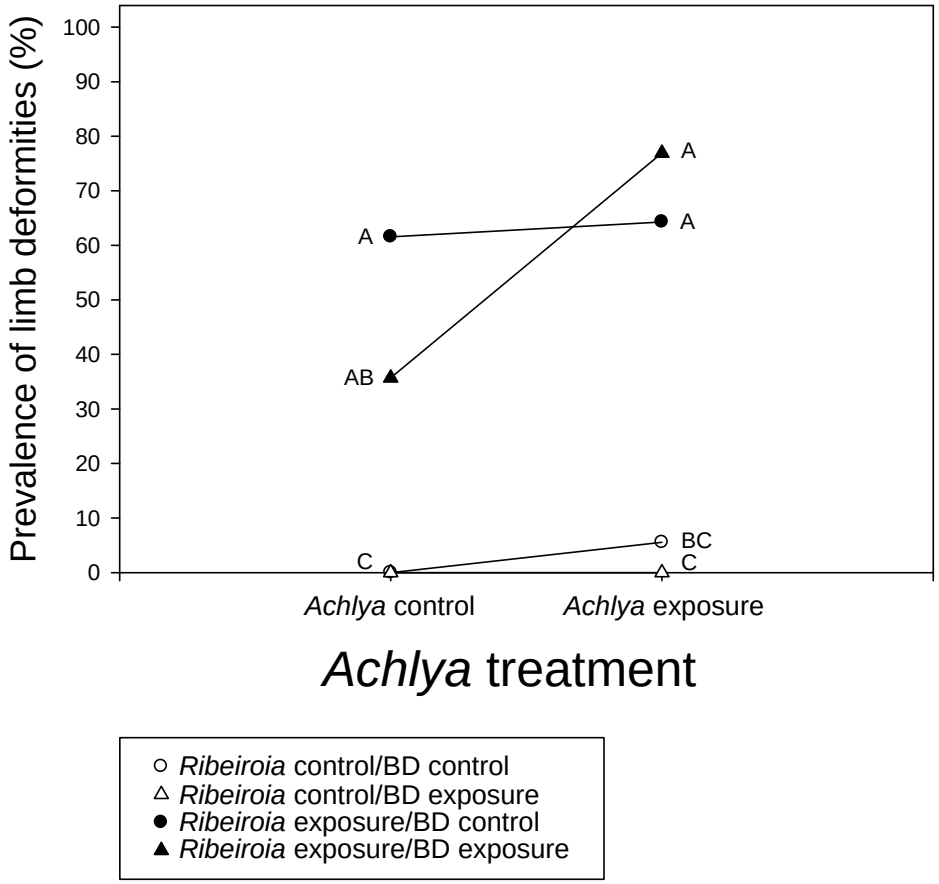

Figure 5.2. Prevalence of limb deformities (percentage of individuals with at least one limb deformity) in *Pseudacris regilla*. Treatment combinations that do not share a letter are significantly different from each other (multiple comparisons of proportions, α = 0.05).

