AN ABSTRACT OF THE DISSERTATION OF

Julia C. Buck for the degree of Doctor of Philosophy in Zoology presented on May 17, 2013.

Title: Environmental Influences on Host-Pathogen Dynamics of the Amphibian Chytrid Fungus.

Abstract approved:

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Andrew R. Blaustein

The causes of the global biodiversity crisis are varied and complex. Anthropogenic threats may act in isolation, or interact additively or synergistically with each other or with natural stressors to affect sensitive taxa. The recent emergence of many infectious diseases in wildlife has brought attention to the role of disease in population declines and species extinctions. Both abiotic and biotic components of the environment may mitigate or exacerbate effects of pathogens on their hosts through direct or indirect mechanisms. The effects of the environment on host-pathogen dynamics are complex, context-dependent, and in need of further examination.

One particularly sensitive group, amphibians, is at the leading edge of the sixth mass extinction. The emerging infectious disease (EID) chytridiomycosis, caused by the fungal pathogen *Batrachochytrium dendrobatis* (Bd), is implicated in population declines and extinctions of amphibians globally. My disseration addresses questions pertaining to environmental influences on disease dynamics of Bd. As described in chapter 1, various abiotic and biotic components of the environment may affect host-

pathogen dynamics of Bd, resulting in changes to the dynamics of Bd transmission and spread.

Chapter 2 examines the influence of an abiotic factor, the insecticide (carbaryl) and three different assemblages of larval Pacific treefrogs (*Pseudacris regilla*) and Cascades frogs (*Rana cascadae*) on host-pathogen dynamics of Bd within a community context. I found separate effects of each treatment on amphibian growth and development, but no interactive effects among the treatments. However, Bd appeared to reduce phytoplankton abundance and increase periphyton biomass, an unexpected result that merited further investigation.

One possible explanation for the results described in chapter 2 is that zooplankton might consume Bd zoospores, the infective stage of the pathogen, a hypothesis that I examine in chapter 3. I conducted laboratory experiments and confirmed the presence of Bd zoospores in the gut of *Daphnia sp.* through quantitative PCR and visual inspection. I discuss conservation implications of this finding.

To determine whether predation on Bd zoospores by zooplankton could reduce infection in amphibians, I conducted a mesocosm experiment, which is described in chapter 4. I found complex effects on species interactions: competition between larval Cascades frogs and zooplankton for phytoplankton resources reduced phytoplankton concentration, zooplankton abundance, and survival of amphibians. These effects were diminished in the presence of Bd, suggesting that zooplankton may have at least partially substituted Bd zoospores for phytoplankton in their diet, thus stimulating competitive release. However, competitive effects between zooplankton and larval

amphibians overshadowed indirect positive benefits of zooplankton predation on Bd zoospores.

In chapter 4, competitive effects between zooplankton and larval amphibians for phytoplankton suggested that host-pathogen dynamics might be affected by the host's supply of resources. Chapter 5 describes a mesocosm experiment that examined how eutrophication might affect Bd-infected Pacific treefrogs and other members of the aquatic community. Nutrient additions caused increased algal growth, which benefitted herbivorous larval amphibians. Larvae exposed to Bd altered their growth, development, and diet, and allocated resources differently than unexposed individuals. However, nutrient supplementation did not alter the response of larval amphibians to Bd.

As described in chapter 6, consideration of hosts and pathogens as functional members of the ecological communities in which they exist can lead to important insights in host-pathogen dynamics. My PhD research may contribute to control measures for the emerging infectious disease chytridiomycosis.

Environmental Influences on Host-Pathogen Dynamics of the Amphibian Chytrid Fungus

by
Julia C. Buck

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Dean of the Graduate School

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

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Julia C. Buck, Author

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CONTRIBUTION OF AUTHORS

Chapter 1: Dr. Andrew Blaustein substantially improved the written manuscript.

Chapter 2: Dr. Andrew Blaustein and Dr. Rick Relyea helped design the experiment, analyze data, interpret results, and substantially improved the written manuscript. Dr. Erin Scheessele provided equipment and assisted with data collection.

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Chapter 6: Dr. Andrew Blaustein substantially improved the written manuscript.

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Chapter 1

Introduction

Julia C. Buck and Andrew R. Blaustein

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Unlike previous mass extinction events, the impending sixth mass extinction is rooted in anthropogenic causes (Barnosky et al. 2011). Current extinction rates are 100-1000 times greater than background extinction rates and are accelerating (May 2010). Identifying and understanding the complex causes of this biodiversity crisis is a pressing challenge. This task is complicated because threats to biodiversity rarely operate in isolation. Rather they tend to interact with one another, often synergistically.

Emerging infectious diseases (EID's) are playing a prominent role in the current biodiversity crisis (Daszak et al. 2000, Smith et al. 2006). For example, fungal and fungal-like diseases are causing severe population declines and extinctions of animals and plants globally (Fisher et al. 2012). The ascomycete fungus *Geomyces destructans* causes white nose syndrome, which threatens bat populations in North America (Foley et al. 2011), and the oomycete *Phytopthora ramorum* is responsible for the sudden oak death epidemic (Grunwald et al. 2012). Epidemiological theory predicts that a pathogen will fade out when its host's population is driven below a threshold density (Anderson and May 1979). Thus, density-dependent infectious diseases rarely cause species extinctions in isolation, although a few documented cases exist (de Castro and Bolker 2005). However, in combination with other stressors, infectious diseases are increasingly recognized as a major cause of population declines and extinctions of wildlife (Smith et al. 2009).

Epidemiologists investigate interactions between hosts and their pathogens, but the influence of the environment in shaping these interactions is important for a more thorough understanding of how a disease is manifested. Both abiotic and biotic components of the environment may modulate effects of pathogens on their hosts through a variety of mechanisms. When hosts are exposed to stressful conditions, they may lose their ability to launch an effective immune response, rendering them susceptible to pathogens (Luebke et al. 1997, Carey et al. 1999, Lafferty and Gerber 2002, Lafferty and Holt 2003, Marcogliese and Pietrock 2011, Kiesecker 2011). Another direct effect may occur if abiotic and biotic stressors affect the pathogen itself, for example, by altering its growth rate. Stressors may also affect host-pathogen dynamics indirectly by changing food web dynamics or causing trophic cascades. Hosts and pathogens are functional members of ecosystems (Lafferty et al. 2006, Lafferty et al. 2008) and changes to community structure may contribute to epidemics. For example, increasing biodiversity may increase (amplification effect) or decrease (dilution effect) disease risk (Keesing et al. 2006). Thus, the effects of abiotic and biotic components of the environment on host-pathogen dynamics are complex, context-dependent, and in need of continuing examination, in light of numerous EID threats (Daszak et al. 2000, Smith et al. 2006).

Although the loss of biodiversity is affecting all taxonomic groups, among vertebrates, amphibians are declining at especially alarming rates. One estimate suggests that extinction rates of amphibians may be 211 times greater than the

background rate of extinction (McCallum 2007) and more than 40% of amphibian species have experienced population declines or extinctions in recent decades (Stuart et al. 2004). The causes for amphibian population declines are complex and varied (Stuart et al. 2004, Blaustein and Kiesecker 2002, Wake and Vredenburg 2008). Anthropogenic threats including habitat loss, invasive species, climate and atmospheric changes, and contaminants may act in isolation, or interact additively or synergistically with each other and with natural stressors including competition, predation, and disease.

Pathogens, some of which are associated with EID's, are playing an especially prominent role in amphibian population declines and extinctions. A variety of infectious agents including viruses, bacteria, oomycetes, and fungi (microparasites) as well as trematodes, nematodes, cestodes, acanthocephalans, mites, and copepods (macroparasites) infect amphibians and may contribute to amphibian population declines and extinctions (Blaustein and Kiesecker 2002, Blaustein et al. 2012). One particular pathogen, the amphibian chytrid fungus, *Batrachochytrium dendrobatidis* (hereafter Bd), first described in 1999 (Longcore et al. 1999), is now widespread globally (Hyatt et al. 2010). Chytridiomycosis is recognized as one of the most significant factors contributing to worldwide amphibian population declines and extinctions (Skerratt et al. 2007, Wake and Vredenburg 2008, Rohr et al. 2008a). The infective stage of the fungus is a motile aquatic flagellated zoospore which encysts in keratinized tissues of amphibian hosts (Longcore et al. 1999, Rosenblum et al. 2008).

There it develops into a sporangium in which new zoospores mature. Once released into the environment, these zoospores may reinfect the same host individual or may infect a different individual. Keratinized tissues of amphibians that are vulnerable to infection include the mouthparts of larval amphibians (Fellers et al. 2001) and the epidermis of juvenile and adult amphibians.

The effects of chytridiomycosis vary with Bd strain, host species, and life history stage (Rachowicz and Vredenburg 2004, Berger et al. 2005, Blaustein et al. 2005, Searle et al. 2011). Most information on mortality comes from studies on post-metamorphic stages of amphibians. After metamorphosis, Bd infection may disrupt cutaneous osmoregulation, which causes an electrolyte imbalance that can lead to cardiac arrest (Voyles et al. 2009). Clinical signs of chytridiomycosis that may appear before death include abnormal posture, lethargy, epidermal sloughing, and loss of righting reflex (Daszak et al. 1999). Among larval amphibians, lethal effects after exposure to Bd are less common (e.g. Rachowicz and Vredenburg 2004, Blaustein et al 2005) and have been postulated to be due to proteases or other lethal toxins released by the pathogen (Berger et al. 1998, Blaustein et al. 2005, Brutyn et al. 2012, McMahon et al. 2013). Keratinized tissues vulnerable to infection in larval amphibians are limited to the mouthparts, and sublethal effects of Bd exposure are more commonly reported for larvae. These effects can include reduced feeding efficiency (Venesky et al. 2009, 2010), and reduced intestinal length (Venesky et al. 2013), which may contribute to reduced growth and development of larval amphibians

exposed to Bd (Parris and Cornelius 2004, Parris and Beaudoin 2004, Parris and Baud 2004). Sublethal effects on larval amphibians may carry over to later life stages.

Questions about the origins of Bd arose soon after its discovery (e.g. Weldon et al. 2004, Rachowicz et al. 2005). The “novel pathogen hypothesis” postulates that recent spread of Bd from introduction sites is causing observed population declines and extinctions of naïve hosts (Rachowicz et al. 2005), while the “endemic pathogen hypothesis” suggests that Bd was globally endemic, but has recently become pathogenic due to anthropogenic changes to the environment or changes in host responses (Rachowicz et al. 2005). Although these hypotheses have sometimes been treated as mutually exclusive (e.g. Rachowicz et al. 2005, Rohr et al. 2008a), geographic spread of the fungus does not preclude a role for cofactors in observed population declines and extinctions (Morell 1999, McCallum 2005). Recent molecular comparisons of Bd isolates from several different continents reveal the existence of a hypervirulent global panzootic lineage (BdGPL) that is spreading geographically and causing amphibian population declines and extinctions in its wake (Fisher et al. 2009, James et al. 2009, Farrer et al. 2011). At the same time, evidence is accumulating that changes to the environment can alter host-pathogen dynamics. Here I briefly review evidence that abiotic and biotic components of the environment can affect host-pathogen dynamics of Bd. I conclude with implications of these findings for amphibian conservation and recommendations for future research.

Abiotic influences

Climate and atmospheric changes

Global climate change may affect amphibians disproportionately compared to other vertebrates because their aquatic habitats, physiological requirements, and limited dispersal abilities make them especially sensitive (Lawler et al. 2010). Increasing temperatures and changes in precipitation may interact additively or synergistically with other stressors, such as diseases. For example, climate change may reduce water depth in amphibian breeding habitats, thus increasing exposure of embryos to ultraviolet –B (UVB) radiation, and consequently, their susceptibility to infection by a pathogenic water mold, *Saprolegnia ferax* (Kiesecker and Blaustein 1995, Kiesecker et al. 2001). Climatic conditions may also influence pathogens directly, a finding with important implications for disease dynamics (Harvell et al. 2002, Acevedo-Whitehouse and Duffus 2009, Brooks and Hoberg 2007). For example, both the trematode parasite *Ribeiroia ondatrae* and its snail intermediate host *Planorbella trivolvis* exhibited increased growth and development at higher temperatures, which may affect pathology of amphibian limb malformations caused by the parasite (Paull and Johnson 2011, Paull et al. 2012).