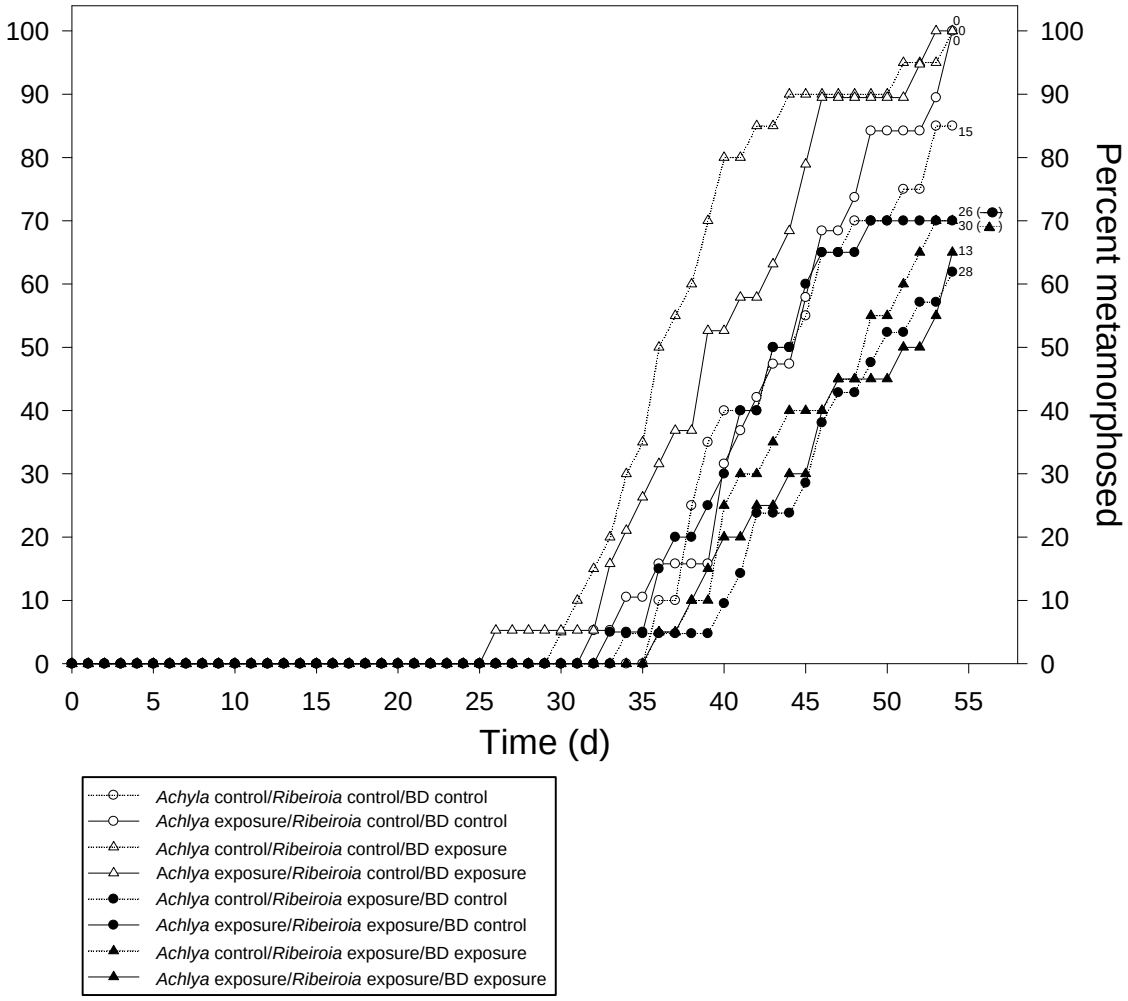

Figure 5.3. Percentage of individuals reaching metamorphosis for *Pseudacris regilla* over time. Figure does not include the individual sacrificed at stage 41.

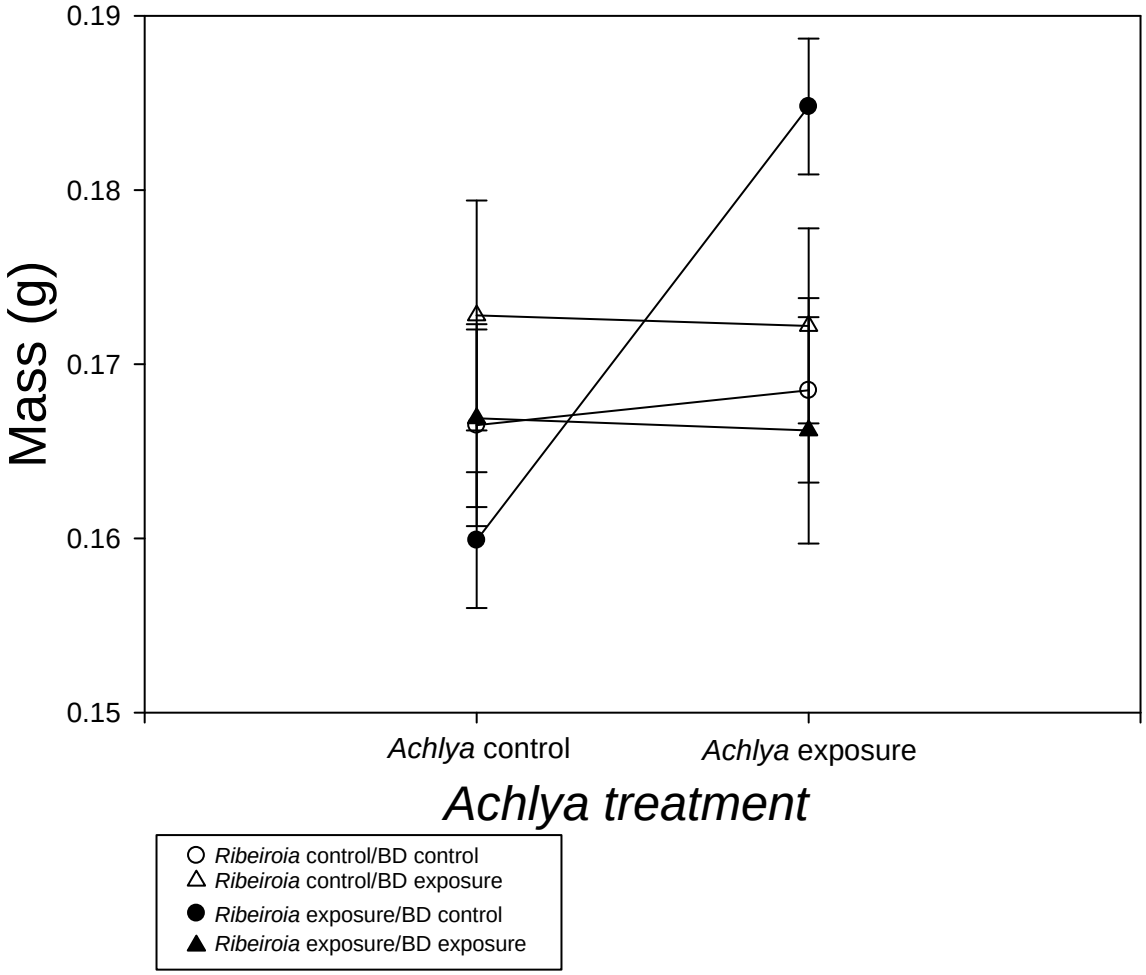

Figure 5.4. Mean mass at metamorphosis ± 1 SE of *Pseudacris regilla*.

Chapter 6. General Conclusions

Research on amphibian diseases and their relationship with amphibian population declines has focused on viruses and the pathogenic fungus *Batrachochytrium dendrobatidis* (BD). Recently, it has been claimed that BD is by far the most likely single cause of amphibian population declines not due to obvious factors such as habitat destruction (Skerrat *et al.*, 2007, but see McCallum 2005). However, focusing on a single pathogen as the cause of these declines is not justified. There are several there are other factors, including other pathogens that may play important roles in the population-level biology of amphibians. If BD is negatively affecting a host population, it does not necessarily preclude other factors, such as environmental stressors or additional pathogens, from affecting the host population directly or by interacting synergistically with BD. Indeed, environmental stressors can make amphibians more susceptible to pathogens (e.g. Kiesecker & Blaustein, 1995, Kiesecker, 2002, Forson & Storfer, 2006a) and BD may be influenced by temperature and climate change (Woodhams *et al.*, 2003, Berger *et al.*, 2004, Pounds *et al.*, 2006, Bosch *et al.*, 2007). Moreover, there are several cases in which an amphibian population has not gone extinct in the presence of BD, but rather persists with BD infections (Retallick, *et al.*, 2004, Daszak *et al.*, 2005, Kriger & Hero, 2006, 2007, Briggs, 2005). These host populations, in which BD appears to be endemic, are at risk from other factors, including other pathogens.