Bd growth is maximized at relatively low temperatures of 17-25^{0}C (Piotrowski et al. 2004). Below this optimal temperature, trade-offs increase fecundity as maturation rate slows and increase infectivity as growth decreases (Woodhams et al. 2008). Corresponding to these findings, field studies have shown that the probability

of infection declines as a host individual spends more time above 25^0C (Rowley and Alford 2013), and the most severe population declines often occur at high elevations and at relatively low temperatures (reviewed by Fisher et al. 2009). For example, Drew et al. (2006) showed that Bd is more likely to be present at sites where the average summer maximum temperature is <30ºC. Pounds et al. (2006) proposed that climate change is shifting temperatures at highland localities toward the thermal optimum for Bd, which may have caused the extinction of an estimated 67% of species within the genus *Atelopus*. Bosch et al. (2007) confirmed this "chytrid thermal-optimum hypothesis", showing that rising temperatures were linked to disease outbreaks in natural populations of amphibians in Spain. Another study showed that temperature variability, rather than warming per-se, might ultimately be responsible for the *Atelopus sp.* extinctions (Rohr and Raffel 2010), a finding which was corroborated by a mesocosm experiment on amphibian larvae (Hamilton et al. 2012a).

UVB radiation has strong negative effects on survival and growth of many aquatic organisms (Bancroft et al. 2007). In amphibians, its effects vary by species, population, and developmental stage, and it may interact synergistically with other stressors including disease (Bancroft et al. 2008). Thus, host-pathogen dynamics of Bd may be altered in the presence of this additional stressor. In a field experiment, tadpoles exposed to UVB radiation exhibited lower infection prevalence than individuals shielded from UVB radiation (Ortiz-Santaliestra et al. 2011). However, other field and laboratory experiments found no interactive effects of UVB radiation

and Bd on larval (Searle et al. 2010) and metamorphic (Garcia et al. 2006) amphibians.

Contaminants

Contaminants such as fertilizers, pesticides, and heavy metals are introduced to aquatic habitats via direct application, agricultural and urban runoff, and atmospheric deposition (Carpenter et al. 1998, Kolpin et al. 2002). They may alter ecosystem function, and are thus considered to be a major threat to aquatic ecosystems (Wilcove and Master 2005). Amphibians may experience lethal or sublethal effects following contaminant exposure, including reduced growth and development, altered behavior, and developmental abnormalities (reviewed by Egea-Serrano et al. 2012). Furthermore, community structure may be altered by contaminants, and trophic cascades involving amphibians may result (Relyea et al. 2005, Relyea and Diecks 2008).

Interactions between contaminants and pathogens may alter disease outcomes. Pesticide exposure may reduce immunocompetence and increase susceptibility of amphibian hosts to diseases (e.g. Carey et al. 1999). For example, Kiesecker (2002) showed through laboratory and field experiments that pesticide exposure reduced the ability of wood frog (*Rana sylvatica*) tadpoles to resist infection by the trematode *Riberoia ondatrae*, leading to increased parasite loads and a greater risk of limb deformities. Simultaneous exposure of tiger salamander (*Ambystoma tigrinum*) larvae

to the pesticide chlorpyrifos and *Ambystoma tigrinum* virus (ATV) resulted in increased mortality compared to either stressor alone (Kerby and Storfer 2009).

Although contaminants may increase susceptibility of amphibians to some diseases, the link between pesticide exposure and increased susceptibility to Bd is tenuous. In a laboratory study, Kleinhenz et al. (2012) found negative impacts of exposure to Bd on activity, survival, and development of larval *P. regilla*, but exposure to four insecticides (either individually or as a mixture) did not change the effects of Bd exposure. Davidson et al. (2007) showed that exposure to the pesticide carbaryl reduced skin peptide defenses of post-metamorphic amphibians, but similar results were not found for post-metamorphic amphibians exposed to the pesticide malathion in the larval stage (Groner et al. in press). Neither of these studies found evidence that pesticide exposure altered the susceptibility of post-metamorphic amphibians to chytridiomycosis, and exposure to the herbicide glyphosate may reduce, rather than increase, morality due to chytridiomycosis (Gahl et al. 2011). While direct effects of pesticides on Bd-susceptibility are variable, indirect effects may also be possible. In chapter 2, we tested the hypothesis that carbaryl alters host-pathogen dynamics of Bd within a community context.

Biotic influences

Competition between hosts

In addition to abiotic factors in the environment, biotic factors may also mitigate or exacerbate effects of pathogens on hosts through direct or indirect mechanisms. Competition is a major structuring force in ecological communities (Schoener 1983, Connell 1983, Gurevitch et al. 1992). Herbivores, including larval anurans, may experience stronger effects of competition than other trophic or taxonomic groups (Gurevitch et al. 1992). Inter- and intraspecific competition between larval amphibians can limit resource availability, which could affect growth and development (Wilbur and Collins 1973). A pathogen that differentially affected competing species could change the outcome of this interaction. For example, Kiesecker and Blaustein (1999) showed that the pathogenic fungus *Saprolegnia ferax* reversed the outcome of competitive interactions between two species of larval anurans.

Bd infects keratinized tissues of larval amphibians, causing mouthpart abnormalities (Fellers et al. 2001) that reduce feeding efficiency (Venesky et al. 2009, 2010). Thus, competition between amphibian hosts may exacerbate these effects by reducing resource availability. However, few studies have examined possible interactions between Bd and inter- or intraspecific competition of hosts. Parris and Cornelius (2004) showed that the outcome of interactions between larval *Bufo fowleri* and *Hyla chrysoscelis* was reversed by Bd infection in experimental mesocosms: interspecific competition was manifested only in the presence of the pathogen. In chapter 2, we examined the hypothesis that different amphibian assemblages (presence

of conspecific versus heterospecific competitors) can alter host-pathogen dynamics of Bd.

Predation on hosts

Predators may also alter the effects of pathogens on hosts. Trophically-transmitted parasites are transmitted upon consumption of their intermediate host by a definitive host (Lafferty 1999). For parasites and pathogens that are not trophically-transmitted, diseased prey are often preferentially selected by predators, which could reduce transmission to healthy individuals in the prey population (e.g. Schaller 1972, Moore 2002). Thus, theory predicts that Bd-infected amphibian larvae should be consumed by predators at a higher frequency than uninfected individuals, as shown by Han et al. (2011) for red-legged frog tadpoles (*Rana aurora).* However, this response may be species-specific: infected leopard frog tadpoles (*R. pipiens)* experienced lower mortality rates due to predation than uninfected tadpoles (Parris et al. 2006). This result might be explained by increased use of refuges by some species of infected larvae in response to predator cues (Han et al. 2011). For other species, however, both chemical and visual predator cues may be needed to elicit predator-avoidance behavior of tadpoles infected with Bd (Parris et al. 2006).

In addition to effects on behavior, predators may alter development rates of larval amphibians (Skelly 1992). If developmental rates are also changed by a pathogen, then the potential for additive or synergistic effects exists. Parris and

Beaudoin (2004) showed that both Bd and predator cues were needed to elicit reduced development rates of tadpoles. Effects of predators on host-pathogen dynamics may also persist through subsequent developmental stages. Groner et al. (in press) showed that amphibians exposed to predator cues as larvae experienced a 20% reduction in hydrophobic skin peptides thought to inhibit Bd infection.

Reservoir hosts and carriers of Bd

A biotic reservoir is a host that becomes infected with a pathogen and can transmit it to other susceptible hosts, but is tolerant to the pathogen (de Castro and Bolker 2005). Reservoir species are of concern in Bd outbreaks because they may spread the fungus geographically and to other species (Reeder et al. 2012). Several species of amphibians may act as reservoir hosts of Bd. Recent retrospective field studies suggest that *Pseudacris regilla* is an efficient vector of Bd in its native range, the western United States (Padgett-Flohr and Hopkins 2009, 2010). This species is a habitat generalist that persists after extirpation of other native species (Reeder et al. 2012) and it carries high infection loads in laboratory studies (Reeder et al. 2012, Gervasi et al. 2013). Invasive species may also serve as reservoir hosts of Bd. The American bullfrog, *Lithobates catesbeianus*, is invasive in the western United States and on several other continents where it is commonly farmed. Bullfrogs can carry and transmit some strains of Bd to other amphibians (Garner et al. 2006, Greenspan et al. 2012), but may be susceptible to other strains of the fungus (Gervasi et al. in press).

Large-scale global trade in this species is hypothesized to have contributed to the geographic spread of Bd (e.g. Mazzoni et al. 2003, Schloegel et al. 2009). Other invasive amphibian species that may act as reservoir hosts for Bd include *Xenopus laevis* (Solis et al. 2011) and *Bufo marinus* (Berger et al. 1998).

Until recently, non-amphibian carriers of Bd were unknown. However, McMahon et al. (2013) detected zoosporangia within the gastrointestinal tract of crayfish, and found pathogen prevalence in crayfish in natural systems of up to 29%. Experiments confirm that crayfish can maintain Bd infection, and transmit the pathogen to amphibians (McMahon et al. 2013).

Other pathogens

While host-pathogen dynamics may be influenced by interactions between hosts and other members of the community, diversity at the level of the pathogen may also influence disease dynamics. Simultaneous infection with multiple parasites or pathogens is common in many systems (Pedersen and Fenton 2007, Telfer et al. 2010), including amphibians (Johnson and Hoverman 2012). For example, Hoverman et al. (2012) found that Bd commonly co-occurs with ranaviruses and trematodes in wetlands in the East Bay region of California, U.S.A. Because parasites within a host are likely to interact strongly (Fenton and Perkins 2010, Hawley and Altizer 2010), coinfection has the potential to profoundly influence disease dynamics. For example, Johnson and Hoverman (2012) investigated coinfection dynamics of six trematode

parasite species in an amphibian host, *P. regilla*. They found evidence that parasite richness reduced overall infection success (a dilution effect), but had variable effects on pathology. In the only experiment examining coinfection dynamics of Bd, Romansic et al. (2011) found that larval *P. regilla* simultaneously exposed to the trematode *Ribeiroia ondatrae* and Bd experienced higher mortality than larvae exposed to either the trematode or the fungus alone.

Skin microbiota

Diversity at the level of the pathogen also includes non-pathogenic microorganisms, which may interact with pathogens (Robinson et al. 2010, Grice and Segre 2011). As part of their innate immune system, amphibians have a well-developed skin microbiota (Becker et al. 2009, Harris et al. 2009a, Harris et al. 2009b, Muletz et al. 2012). Normal microbiota on the skin of amphibians may compete with pathogenic organisms, and have the potential to exclude them (Belden and Harris 2007). These bacteria may suppress chytridiomycosis by producing inhibitory metabolites or regulating the production of antimicrobial peptides (AMPs) and lysozymes (Harris et al. 2009 a, 2009b, reviewed by Rollins-Smith 2009, Rollins-Smith et al. 2011). For example, two genera of bacteria, *Pseudomonas* spp. and *Janthinobacter* spp. can produce anti-chytrid metabolites such as violacein, which may mitigate effects of Bd on amphibian hosts (Lauer et al. 2007, 2008, Woodhams et al. 2007, Becker et al. 2009, Harris et al. 2009a, 2009b). Bioaugmentation adds

beneficial (probiotic) strains of bacteria to individual hosts or to the environment to mitigate disease (Fuller 1989, Verscheure et al. 2000). This strategy is currently being employed to mitigate the effects of Bd on amphibians (Harris et al. 2009a, Vredenburg et al. 2011, reviewed by Bletz et al. 2013).

Other species

Pathogens may also interact with other species in the aquatic community to alter disease dynamics. For example, as we show in chapter 3, cladoceran zooplankton ingest Bd zoospores and are predicted to digest them (Beakes et al. 1988, Kagami et al. 2007, Gleason et al. 2009). This may limit the numbers of infective zoospores in the water column (Woodhams et al. 2011, Hamilton et al. 2012), which could protect amphibians from Bd infection, a hypothesis we test in chapter 4.

Reduced foraging efficiency of larval amphibians infected with Bd (Venesky et al. 2009, 2010) suggests that negative effects of Bd infection in larval growth and development could be overcome if algal resources are abundant, a hypothesis we test in chapter 5.