My thesis explored how amphibians are affected by water molds, a group of pathogens that may contribute to amphibian population declines. *Saprolegnia* has gained attention as a factor that may play a role in amphibian population declines because it contributes to massive mortality of amphibian embryos (Blaustein *et al.*, 2004, Kiesecker

& Blaustein, 1995). Most research on the effects of water molds on amphibians has concentrated on the embryonic life history stage. To help gain a more complete evaluation of how these pathogens influence amphibian populations, I tested the effects of water molds on post-embryonic amphibians.

The results of Chapters 2, 3, and 4 demonstrate that *Saprolegnia* can kill amphibians at the larval and newly-metamorphosed juvenile life stages. These results suggest that possible effects on post-embryonic life stages need to be investigated to more fully understand the population-level effects of *Saprolegnia* on amphibians. Because mortality of post-embryonic life stages may contribute to population declines in amphibian species (Biek *et al.*, 2002, Vonesh & De la Cruz, 2002a,b), mortality of larvae and newly-metamorphosed juveniles from water molds may intensify the effects of these pathogens on amphibian populations. Thus, water molds deserve further attention from amphibian researchers.

Synergistic effects of environmental stressors and pathogens may greatly influence the dynamics of amphibian population declines (e.g. Kiesecker & Blaustein, 1995, Taylor *et al.*, 1995, Christin *et al.*, 2002). However, the results of Chapter 2 suggest that the environmental contaminant nitrate and *Saprolegnia* had less-than-additive effects on survival of *Rana aurora* (red-legged frog) larvae. *Saprolegnia* killed *R. aurora* larvae, but only when nitrate was not added, consistent with the addition of nitrate preventing *Saprolegnia* from affecting survival in larvae of this species. Such a less-than-additive interaction could have resulted from direct negative effects of nitrate on *Saprolegnia*, nitrate-induced shift of *Saprolegnia* from parasitism to saprobism, or by a nitrate-induced increase in immune response by *R. aurora*. This study elucidates the

range of complexity possible in the disease ecology of amphibian disease ecology (Blaustein & Kiesecker, 2002).

In contrast to the less-than additive interaction found in Chapter 2, no interaction affecting survival of amphibian larvae was found between predator cues and *Saprolegnia* in Chapter 4. However, the presence of synergistic effects of predator cues and environmental contaminants on amphibians is context dependent, depending on the type of chemical, species of predator, and species of amphibian (Relyea, 2003, 2004, 2005). Thus, our investigation of possible synergism between predator cues and pathogens should be considered a first step toward evaluating the importance of predator cues in amphibian diseases. It is possible that there may be synergistic interactions between predator cues and amphibian pathogens in other ecological contexts. Likewise, Chapter 5 tested for but did not find between-pathogen interactions among BD, the pathogenic water mold *Achlya flagellata*, and the parasitic trematode *Ribeirioa*. However, the presence or absence of such interactions may likewise depend on ecological context. Thus, investigations of such interactions in other amphibian host-pathogen systems are warranted.