Conclusions

The “novel pathogen hypothesis” and the “endemic pathogen hypothesis” postulate different origins of Bd and different ultimate causes for observed population declines and extinctions. Distinguishing between these hypotheses is important

because disease management strategies may change depending on which hypothesis is correct (Rachowicz et al. 2005). However, geographic spread of the fungus does not preclude a role for cofactors in observed amphibian population declines and extinctions (Morell 1999, McCallum 2005). Abiotic and biotic factors including climate change and UVB radiation, contaminants, competitors, predators, carriers, other pathogens, skin microbiota, and other species have the potential to modulate host-pathogen dynamics through direct and indirect mechanisms.

Future directions

Abiotic and biotic influences on host-pathogen dynamics of Bd have been investigated from the molecular level (e.g. Davidson et al. 2007) to the level of the amphibian species assemblage (e.g. Searle et al. 2011), yet relatively few studies have investigated host-pathogen dynamics of Bd within a community context. Those that have done so (e.g. Parris and Baud 2004, Parris and Beaudoin 2004, Parris and Cornelius 2004, Hamilton et al. 2012) have used outdoor experimental mesocosms, revealing interesting and unexpected results. The value of experimental mesocosms for revealing ecological patterns and processes has been discussed (Carpenter 1996, Skelly and Kiesecker 2001, Skelly 2002, Chalcraft et al. 2005, Skelly 2005). Although field experiments in natural habitats are ideal venues for studying many ecological interactions, manipulation of pathogens in natural systems is often not possible. Experimental mesocosms allow one to manipulate a pathogen under more realistic

conditions than laboratory experiments and thus are an ideal compromise between field experiments in natural habitats and laboratory experiments. Furthermore, mesocosm experiments offer greater opportunities for sophisticated experimental designs than field or laboratory experiments.

Table 1.1. Summary of additional stressors with the potential to interact with Bd.

1	Temperature	Piotrowski et al. 2004
2		Woodhams et al. 2008
3		Drew et al. 2006
4		Pounds et al. 2006
5		Bosch et al. 2007
6		Walker et al. 2010
7		Rohr and Raffel 2010
8		Hamilton et al. 2012a
9		Rowley and Alford 2013
10	UV-B radiation	Ortiz-Santaliestra et al. 2011
11		Searle et al. 2010
12		Garcia et al. 2006
13	Contaminants	Davidson et al. 2007
14		Kleinhenz et al. 2012
15		Groner et al. in press
16		Gahl et al. 2011
17	Competition	Parris and Cornelius 2004
18	Predation	Han et al. 2011
19		Parris et al. 2006
20		Parris and Beaudoin 2004
21		Groner et al. in press
22	Carriers	Padgett-Flohr and Hopkins 2009
23		Padgett-Flohr and Hopkins 2010
24		Garner et al. 2006

Table 1.1. (Continued)

25		Greenspan et al. 2012
26		Solis et al. 2011
27		McMahon et al. 2013
28	Other pathogens	Hoverman et al. 2012
29		Romansic et al. 2011
30		Woodhams et al. 2007
31	Skin microbiota	Becker et al. 2009
32		Harris et al. 2009a
33		Harris et al. 2009b
34	Other species	Hamilton et al. 2012b

Table 1.1. (Continued)

Amphibian species	**Venue**
pathogen grown in liquid medium	laboratory
pathogen grown in liquid medium	laboratory
many	field (observation) - Australia
many	field (observation) - South America
many	field (observation) - Spain
Alytes obstetricans	field (observation) - Spain
many	field (observation) - South America
Rana aurora, Pseudacris regilla	mesocosm
Litoria lesueuri, L. serrata, L. nannotis	field (observation) - Australia
Bufo bufo	field (experiment) - Spain
Rana cascadae	laboratory and mesocosm
P. regilla, R. cascadae, A. boreas	laboratory
Rana boylii	laboratory
P. regilla	laboratory
Lithobates sylvaticus	laboratory
L. sylvaticus	laboratory
Bufo fowleri, Hyla chrysoscelis	mesocosm
many	laboratory
Rana pipiens	laboratory
H. chrysoscelis	mesocosm
L. sylvaticus	laboratory
many	field (observation) - North America
many	field (observation) - North America
L. catesbeianus	field (observation) - several continents

Table 1.1. (Continued)

L. sylvaticus, L. catesbeianus	laboratory
X. laevis	field (observation) - South America
many	laboratory, field (observation) - North America
many	field (observation) - North America
P. regilla	laboratory
Rana muscosa	field (observation)
Plethodon cinereus	laboratory
R. muscosa	laboratory
Plethodon cinereus	laboratory
R. aurora	laboratory and mesocosm

Table 1.1. (Continued)

Pathogen growth maximized at 17-25^{o}C. Higher temperatures lethal.
Zoospore production and infectious period maximized at 7-10^{o}C, encystment and development maximized at 17-25^{o}
Bd presence more likely at sites where average summer maximum temperature is <30^{o}C
Timing of *Atelopus* extinctions assiciated with changes in air temperatures and sea surface temperature.
Rising temperature linked to chytridiomycosis outbreaks.
Outbreaks of fatal chytridiomycosis associated with high elevations and low temperatures.
Temperature variability due to global El Niño climatic events drove *Atelopus* extinctions.
Temperature variability interacted with Bd presence to favor competing *P. regilla* over *R. aurora*
Host's probablity of infection declined with increased time spent above 25^{o}C
Infection prevalence lower in tadpoles exposed to natural UVB radiation compared those shielded from radiation.
No interactive effects of UVB and Bd.
No interactive effects of UVB and Bd.
Skin peptide defenses reduced by carbaryl exposure.
No interactive effects of insecticides and Bd.
No interactive effects of malathion and Bd.
Trend: Glyphosate-based herbicide reduced Bd-induced mortality.
Interspecific competitors magnified negative effects of Bd on growth and development.
Tadpoles more active and sought refuge more frequently when exposed to predator cues.
Predator avoidance behavior of infected tadpoles required visual and chemical predator cues.
Tadpole development rate negatively affected by the interaction of Bd and predators.
Predator cues decreased production of hydrophobic skin peptides.
P. regilla is an efficient vector of Bd.
P. regilla is an efficient vector of Bd.
L. catesbeianus is an efficient vector of Bd.

Table 1.1. (Continued)

L. catesbeianus can transmit Bd to *L. sylvaticus.*
X. laevis is an efficient vector of Bd.
Bd zoosporangia occur within crayfish gastrointestinal tracts with prevalence up to 29%. They can transmit Bd to amphibian hosts.
Amphibian pathogen coocurrence is common in natural systems.
Larvae exposed to Riberoia and Bd exhibited higher mortality compared to individuals exposed to each pathogen separately.
Differences in skin peptide defenses of amphibians from sites where Bd is endemic vs. newly introduced.
Adding probiotics to amphibian skin decreased negative effects of chytridiomycosis.
Adding probiotics to amphibian skin decreased negative effects of chytridiomycosis.
Adding probiotics to amphibian skin decreased negative effects of chytridiomycosis.
Daphnia decreased Bd genomic equivalents detected in the water column.

Chapter 2

The effects of multiple stressors on wetland communities: pesticides, pathogens, and competing amphibians

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Abstract

Anthropogenic effects have propelled us into what many have described as the sixth mass extinction, and amphibians are among the most affected groups. The causes of global amphibian population declines and extinction are varied, complex, context-dependent and may involve multiple stressors. However, experimental studies examining multiple factors contributing to amphibian population declines are rare. Using outdoor mesocosms containing zooplankton, phytoplankton, periphyton and tadpoles, we conducted a 2 x 2 x 3 factorial experiment that examined the separate and combined effects of an insecticide and the fungal pathogen *Batrachochytrium dendrobatidis* (Bd) on three different assemblages of larval pacific treefrogs (*Pseudacris regilla*) and Cascades frogs (*Rana cascadae*).

Larval amphibian growth and development were affected by carbaryl and the amphibian assemblage treatment, but only minimally by Bd. Carbaryl delayed metamorphosis in both amphibian species and increased the growth rate of *P. regilla*. Carbaryl also reduced cladoceran abundance, which, in turn, had positive effects on phytoplankton abundance but no effect on periphyton biomass. Substituting 20 intraspecific competitors with 20 interspecific competitors decreased the larval period but not the growth rate of *P. regilla*. In contrast, substituting 20 intraspecific competitors with 20 interspecific competitors had no effect on *R. cascadae*. Results of real-time quantitative polymerase chain reaction (qPCR) analysis confirmed infection of Bd-exposed animals, but exposure to Bd had no effects on either species in

univariate analyses, although it had significant or nearly significant effects in several multivariate analyses. In short, we found no interactive effects among the treatments on amphibian growth and development. We encourage future research on the interactive effects of pesticides and pathogens on amphibian communities.

Introduction

A current challenge in ecology is to identify multiple and complex causes of species extinctions (May 2010). At the forefront of the biodiversity crisis are amphibians (Blaustein and Kiesecker 2002, Wake and Vredenburg 2008), with recent estimates suggesting that over 40% of amphibian species are experiencing population declines and extinctions (Stuart et al. 2004). The causes of global amphibian population declines are varied and complex. Stressors may operate independently of one another or synergistically (Blaustein and Kiesecker 2002, Hayes et al. 2010, Blaustein et al. 2011). Identifying interactions between multiple stressors through experimental investigations may suggest how future declines can be minimized.

With increasing land-use change, contaminants are a ubiquitous threat in amphibian breeding habitats. One recent survey determined that 30-60% of shallow groundwater and 60-95% of streams across different land-use categories in the U.S.A. are contaminated with at least one pesticide (Gilliom 2007). Contaminants in high doses can have direct lethal effects on amphibians. More often, however, concentrations of contaminants in natural breeding sites are not lethal to amphibians

(e.g. Davidson 2004). Sublethal concentrations can cause changes in immune response, reproduction, physiology, morphology and behavior (reviewed in Relyea and Hoverman 2006), and can also affect amphibians indirectly, often through effects on the food web (Boone and Semlitsch 2001, Relyea et al. 2005, Relyea and Diecks 2008). When presented in a community context (i.e. in the presence of predators and competitors) or in the presence of other anthropogenic stressors including different contaminants, co-factors can become more deadly to amphibians than when presented alone (Relyea 2003, Relyea 2009).

Carbaryl (1-naphthyl-*N*- methylcarbamate; commercial name, Sevin) is a broad-spectrum insecticide used to control various arthropods in a variety of agricultural and urban settings across the U.S.A. It is an acetylcholinesterase inhibitor, interfering with the nervous system of exposed insects. In the Pacific Northwest, U.S.A., carbaryl is applied to apples, cherries and a variety of other agricultural crops (Williams unpubl. data), and is also applied to forests at higher elevations against bark beetles (*Scolytidae*) (Hastings et al. 2001). Carbaryl may enter amphibian habitats through direct aerial spraying, surface runoff and erosion, and it can enter high elevation habitats through aerial drift from agricultural areas at lower elevations (e.g. LeNoir et al. 1999). For example, in California, U.S.A., pesticides, presumably applied in the San Joaquin Valley lowlands, have been discovered in frogs inhabiting high altitude areas in the Sierra Nevada (e.g. Cory et al. 1970), and depressed cholinesterase activity in tadpoles has been documented in this area (Sparling et al.

2001). Davidson et al. (2001, 2002) and Davidson (2004) found correlative evidence for population declines of several species of the genus *Rana* at sites downwind of pesticide application in the Sierra Nevada Mountain Range of California, U.S.A. Of 64 classes of pesticides considered, the cholinesterase-inhibiting pesticides (including most organophosphates and carbamates) were most strongly implicated in population declines. Although fewer studies have examined atmospheric transport of pesticides to the Cascade Range of Oregon, U.S.A., predominant wind direction patterns indicate that pesticides applied in the Central Valley of California and in the Willamette Valley of Oregon are likely to be transported there (Stanley 2009). Despite compelling evidence that pesticide application is associated with downwind amphibian population declines, pesticide concentrations measured at these sites are orders of magnitude below lethal concentrations determined through laboratory experiments (Davidson 2004), suggesting a role for co-factors in these population declines.

A growing body of literature suggests that pathogens such as water molds, several viruses, and the fungus *Batrachochytrium* have contributed significantly to global amphibian population declines (e.g. Daszak et al. 1999, Blaustein and Kiesecker 2002, Daszak et al. 2003). Chytridiomycosis, an emerging infectious disease of amphibian caused by the fungus *Batrachochytrium dendrobatidis* (hereafter Bd), is considered to be among the greatest disease threats to global amphibian biodiversity (Skerratt et al. 2007, Wake and Vredenburg 2008, Rohr et al. 2008). The pathogen occurs on every continent inhabited by amphibian, and is implicated in the

recent decline of several hundred species (Skerratt et al. 2007). Aquatic zoospores of the fungus infect keratinized tissue of larval, juvenile and adult amphibians. Susceptibility varies with species (e.g. Blaustein et al. 2005) and although larval mortality is rare, presumably because the infection is restricted to the keratinized mouthparts (Longcore et al. 1999), Bd can cause a variety of sublethal effects on growth, development and behavior (Parris and Cornelius 2004, Blaustein et al. 2005, Parris et al. 2006). Although Bd is associated with numerous population declines and extinction, some populations persist in spite of pathogen presence (e.g. Retallick et al. 2004, Briggs et al. 2005, Daszak et al. 2005, Briggs et al. 2010). Furthermore, surveys of museum specimens show that individuals in many populations were infected for years preceding population declines (e.g. Ouellet et al. 2005). Taken together, this evidence suggests that co-factors may have played a role in population declines for which Bd is the only identifiable proximate cause.

Many recent studies have called for an examination of possible interactive effects of multiple stressors on amphibians (agricultural contaminants and Bd in particular; Collins and Storfer 2003, Davidson 2004, Rollins-Smith et al. 2006). One study looked for interactive effects between carbaryl and Bd (Davidson et al. 2007). It has been suggested that sublethal concentrations of contaminants could negatively affect the immune system of larval amphibians, making them more susceptible to infection (Taylor et al. 1999, Kiesecker 2002, Gilbertson et al. 2003, Christin et al. 2004). For example, exposure to the contaminants chlorpyrifos and atrazine increases

larval mortality and susceptibility of salamanders to *Ambystoma tigrinum* virus (Kerby and Storfer 2009). Skin peptide defenses thought to inhibit the growth of Bd are negatively affected by the pesticide carbaryl (Rollins-Smith et al. 2006, Davidson et al. 2007). The limited research has thus far not detected a synergism between any pesticide and Bd, although interactive effects between pesticides and pathogens have not been investigated under more natural conditions.

Natural biotic stressors such as competition may also have negative effects on amphibians. At high densities of interspecific and intraspecific competitors, for example, amphibians may experience delayed metamorphosis, as well as reduced survival and growth due to competition for resources (Skelly 1997, Skelly and Kiesecker 2001). Recent research has focused on interactions between natural biotic stressors and anthropogenic abiotic stressors, especially pesticides, in amphibian habitats (e.g. Relyea 2003, Boone and Semlitsch 2001). A growing body of literature focuses on interactions between natural biotic stressors and diseases (e.g. Kiesecker and Blaustein 1999, Parris and Cornelius 2004). Among other possibilities, the strength and direction of pairwise competitive interactions between species may be context-dependent; they may change in the presence of other stressors, especially if species are differentially affected by direct effects of the other stressors (Kiesecker and Blaustein 1999, Boone and Semlitsch 2001).

We examined the interactive effects of the pesticide carbaryl, Bd, and amphibian assemblage through a 2 x 2 x 3 factorial experiment. We used technical

grade carbaryl as a representative contaminant in this experiment because its direct toxic effects on amphibians have been established (Relyea and Mills 2001); LC50 estimates vary from 1 to 18 mg L^{-1} (Marian et al. 1983, Bridges 1997, Zaga et al. 1998). Furthermore this broad-spectrum insecticide is widely applied due to its rapid breakdown and low toxicity to mammals, and upwind application of cholinesterase-inhibiting pesticides including carbamates was identified as being associated with amphibian population declines (Davidson et al. 2001, Davidson et al. 2002, Davidson 2004).

We used Bd as a representative pathogen in this experiment because chytridiomycosis is the foremost disease threatening amphibian populations worldwide (Rohr et al. 2008), and it occurs on the west coast of the U.S.A., in populations of the Pacific treefrog, *Pseudacris regilla* (Pagett-Flohr and Hopkins 2010) and the Cascades frog, *Rana cascadae* (Pearl et al. 2009), among other species. Its direct effects on larval amphibians are increasingly known from laboratory studies. Infected larvae usually experience reduced growth and delayed metamorphosis, although effects vary widely by species (Parris and Baud 2004, Parris and Beaudoin 2004, Parris and Cornelius 2004, Blaustein et al. 2005).

We used larvae of the *P. regilla* and *R. cascadae* because they commonly co-occur at breeding sites in the Cascade Range of Oregon (Nussbaum et al. 1983). *P. regilla* is a habitat generalist (Nussbaum et al. 1983) and is one of the most common amphibian species across most of its range. In contrast, *R. cascadae* inhabits moderate

to high elevation sites throughout the Cascade Range. Recently, population declines have been reported in the southern portion of the species' range (e.g. Fellers et al. 2007), and may be associated with upwind pesticide application (Davidson 2004). Its IUCN (Red List) status is "near threatened". These two species compete for periphyton, phytoplankton, and detrital resources, and they have larval periods of similar duration (Nussbaum et al. 1983; Kiesecker and Blaustein 1999). Furthermore, they exhibit differential susceptibility to Bd: *P. regilla* is less susceptible than *R. cascadae* (Blaustein et al. 2005, Garcia et al. 2006), and *R. cascadae* larvae are more likely to exhibit mouthpart abnormalities (Blaustein et al. 2005). Padgett-Flohr and Hopkins (2009, 2010) suggest that *P. regilla* may be a resistant carrier of Bd, effectively vectoring it to ephemeral habitats where it may infect more susceptible species.

We predicted that carbaryl would reduce the abundance of zooplankton (Mills and Semlitsch 2004, Relyea 2005, Relyea 2009), increase the abundance of phytoplankton (Hanazato and Yasuno 1987, Boone and James 2003), reduce the biomass of periphyton (Distel and Boone 2009), and have a negative impact on the growth and development of larval amphibians (Mills and Semlitsch 2004). We predicted that Bd would extend the larval period and reduce growth rate of amphibians, but would not affect survival (Parris and Baud 2004, Parris and Beaudoin 2004, Parris and Cornelius 2004, Blaustein et al. 2005). We predicted that at the highest density of *R. cascadae*, the superior competitor (Kiesecker and Blaustein

1999), periphyton biomass would be reduced, which would extend the larval period and reduce survival and growth rate of larval amphibians (Skelly 1997, Skelly and Kiesecker 2001). Furthermore, we hypothesized that carbaryl, Bd and amphibian assemblage would have interactive effects on larval period, survival and growth rate of larval amphibians.

Methods

We manipulated the presence of carbaryl, Bd and the amphibian assemblage in artificial ponds. The experiment took place at the Lewis-Brown Horticulture Research Farm near Corvallis, Oregon, U.S.A., and ran from 24 July to 13 August 2009. Experimental units consisted of plastic wading pools 1.5 m in diameter filled with about 120 L of tap water (pH = 8) on 19 April and covered with screen lids. On 20 April, 36 g of leaf litter, which created habitat heterogeneity and 3 g of rabbit food (Purina, St. Louis, MO, U.S.A.), which served as a nutrient source, were added to each pool. On 24 April, all pools were inoculated with zooplankton, phytoplankton and periphyton collected from 10 natural ponds in the area. This experiment employed a completely randomized 2 x 2 x 3 factorial design. We crossed carbaryl (absent or present at a nominal concentration = 10 ppb) with Bd (absent or present) and three different assemblages of tadpoles using a substitutive design (*P. regilla* alone, *R. cascadae* alone or *P. regilla* and *R. cascadae* combined). The resulting 12 treatments

were replicated 4 times each for a total of 48 experimental units. We controlled for density between single-species and combined-species treatments.

Egg masses of *P. regilla* (25 egg masses) and *R. cascadae* (5 egg masses) were collected within 48 hours after oviposition between 15 May and 17 May from Site 1, a natural pond in the Cascade Range (elevation = 1140 m). Eggs were hatched and tadpoles were reared in outdoor holding tanks adjacent to the experimental site. Remnants of egg masses including any unhatched eggs were removed from the holding tanks on 28 May. Tadpoles in holding tanks were fed rabbit food ad libitum. On 14 June 40 tadpoles of Gosner stage 25-27 were added to each experimental pool (Gosner 1960) in three different assemblages: 40 *P. regilla,* 40 *R. cascadae* or 20 of each species. The initial mass of the tadpoles (mean ± 1 SE) was 47 ± 22 mg for *P. regilla* and 88 ± 14 mg for *R. cascadae.*

Ten days later, on day 1 of the experiment and every week thereafter for 7 wks, pools assigned to the carbaryl treatment received an application of technical grade carbaryl to achieve a nominal concentration of 10 ppb, which is 0.06 to 1% of LC50 estimates for tadpoles (Marian et al. 1983, Bridges 1997, Zaga et al. 1998), and is well within ecologically-relevant levels (maximum expected concentration in wetlands = 4.8 mg L^{-1}; Norris et al. 1983, Peterson et al. 1994). This type of "press" application probably approximates real-world pesticide applications more closely than single "pulse" applications, especially for high elevation amphibian habitats where aerial drift of pesticides from distant agricultural areas represents the greatest source. The

frequency of application of the pesticide was within the range of other studies examining press disturbances of pesticides (Hanazato and Yasuno 1990, Boone et al. 2001, Relyea and Diecks 2008). Additionally, the frequency of application of the pesticide in this study (7 d) was within the recommended application rate for the insecticide Sevin (7 to 10 d, or as needed).

We created a carbaryl stock solution by dissolving 30 mg of technical grade carbaryl (Chem Service, West Chester, PA, U.S.A.) into 75 mL of 100% ethanol. We added 3 mL of this solution to each pool in the carbaryl treatment. To ensure that any effects observed were due to carbaryl rather than ethanol, 3 mL of 100% ethanol was added to each pool not dosed with carbaryl as a vehicle control. After the chemicals were applied, all pools were thoroughly stirred to ensure uniform exposure of all animals. One hour after dosing, a water sample of 10 mL was collected from each experimental pool. Water samples were pooled by pesticide treatment (carbaryl or no carbaryl), frozen in pre-cleaned amber glass jars, and the samples from the first and last weeks were shipped to Mississippi State Chemical Laboratory (Mississippi State, MS, U.S.A.) for independent analysis of contaminant concentration using high pressure liquid chromatography. Results of these analyses indicated that actual concentrations were 5 and 3 ppb, respectively. Thus, the actual concentrations were, on average, 40% of the nominal concentration and did not accumulate with multiple applications. The half-life of carbaryl is 3.2 hrs at pH=9 and 12.1 d at pH=7. pH in our mesocosms averaged 7.9, but because carbaryl was reapplied on a weekly basis,

observed effects are likely to be due to the insecticide itself, rather than its breakdown product, 1-naphthol.

On day 1 of the experiment (24 June) and every 2 wks thereafter for a total of four inoculations, Bd inoculate was added to pools assigned to the Bd treatment. The fungus was grown in pure culture on plastic Petri plates (10 cm-diameter; Fisherbrand, Santa Clara, CA, U.S.A.) with standard TGhL nutrient agar medium (Becton, Dickinson and Company, Sparks, MD, U.S.A; Longcore et al. 1999). Plates were inoculated with liquid culture of Bd isolate JEL 274, originally isolated from *Anaxyrus boreas* from Colorado and incubated at 22 ˚C for 8 d prior to use. A broth containing Bd scraped from 50 flooded plates was diluted to 800 mL and 30 mL of this broth was added to each pool in the Bd treatment. A small sample of this broth was examined in the laboratory with the use of a hemocytometer to determine zoospore concentration. Average zoospore concentration in mesocosms following Bd inoculation was 20,000 zoospores L^{-1}. A broth containing water from 50 flooded control plates was diluted to 800 mL and 30 mL of this broth was added to each pool in the Bd control treatment.

To measure how the treatments affected the other members of the pond community, we sampled the zooplankton, phytoplankton and periphyton from each mesocosm on days 11-12 and on day 32. To measure the abundance of zooplankton, a 1.5-cm tube sampler that held approximately 30 mL of water was plunged vertically through the water column and sealed near the bottom of the pool. Three samples were taken on opposite sides of each pool and in the center and pooled. This procedure was

repeated three times for each pool. Water samples were then filtered through 150 μm mesh (Florida Aquatic Nurseries, Ft. Lauderdale, FL, U.S.A.) and zooplankton from each sample were pooled and preserved in 30% ethanol for later quantification. Zooplankton were identified to the level of copepods and cladocerans because past research has shown that the two groups differ in their susceptibility to insecticides, but species within each group are similar in their sensitivities (Relyea 2005, Relyea and Diecks 2008). Furthermore, cladocerans are relatively indiscriminant filter feeders, consuming smaller food particles than the more selective copepods (Sommer and Sommer 2006), so shifts in the relative composition of the zooplankton could change the size distribution of the phytoplankton.