Although no between-pathogen interactions were found, the results of Chapter 5 include some interesting main effects of *Ribeiroia* on *P. regilla* (Pacific treefrog) larvae. *Ribeiroia* caused mortality and increased frequency of limb deformities, consistent with the results of Johnson *et al.* (1999). The range of deformities produced by *Ribeiroia* in our study was similar to that found by Johnson *et al.* (1999). Many of the deformities were severe, such as missing or partially missing limbs, abnormally small limbs, and extra limbs. Severe deformities like these are likely to impair survival in nature and may

result in population-level declines (Johnson *et al.*, 2005). Our study adds to the evidence that *Ribeiroia* causes limb deformities in amphibians. In addition, we further demonstrate that this pathogen causes a wide range of deformities that includes not only extra limbs and extra limb elements, but also missing and partially missing limbs and toes, shortened limbs and digits, abnormally small limbs, joint deformities, skin webbings, finger-like projections, and bumps. Skelly *et al.* (2007) found a low frequency (5%) of extra limbs or limb elements among the deformities in Vermont amphibians, and used this to argue against *Ribeiroia* being a causative factor in these deformities. However, in our study, relatively few of the deformities produced by *Ribeiroia* involved extra limbs or limb elements. Frequency of extra limbs and limb elements in *Ribeiroa*-exposed individuals ranged from 5.3 to 14.3% of across different treatments, lower than in previous experimental studies of *Ribeiroia* and *P. regilla*. Thus, the relative frequency of the different types of limb deformities caused by *Ribeiroia* may be context-dependent. In light of our results, low percentages of extra limbs or limb elements should not be interpreted as strong evidence against *Ribeiroia* being involved.

In addition, we found that *Ribeiroia* decrease rate of development in *P. regilla* hosts. There was an overall effect of slower development in *Ribeiroia*-exposed *P. regilla* compared to unexposed controls. Such delayed development may be caused by *P. regilla* shifting resources from development to healing of skin wounds caused by entry of *Ribeiroia* cercaria and/or shifting resources to an immune response to *Ribeiroia* infection. *Ribeiroia* appears to cause limb deformities in amphibians, including *P. regilla*, only if it infects during early hindlimb development (Schotthoefer *et al.*, 2003, Bowerman & Johnson, 2003). Thus, delayed development may lengthen this critical window in

development, which may allow more cercaria to infect during this window and lead to a higher prevalence of deformities, more severe deformities, and a greater number of limb deformities per deformed individual. This could have interesting consequences for *Ribeiroia* and its hosts. Limb deformities may greatly aid transmission of *Ribeiroia* from amphibians to their vertebrate definitive hosts which prey on amphibians (Johnson *et al.*, 2005). Therefore, delayed development that intensifies deformities may contribute to such transmission.

In addition, among *P. regilla* that did not die or display any limb deformities, development was slower in *Ribeiroia*-exposed individuals compared to unexposed controls. This suggests that *Ribeiroia*-exposed *P. regilla* that escape both death and severe limb deformities have delayed development. This has potentially lethal consequences for amphibian larvae in temporary aquatic habitats, since larvae that fail to metamorphose before the habitat dries up will die. Thus, *Ribeiroia*-induced delayed development amphibian individuals may increase the population-level effects of *Ribeiroia* on amphibian hosts, especially those that breed in temporary ponds and streams.

Chapter 5 also describes an interesting main effect of BD. Exposure to BD sped up development in *P. regilla* larvae. However, there was no evidence of BD infection. This dual result is consistent with *P. regilla* responding to the presence of BD by accelerating development. Hastened metamorphosis after exposure to BD may be advantageous because earlier dispersal from larval habitats may reduce the risk of infection with BD or reduce BD infection load. Our results concerning BD are particularly relevant to efforts to determine the effects of BD on amphibian populations,

since these results are consistent with BD producing a biologically significant effect in amphibians that it does not succeed in infecting.

Overall, my thesis highlights that further research is needed on a variety amphibian pathogens, not just BD. Although is highly appropriate that measures be taken to prevent the potential spread of BD to naïve amphibian populations (Skerrat *et al.*, 2007), a variety of pathogens may also play a role in the global decline of amphibians. Indeed, research is still in the early stages for some pathogens that are associated with massive mortality of amphibians (Raffell, 2006, Davis *et al.*, 2007). If their potential effects on amphibian populations are ignored, managers may miss valuable opportunities to conserve amphibian biodiversity.

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