To measure the abundance of phytoplankton, the three water samples from each mesocosm (25 mL each) described above were filtered through a Type A/E 25 mm GF/F filter (Pall Corporation, Port Washington, NY, U.S.A.). Filtering was conducted under full shade to minimize chlorophyll breakdown and filters were stored in 25 mL centrifuge tubes on ice. Samples were stored at -20 ˚C for 4 d before chlorophyll extraction. Following the Welschmeyer method, chlorophyll-*a* was extracted with 10 mL of 90% acetone, agitated, and incubated for 24 hrs at -20 ˚C (Welschmeyer 1994). A Turner Designs fluorometer (model TD-700, Sunnyvale, CA, U.S.A.) was used to take fluorescence measurements, and chlorophyll-*a* concentration was calculated as the mean value of the three replicates from each mesocosm.

Two periphyton samplers, consisting of glass microscope slides mounted vertically on a small Styrofoam block, were deployed in each pool on day 1 of the experiment. The periphyton on both sides of one slide was scraped into a Petri plate using a straight-edge razor blade on each community sampling day. Contents of the Petri plate were filtered through a Type A/E 25 mm GF/F filter that had been previously dried for 24 hrs at 60 ˚C and weighed. To determine periphyton biomass, filters were dried again for 24 hrs at 60 ˚C and reweighed.

iButton temperature probes (Maxim, Sunnyvale, CA, U.S.A.) were deployed in ten pools on 24 May. Each probe logged temperature every hour over the course of the experiment. Dissolved oxygen and pH measurements were taken using digital meters (Oakton Instruments, Vernon Hills, IL, U.S.A.) on days 5 and 51 of the experiment.

On day 31, ten individuals from each pool (10 *P. regilla* or 10 *R. cascadae* from single-species pools or 5 of each species from combined-species pools, hereafter termed “tadpoles”) were haphazardly chosen, euthanized using an overdose of MS-222, preserved in 90% ethanol and subsequently weighed and staged (Gosner 1960). Measures of performance for tadpoles from each mesocosm included mean daily growth rate (mass ÷ 31 d), mean Gosner stage and mean infection level.

All remaining individuals were euthanized and preserved upon emergence from the pools at stage 45-46 (hereafter termed “metamorphs”) and were subsequently weighed (Gosner 1960). The first *P. regilla* metamorph was observed on day 28 of the experiment and the first *R. cascadae* metamorph was observed 2 d later. Following the

initial observation of a metamorph, pools were checked daily for metamorphs until the end of the experiment on day 51. At that time, all remaining individuals (< 2% of all individuals added to mesocosms) were preserved, regardless of Gosner stage; these individuals were excluded from statistical analyses. Measures of performance for metamorphs from each mesocosm were larval period, mean daily growth rate (mass at metamorphosis divided by the number of days from the addition of tadpoles to the mesocosms until metamorphosis) and survival to metamorphosis (the proportion of larvae surviving to metamorphosis from those initially added to each tank, excluding the 10 individuals sampled as tadpoles from each tank).

We used real-time quantitative polymerase chain reaction (qPCR) following the methods of Boyle et al. (2004) to confirm the infection status of five of the individuals sampled as tadpoles from single-species pools and all ten of the individuals sampled as tadpoles from multiple-species pools. The mouthparts of preserved tadpoles were dissected and each sample was run in triplicate against a Bd standard titration from 10^{-1} to 10^{2} zoospores on an Applied Biosystems StepOne Plus real-time PCR machine (Applied Biosystems, Inc., CA, U.S.A.). The experimenter was unaware of the treatment from which each sample originated at the time of qPCR analysis. A tadpole was considered infected if two of three replicates tested positive and replicates were averaged for each sample.

We recognize the risk of releasing contaminants into the environment when conducting manipulative studies involving pesticides and pathogens in outdoor

experimental tanks (Parris & Beaudoin, 2004). However, such studies are important for understanding complex interactions between various stressors affecting populations, and constitute the most ecologically realistic way of establishing causal mechanisms. We took precautions to minimize the risk of environmental contamination. Each tank was covered with a tightly-fitting screen lid to prevent escape of metamorphic amphibians, colonization by aquatic insects and use of pools as a water source by mammals and birds. Water levels were kept low to prevent overflow. All equipment was thoroughly disinfected with 10% bleach (6% sodium hypochlorite) solution during and after the experiment and all used Petri plates were autoclaved. After the conclusion of the experiment, bleach was added to tanks to yield a 10% solution, which is sufficient to kill Bd. After breakdown (4 wks), sodium bicarbonate was added to each tank to raise the pH to 10, which encourages rapid breakdown of carbaryl. Tanks were emptied and scrubbed 8 wks after the conclusion of the experiment.

Statistical analyses

We conducted statistical analyses in R and S-plus to test for effects of treatments. Due to non-independence of individuals within tanks, mean values per tank were used as the unit of analysis for all variables. Most response variables met the parametric assumptions, although survival to metamorphosis was arcsine transformed.

The response variables were analyzed using a series of multivariate analysis of variance (MANOVA) tests. Separate MANOVAs were conducted for each amphibian species. For tadpoles, the response variables were daily growth rate and developmental stage (Gosner 1960). For metamorphs, the response variables were daily growth rate, larval period and survival. Whenever a multivariate effect was significant, we conducted subsequent univariate analyses of variance (ANOVAs).

We also examined the effects of the treatments on the other members of the community (copepods, cladocerans, phytoplankton and periphyton). For all four groups, we log-transformed the data to meet parametric assumptions. We conducted separate MANOVAs for each of the two samples. Whenever a multivariate effect was significant, we conducted subsequent ANOVAs on each of the response variables.

Results

Our first analysis examined the growth and development of *P. regilla* tadpoles (Table 2.1, Figs. 2.1, 2.3). There were significant multivariate effects of amphibian assemblage, Bd and carbaryl as well as significant assemblage-by-carbaryl and assemblage-by-Bd-by-carbaryl interactions. Subsequent univariate analyses indicated that carbaryl increased tadpole growth by approximately 0.006 mg d^{-1}. The remaining main effects and interactions were not significant at the univariate level.

The second analysis examined the growth rate, development, and survival of *P. regilla* metamorphs (Table 2.2, Figs. 2.2, 2.3). There were significant multivariate

effects of assemblage and carbaryl; there was no effect of Bd and there were no significant interactions. Subsequent univariate tests indicated that substituting in 20 *R. cascadae* tadpoles decreased the larval period of *P. regilla* by approximately 0.7 d. Carbaryl increased growth rate of *P. regilla* by approximately 0.001 mg d^{-1} and increased the larval period by approximately 2 d.

The third analysis examined the growth and development of *R. cascadae* tadpoles (Table 2.3, Figs. 2.1, 2.3). The MANOVA found no significant main effects or interactions. As a result, we did not conduct any subsequent univariate analyses.

The final amphibian analysis examined the growth, development and survival of *R. cascadae* metamorphs (Table 2.4, Figs. 2.2-2.3). There was a multivariate effect of carbaryl but no other main effects or interactions. The subsequent univariate analysis indicated that carbaryl increased the larval period of *R. cascadae* by approximately 0.0006 mg d^{-1}, but there were no effects on growth or survival.

The qPCR analysis on the dissected mouthparts of tadpoles revealed that the majority of individuals exposed to Bd harbored infections by day 31, while none of the unexposed individuals showed infection, thus confirming infection of Bd-exposed animals. Species exhibited similar levels of infection. A univariate ANOVA indicated that neither carbaryl, amphibian assemblage, nor their interaction, had an effect on infection levels of either species (Table 2.7).

We also tested for effects of treatment variables on the other trophic groups in the community. Our first analysis examined the effects of amphibian assemblage, Bd,

carbaryl and their interactions on cladocerans, copepods, phytoplankton and periphyton during the first sample (days 11-12; Table 2.5, Figs. 2.4-2.7). We found a multivariate effect of carbaryl and an assemblage-by-Bd interaction. Subsequent univariate analyses indicated that carbaryl decreased cladoceran abundance by about 50 times, resulting in more than twice the concentration of phytoplankton. There was a nearly significant assemblage-by-Bd interaction on both phytoplankton abundance and periphyton biomass in the univariate analyses: presumably, Bd-exposed *R. cascadae* consumed less periphyton and more phytoplankton than unexposed counterparts, but the opposite was true of *P. regilla*. Together, these nearly significant effects probably drove the significant multivariate effect. The remaining main effects and interactions were not significant at the univariate level.

Our second analysis examined the same response variables during the second sample (day 32; Table 2.6, Figs. 2.4-2.7). We found a significant multivariate effect of carbaryl. Subsequent univariate analyses indicated that carbaryl continued to cause a decrease in the abundance of cladocerans, by about 14 times. The increase in phytoplankton was no longer significant because phytoplankton in the carbaryl-free mesocosms had increased by the second sample to be similar in abundance. The remaining main effects and interactions were not significant at the univariate level.

We measured temperature (continuously over the experiment), dissolved oxygen (two occasions) and pH (two occasions). Average weekly temperatures ranged from 15.01ºC to 18.38ºC, which is suitable for growth of Bd (Piotrowski 2004).

Average dissolved oxygen and pH were 16.8 ppm and 7.94 respectively on day 5 and 6.5 ppm and 7.97 respectively on day 51. We found no effects of amphibian assemblage, Bd or carbaryl treatments on any abiotic variable.

Discussion

We demonstrated effects of amphibian assemblage, carbaryl and Bd on larval period, growth rate and survival of amphibians. Treatments differentially affected *P. regilla* and *R. cascadae*, and effects were dependent upon developmental stage. We also demonstrated effects of treatments on the aquatic community, including zooplankton and algae.

We hypothesized that manipulating the amphibian assemblage would affect growth rate and larval period of both amphibian species. We controlled for density by adding 40 larvae to each pool (40 *P. regilla*, 40 *R. cascadae* or 20 of each species). Kiesecker and Blaustein (1999) established that *R. cascadae* is a superior competitor to *P. regilla*. In our experiment, *P. regilla* was negatively affected by the replacement of 20 intraspecific competitiors with 20 superior *R. cascadae* competitors. When *R. cascadae* were added, the larval period of *P. regilla* increased. In contrast, *R. cascadae* was unaffected by the replacement of 20 intraspecific competitiors with 20 inferior *P. regilla* competitors. The amphibian assemblage did not affect the other members of the aquatic community (zooplankton, algae), except for a nearly significant assemblage-by-Bd interaction on phytoplankton, discussed below.

The insecticide carbaryl increased the growth rate and larval period of *P. regilla* and increased the larval period of *R. cascadae*. Effects of carbaryl on growth rate and larval period of tadpoles in mesocosms are quite mixed (Boone and Semlitsch 2001, Boone and Bridges 2003, Boone et al. 2004, Relyea 2006), but cases of increased growth rate are hypothesized to be caused by a short-term increase in periphyton biomass caused by a pesticide-induced reduction in tadpole foraging activity (Boone et al. 2005, Distel and Boone 2009). As carbaryl breaks down and its negative effects on tadpole foraging diminish, tadpoles may benefit from the overabundance of food resources.

We hypothesized that growth and development of tadpoles would be negatively affected by exposure to carbaryl due to a trophic cascade involving zooplankton: carbaryl is known to reduce the abundance of herbivorous cladocerans (Mills and Semlitsch 2004, Relyea 2005, Relyea 2009), thus increasing phytoplankton concentration (Hanazato and Yasuno 1987, Boone and James 2003). Given enough time, the bloom of phytoplankton can lead to a reduction in periphyton biomass due to competition for nutrients and light, which could negatively impact tadpole growth and development (Mills and Semlitsch 2004, Distel and Boone, 2009). We confirmed that carbaryl reduced cladoceran abundance by about 50 times, and a phytoplankton bloom (more than twice the concentration) was detected in carbaryl mesocosms at the first sample. However, we did not detect changes in periphyton biomass, probably because the tadpoles metamorphosed before the trophic cascade could fully develop (i.e. it

appears to require more than 30 d; Relyea and Diecks 2008), and *P. regilla* tadpoles grew larger, not smaller, in the presence of carbaryl. One possible explanation for the observed pattern is that *P. regilla* were able to utilize the abundant phytoplankton resources after cladoceran abundance was severely decreased by carbaryl. Whiles et al. (2010) determined via fatty acid analysis that rasping tadpoles exhibit high dietary plasticity and that phytoplankton may contribute significantly to their diet, a suggestion put forth previously by Altig et al. (2007).

Interestingly, previous studies that documented increased growth and delayed metamorphosis of amphibians in response to carbaryl applied the insecticide at concentrations that were 100 to 700 times greater than our study. This suggests that even at much lower concentrations (0.06 to 1% of the LC50 estimate for tadpoles), tadpoles may be affected (Relyea and Edwards 2010).

We found no effect of Bd on the growth and development of amphibians, although qPCR analysis confirmed infection of the majority of Bd-exposed individuals. We did, however, detect a significant multivariate effect of Bd on *P. regilla* tadpoles. Our results confirm previous findings that the effects of Bd on certain species of infected larval amphibians may be minimal (Blaustein et al. 2005). Previous studies have shown varied effects on larvae of different species after exposure to Bd. These include effects on growth and development, as well as mortality in some species, and the magnitude of these effects may vary among species (Parris and Baud 2004, Parris and Beaudoin 2004, Parris and Cornelius 2004, Blaustein et al. 2005).

One possible mechanism that has been suggested is that keratinized mouthparts of infected larvae may become disfigured by Bd, thus inhibiting normal feeding behaviour (Fellers et al. 2001, Rachowicz and Vredenburg 2004, Venesky and Parris 2009). In our experiment, abundant algal resources and weak competition among the tadpoles may have provided excellent growth conditions for tadpoles, thereby allowing them to avoid clinical signs of infection (extended larval period, reduced growth rate, reduced survival) in spite of their infection status. Although larvae may fail to exhibit clinical signs of infection, infected tadpoles in natural populations may serve as important reservoirs for the pathogen, thereby increasing the infection risk for conspecifics and heterospecifics of all developmental stages (Briggs et al. 2010).

A nearly significant interaction between amphibian assemblage and Bd on phytoplankton concentration ($p=0.056$) and periphyton biomass ($p=0.063$) during the first sample was unexpected and suggests that under certain circumstances, Bd can somehow reduce phytoplankton abundance and increase periphyton biomass. One hypothesis is that infected tadpoles may be less effective at scraping periphyton, due to degradation of keratinized tissues of the mouthparts, thereby forcing a switch to a diet composed largely of phytoplankton. Indeed, tadpoles of both species were observed to spend a significant amount of time in the water column, presumably foraging on suspended algal particles. The significant assemblage-by-Bd interaction in the multivariate analysis could be explained if this switch was more pronounced in *R.*

cascadae than in *P. regilla*, as would be suggested by previously reported patterns of keratin loss in infected individuals of these species (Blaustein et al. 2005).

Interestingly, our data on tadpole growth rate and larval period support the idea that Bd infection may influence consumption of phytoplankton and periphyton. Figure 2 shows that in the absence of Bd and the presence of *R. cascadae* competitors, the effect of carbaryl extending the larval period of *P. regilla* disappeared. One interpretation is that Bd-exposed *R. cascadae* competitors are hampered by their infections, thus allowing *P. regilla* to capitalize on the carbaryl-induced abundance of phytoplankton and metamorphose earlier. Indeed, Figure 1 shows that *R. cascadae* achieved the fastest growth in the absence of Bd and the presence of carbaryl and *P. regilla*.

Although we demonstrated main effects of carbaryl and amphibian assemblage, main effects of Bd and interactive effects between the treatments were not detected. Despite the differences between immune function of larval and metamorphic amphibians, both the current study and Davidson et al. (2007) failed to demonstrate interactive effects between carbaryl and Bd. The Davidson et al. (2007) study differed from ours in that carbaryl was applied as a “pulse treatment” (a single high-dose application) rather than a “press treatment” (multiple applications of lower concentration) (Relyea and Diecks 2008). To explain the absence of interactive effects of carbaryl and Bd, Davidson et al. (2007) hypothesized that 1) while immune function may have been reduced by carbaryl, it may not have been reduced to the

extent that susceptibility to Bd increased, 2) immune function may have recovered after a one-time application of carbaryl, or 3) carbaryl and Bd may have affected different aspects of immune function. Hypotheses 1 and 3 seem plausible in terms of the results of our study, but carbaryl was applied on a weekly basis in our experiment, so recovery of immune function after a carbaryl dose seems unlikely. Previous studies have documented interactive effects between pesticides and interspecific competition (Mackey and Boone 2009) and Bd and interspecific competition (Parris and Cornelius 2004, Han 2008), although not at the low concentrations of carbaryl and Bd tested in our experiment.

We found evidence suggesting that phytoplankton contributed to the diet of tadpoles in this experiment. *P. regilla* appeared to benefit from the overabundance of phytoplankton caused by carbaryl-induced mortality of zooplankton, and Bd-infected *R. cascadae* may have switched from a diet composed primarily of periphyton to one rich in phytoplankton. Although inconclusive, both results suggest that a closer examination of phytoplankton as an important component of the larval anuran diet is warranted, as has been previously suggested (e.g. Altig et al. 2007, Whiles et al. 2010).

We encourage future studies examining possible interactive effects between Bd and other pesticides. Furthermore, we find mesocosms to be an ecologically realistic and tractable venue for studies examining the interaction between community structure and host-pathogen dynamics.

Acknowledgements

We thank Caroline Scott, Phyllis Buck and Evan Scheessele for assistance executing the experiment. Members of the Blaustein and Betts laboratories, and especially Catherine Searle, provided invaluable advice regarding experimental design and execution, data analysis, as well as comments on the manuscript. Aaron Stoler provided advice on community sampling protocol. We also thank the Tanguay, Spatafora, B. Menge and Borer/Seabloom laboratories, Laura McMullen, Joe Tyburczy and Kate Boersma for use of equipment and protocol and Scott Robbins and Don Hinds-Cook for assistance at the Horticulture Farm. We thank M. Boone and two anonymous reviewers for their insightful comments on this manuscript. This material is based upon work supported under a National Science Foundation Graduate Research Fellowship to J.C. Buck.

Table 2.1. Results of a MANOVA on the effects of amphibian assemblage, Bd, and carbaryl on the daily growth rate and developmental stage (Gosner 1960) of *P. regilla* tadpoles. Subsequent univariate tests (P-values) were conducted for all significant multivariate effects. Bold P-values are significant at $P < 0.05$.

A. Multivariate	df	Wilks' F	P
Assemblage	2,14	8.49	**<0.001**
Bd	2,14	8.17	**0.01**
Carbaryl	2,14	22.0	**<0.001**
Assemblage * Bd	2,14	2.93	**0.027**
Assemblage * Carbaryl	2,14	5.85	**0.003**
Bd * Carbaryl	2,14	0.92	0.815
Assemblage * Bd * Carbaryl	2,14	3.43	**0.018**

B. Univariate	Growth rate	Gosner stage
Assemblage	0.072	0.191
Bd	0.366	0.549
Carbaryl	**<0.001**	0.107
Assemblage * Bd	0.690	0.536
Assemblage * Carbaryl	0.141	0.743
Assemblage * Bd * Carbaryl	0.917	0.586

Table 2.2. Results of a MANOVA on the effects of amphibian assemblage, Bd, and carbaryl on the daily growth rate, larval period and survival of *P. regilla* metamorphs. Subsequent univariate tests (P-values) were conducted for all significant multivariate effects. Bold P-values are significant at $P < 0.05$.

A. Multivariate	Df	Wilks' F	P
Assemblage	3,14	5.38	**0.013**
Bd	3,14	2.87	0.077
Carbaryl	3,14	14.3	**<0.001**
Assemblage * Bd	3,14	0.31	0.816
Assemblage * Carbaryl	3,14	1.90	0.179
Bd * Carbaryl	3,14	0.47	0.706
Assemblage * Bd * Carbaryl	3,14	3.18	0.060

B. Univariate	Growth rate	Larval period	Survival
Assemblage	0.677	**0.035**	0.625
Carbaryl	**0.039**	**<0.001**	0.145

Table 2.3. Results of a MANOVA on the effects of amphibian assemblage, Bd, and carbaryl on the daily growth rate and developmental stage (Gosner 1960) of *R. cascadae* tadpoles. Bold P-values are significant at $P < 0.05$.

A. Multivariate	Df	Wilks' F	P
Assemblage	2,14	1.27	0.310
Bd	2,14	0.96	0.406
Carbaryl	2,14	2.70	0.100
Assemblage * Bd	2,14	0.58	0.571
Assemblage * Carbaryl	2,14	2.22	0.143
Bd * Carbaryl	2,14	0.61	0.556
Assemblage * Bd * Carbaryl	2,14	0.12	0.884

Table 2.4. Results of a MANOVA on the effects of amphibian assemblage, Bd, and carbaryl on the daily growth rate, larval period and survival of *R. cascadae* metamorphs. Subsequent univariate tests (P-values) were conducted for all significant multivariate effects. Bold P-values are significant at $P < 0.05$.

A. Multivariate	Df	Wilks' F	P
Assemblage	3,14	2.12	0.143
Bd	3,14	0.71	0.560
Carbaryl	3,14	5.24	**0.012**
Assemblage * Bd	3,14	0.69	0.576
Assemblage * Carbaryl	3,14	3.25	0.054
Bd * Carbaryl	3,14	0.36	0.786
Assemblage * Bd * Carbaryl	3,14	0.19	0.903

B. Univariate	Growth rate	Larval period	Survival
Carbaryl	0.249	**0.021**	0.097

Table 2.5. Results of a MANOVA on the effects of amphibian assemblage, Bd, and carbaryl on the abundance of cladocerans, copepods, phytoplankton, and periphyton early in the experiment (day 11-12). Subsequent univariate tests (P-values) were conducted for all significant multivariate effects. Bold P-values are significant at $P < 0.05$.

A. Multivariate	df	Wilks' F	P
Assemblage	8,66	0.65	0.062
Bd	4,33	0.82	0.154
Carbaryl	4,33	0.20	**<0.001**
Assemblage * Bd	8,66	0.63	**0.042**
Assemblage * Carbaryl	8,66	0.78	0.361
Bd * Carbaryl	4,33	0.98	0.965
Assemblage * Bd * Carbaryl	8,66	0.89	0.845

B. Univariate	Cladocerans	Copepods	Phytoplankton	Periphyton
Carbaryl	**<0.001**	0.095	**0.001**	0.388
Assemblage * Bd	0.132	0.193	0.056	0.063

Table 2.6. Results of a MANOVA on the effects of amphibian assemblage, Bd, and carbaryl on the abundance of cladocerans, copepods, phytoplankton, and periphyton late in the experiment (day 32). Subsequent univariate tests (P-values) were conducted for all significant multivariate effects. Bold P-values are significant at $P < 0.05$.

A. Multivariate	df	Wilks' F	P
Assemblage	8,66	0.44	0.892
Bd	4,33	2.51	0.060
Carbaryl	4,33	8.52	**<0.001**
Assemblage * Bd	8,66	0.47	0.872
Assemblage * Carbaryl	8,66	0.85	0.560
Bd * Carbaryl	4,33	0.06	0.994
Assemblage * Bd * Carbaryl	8,66	1.09	0.380

B. Univariate	Cladocerans	Copepods	Phytoplankton	Periphyton
Carbaryl	**<0.001**	0.236	0.073	0.617

Table 2.7. Results of two ANOVAs on the effects of amphibian assemblage and carbaryl on infection prevalence of *P. regilla* and *R. cascadae*. P-values are reported.

Univariate	*P. regilla*	*R. cascadae*
Assemblage	0.621	0.834
Carbaryl	0.858	0.572
Assemblage * Carbaryl	0.052	0.723

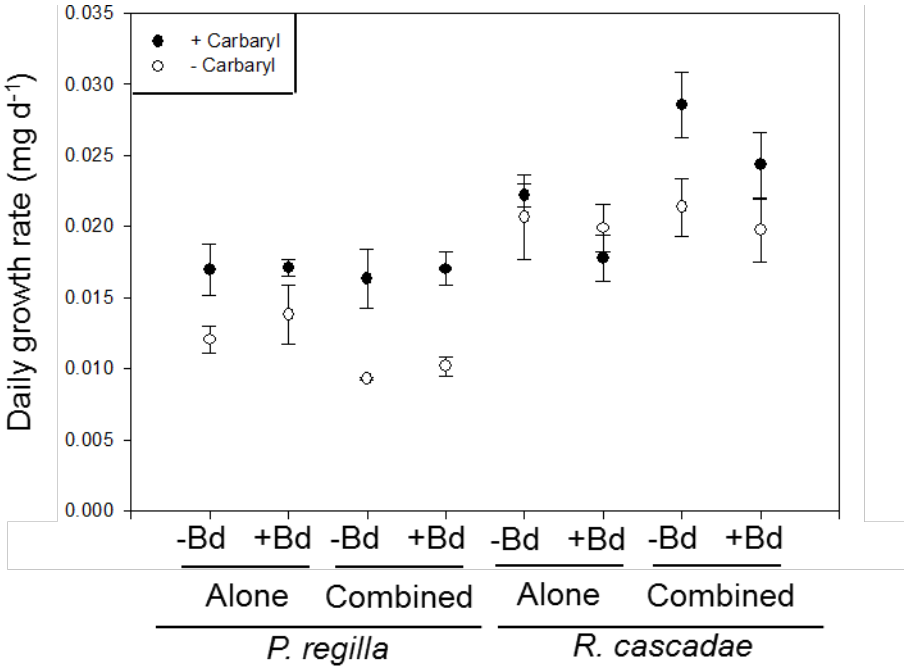

Figure 2.1. The effects of amphibian assemblage, Bd, and carbaryl on daily growth rate of tadpoles for *P. regilla* alone, *P. regilla* when combined with *R. cascadae*, *R. cascadae* alone, and *R. cascadae* when combined with *P. regilla*. Values plotted are means ± 1SE.

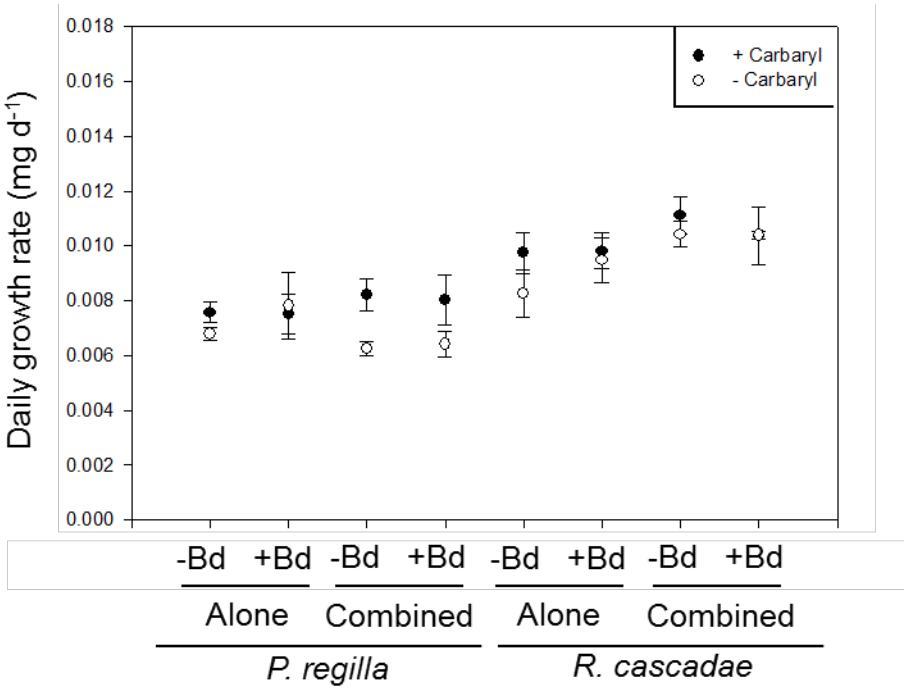

Figure 2.2. The effects of amphibian assemblage, Bd, and carbaryl on daily growth rate of metamorphs for *P. regilla* alone, *P. regilla* when combined with *R. cascadae*, *R. cascadae* alone, and *R. cascadae* when combined with *P. regilla*. Values plotted are means ± 1SE.

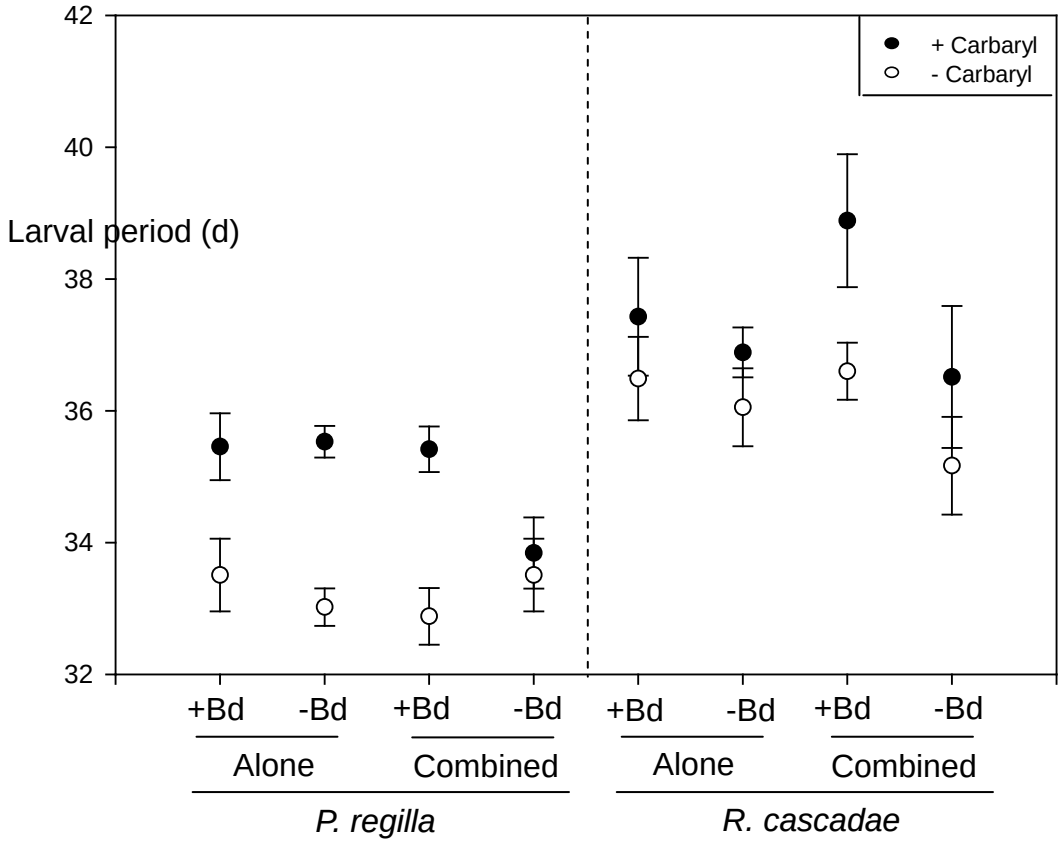

Figure 2.3. The effects of amphibian assemblage, Bd and carbaryl on the larval period of amphibians for *P. regilla* alone, *P. regilla* when combined with *R. cascadae*, *R. cascadae* alone and *R. cascadae* when combined with *P. regilla*. Values plotted are means ± 1SE.

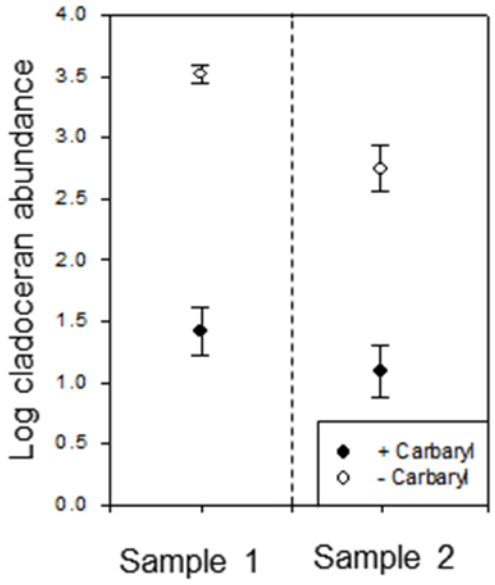

Figure 2.4. The effect of carbaryl on log cladoceran abundance at the time of first sampling (days 11-12) and second sampling (day 32). Values plotted are means ± 1 SE.

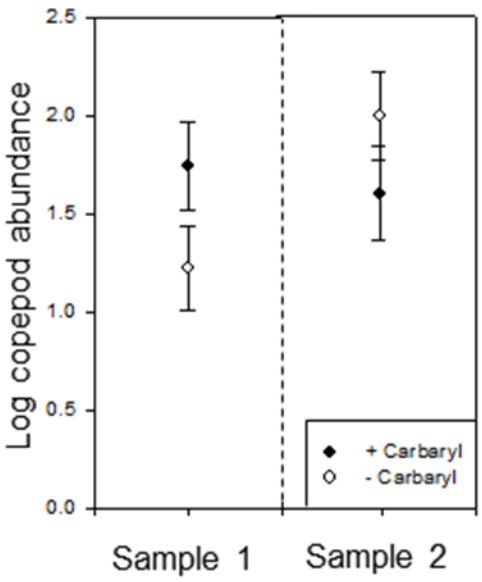

Figure 2.5. The effect of carbaryl on log copepod abundance at the time of first sampling (days 11-12) and second sampling (day 32). Values plotted are means ± 1 SE.

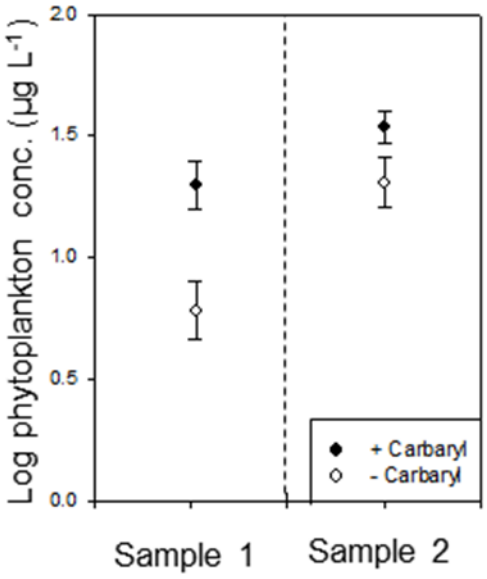

Figure 2.6. The effect of carbaryl on log phytoplankton concentration at the time of first sampling (days 11-12) and second sampling (day 32). Values plotted are means ± 1 SE.

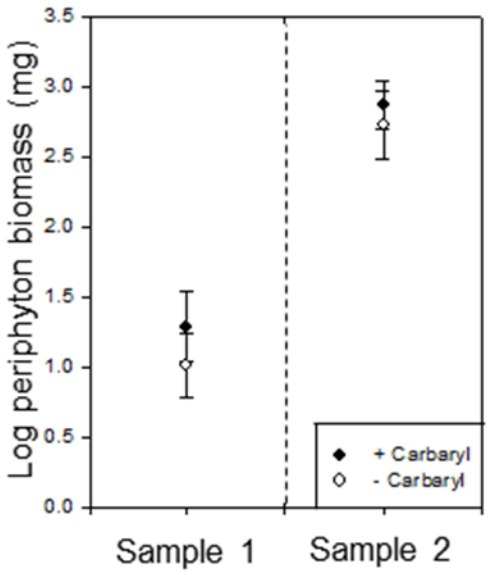

Figure 2.7. The effect of carbaryl on log periphyton biomass at the time of first sampling (days 11-12) and second sampling (day 32). Values plotted are means ± 1 SE.

Chapter 3

Predation by zooplankton on *Batrachochytrium dendrobatidis*: Biological control of the deadly amphibian chytrid fungus?

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Abstract

Batrachochytrium dendrobatidis (hereafter Bd), a fungal pathogen of amphibians, causes the disease chytridiomycosis which is responsible for unprecedented population declines and extinctions globally. Host defenses against chytridiomycosis include cutaneous symbiotic bacteria and anti-microbial peptides, and proposed treatment measures include use of fungicides and bioaugmentation. Efforts to eradicate the fungus from localized areas of disease outbreak have not been successful. Instead, control measures to mitigate the impacts of the disease on host populations, many of which are already persisting with Bd in an endemic state, may be more realistic. The infective stage of the fungus is an aquatic zoospore, 3-5µm in diameter. Here we show that zoospores of Bd are consumed by the zooplankter *Daphnia pulex*. This species inhabits amphibian breeding sites where Bd transmission occurs, and consumption of Bd zoospores may lead to effective biological control of Bd.

Introduction

As part of an overall "biodiversity crisis", amphibians are undergoing population declines and extinctions at unprecedented rates (Stuart et al. 2004, McCallum 2007). Emerging infectious diseases such as chytridiomycosis, caused by the fungus Bd, are playing a prominent role in these declines (Mendelson et al. 2006). The impact of chytridiomycosis on amphibians has been called "the most spectacular

loss of vertebrate biodiversity due to disease in recorded history" (Skerratt et al. 2007). Efforts to treat chytridiomycosis *in vitro*, including treatment with anti-fungal compounds and supplementation of natural cutaneous microbes (bioaugmentation), have met with varied success (Lubick 2010, Woodhams et al. 2011). Although eradication of chytridiomycosis has been attempted in some natural populations (Lubick 2010, Woodhams et al. 2011), it has not been successful, and eradication may not be a realistic goal. Instead, Woodhams et al. (2011) proposed that control measures should seek to mitigate the effects of the pathogen on host populations, many of which are already persisting with Bd in an endemic state.

Parasites commonly function as prey within ecosystems (Johnson et al. 2010), and we suggest that biological control through predation may be effective in controlling Bd. The infective stage of Bd is a free-living aquatic flagellated zoospore, 3-5µm in diameter (Longcore et al. 1999), which is within the size range of preferred prey items of cladocerans such as *Daphnia spp.* Abundant in lentic habitats globally, *Daphnia* are selective filter feeders consuming nanoplanktonic algae, bacteria, fungi, protozoa, and detritus 1-100µm in size (Thorp and Covich 2010). Kagami et al. (2004; 2007) showed that a *Daphnia galeata* × *hyalina* population benefitted from consumption of zoospores of a chytrid fungus, *Zygorhizidium planktonicum*, thereby protecting phytoplankton hosts from infection. Furthermore, Ibelings et al. (2011) suggest that zooplankton may also benefit from a mixed phytoplankton community if chytrid infection reduces the dominant inedible phytoplankton species. A negative

correlation between *Daphnia* abundance and Bd zoospore density over a 3-day experimental trial has been reported (Woodhams et al. 2011). Although this suggests the possibility of predation of Bd zoospores by *Daphnia*, this was not confirmed. Here, we experimentally tested the hypothesis that *Daphnia pulex* consume Bd zoospores.

Methods

Daphnia pulex were collected from a self-contained covered outdoor culture and were transported to a laboratory maintained at 21.5-23.3ºC. Bd was grown in pure culture on plastic Petri plates (10 cm-diameter) with standard TGhL nutrient agar medium (Longcore et al. 1999). Plates were inoculated with liquid culture of Bd isolate JEL 274, originally isolated from *Anaxyrus boreas* toads from Colorado, and incubated at 22 ˚C for 9 d prior to use. We conducted two experiments to determine whether Bd could be detected in the gut of *D. pulex*.

Visual confirmation

Nile red (Fisher Scientific), a lipophilic fluorescent stain, was dissolved in dimethyl sulfoxide (DMSO) and added to standard TGhL nutrient agar medium at a concentration of 500 µg L^{-1}. Bd was cultured on plates poured from this agar. Visual examination indicated that the stain was taken up by the fungus. A broth containing Bd scraped from flooded plates was diluted to achieve a concentration of 1.2×10^5 zoospores mL^{-1}, and 1 mL of this broth was filtered through a Whatman GF/F filter to

eliminate excess stain. To dislodge zoospores, the filter was washed in a 500mL plastic cup containing 200mL dechlorinated water. *D. pulex* (n=10) that had been starved for 24h prior to the experiment were exposed individually in plastic cups at a concentration of 600 zoospores mL^{-1}. Starved control *D. pulex* were exposed to Bd grown on standard TGhL nutrient agar medium lacking the stain (Bd control, n=10), and to a control inoculation from plates containing Nile red (stain control, n=10), both filtered through a Whatman GF/F filter. After 3.5 hours, all individuals were preserved in 90% ethanol and viewed under an Olympus Vanox AH2 fluoroscope. Images were captured with an Olympus DP72 digital camera.

qPCR confirmation

Live *D. pulex* that had been starved for 24h prior to the experiment (n=12) and starved *D. pulex* killed with 90% ethanol (n=12) were exposed individually in 500 mL plastic cups filled with 200 mL of dechlorinated water to Bd at a concentration of 600 zoospores mL^{-1}. Starved *D. pulex* (n=12) were exposed to a control inoculation. After 3.5 hours, all animals were preserved in 90% ethanol. The guts of all individuals exposed to Bd and three randomly chosen control individuals were extracted with the use of a dissecting microscope. 30-40mg of Zirconium/silica beads measuring 0.5mm diameter (Biospec products) were added to a vial containing the gut, and the vial was alternately homogenized in a BBX24W-Bullet Blender (Next Advance) for 45 sec and centrifuged 5 times. 60µL Prepman Ultra (Applied Biosystems) was added and vials were heated to 100ºC for 10 min, cooled for 2 minutes, and the supernatant was

extracted and diluted to a 10% solution. Real-time quantitative PCR (qPCR) was conducted on an Applied Biosystems StepOne Plus real-time PCR machine (Applied Biosystems, Inc., CA, USA) according to methods of Boyle et al. 2004. Each sample was analyzed in triplicate against a Bd standard titration from 10^{-1} to 10^{2} zoospores, and the average number of genome equivalents per individual was calculated.

Results

Visual confirmation

When viewed under a fluoroscope, the gut of individual *Daphnia* exposed to Bd grown on plates containing the stain appeared intensely fluorescent red (Fig1). Guts of individuals from the Bd control and stain control treatments did not fluoresce.

qPCR confirmation

A Wilcoxon rank-sum test indicated that guts of *D. pulex* exposed to Bd while alive contained significantly more zoospore equivalents than guts of those exposed to Bd after death ($W=191$, $p=0.0001$, Fig 2). qPCR confirmed that guts of unexposed individuals contained no Bd.

Discussion

Our study demonstrates consumption of Bd zoospores by the zooplankter *D. pulex* and supports the potential for biological control of Bd by zooplankton as discussed in Woodhams et al. (2011). Vredenburg et al. (2010) and Briggs et al.

(2010) suggested that Bd infection results in host mortality once a threshold density of sporangia (infection intensity) is reached, implying that control may be achieved by limiting the number of Bd zoospores. We suggest that zooplankton such as *D. pulex* may effectively limit the number of infective Bd zoospores and may be a useful means of biological control for chytridiomycosis. Moreover, we suggest that the threat of Bd to amphibians would be lower in systems containing dense populations of zooplankton if the species of zooplankton fed on Bd. Furthermore, it may be possible to augment the numbers of Bd -eating zooplankton in natural systems for effective biological control, although previous species introductions for biological control have met with varied success (Cory and Myers 2000). These suggestions should be examined in natural systems for a more thorough understanding of how Bd may be controlled via zooplankton.

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a. b.

Figure 3.1. Daphnia pulex after consuming zoospores stained with Nile red. (a) Brightfield image. (b) Fluorescent image with sensitivity = 204.78ms.

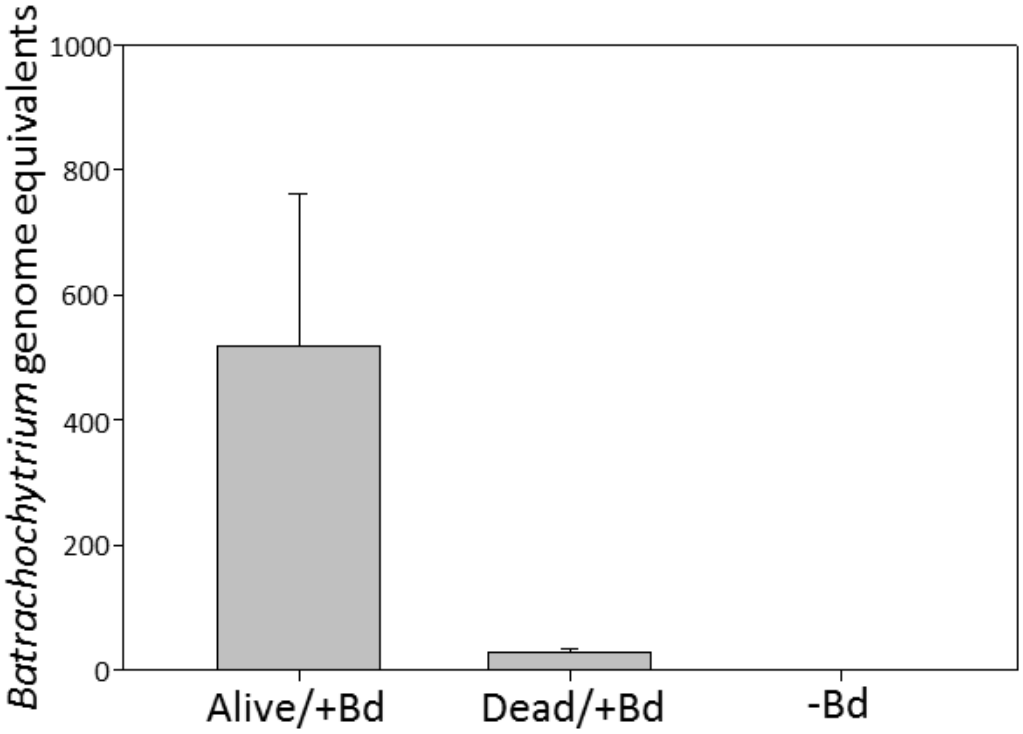

Figure 3.2. Zoospore equivalents in the extracted guts of live *D. pulex* exposed to *Batrachochytrium*, killed *D. pulex* exposed to Bd, and live *D. pulex* exposed to a sham inoculation (control). Wilcoxon rank-sum test: W=191, p=0.0001.

Chapter 4

Trophic dynamics in an aquatic community: Interactions among primary producers, grazers, and a pathogenic fungus

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Abstract

Food webs provide a useful framework for investigating direct and indirect effects of stressors on species and communities. As functional members of ecological communities, infectious agents are increasingly incorporated into food web analyses. Free-living stages of parasites and pathogens may be consumed by predators, leading to important changes in community structure, ecosystem function, and disease risk. For example, zooplankton prey on the infectious stage of the amphibian chytrid fungus, *Batrachochytrium dendrobatidis* (Bd), which may have profound consequences because the fungus is associated with amphibian population declines and extinctions worldwide. We investigated interspecific interactions among amphibian larvae, zooplankton, and Bd in a fully factorial experimental regime in outdoor mesocosms. We crossed the presence/absence of *Rana cascadae* tadpoles with the presence/absence of zooplankton, and the presence/absence of Bd. We measured growth, development, survival, and infection status of amphibians and took weekly measurements of zooplankton abundance, phytoplankton concentration, and periphyton biomass. We hypothesized that zooplankton would benefit larval amphibians 1) directly, through consumption of Bd zoospores, or 2) indirectly, through a trophic cascade.

Zooplankton presence did not influence Bd infection status or infection intensity of larval amphibians in our experiment. However, we found complex effects on species interactions: competition between larval amphibians and zooplankton for

phytoplankton resources reduced phytoplankton concentration, zooplankton abundance, and survival of amphibians. Competitive effects were diminished in the presence of Bd, suggesting that zooplankton may have at least partially substituted Bd zoospores for phytoplankton in their diet, thus stimulating competitive release. However, competitive effects between zooplankton and larval amphibians overshadowed indirect positive benefits of zooplankton predation on Bd zoospores. This result represents a complex interaction between competition and predation.

Introduction

To meet the mandate to study the varied and complex causes of biodiversity loss, several experimental approaches have been employed. One approach is to conduct laboratory experiments investigating the effects of a single stressor on a species of concern. This approach can isolate direct effects and provide useful baseline data for risk assessment and conservation efforts. However, in nature, species live in complex environments that include a variety of abiotic and biotic factors such as resources, competitors, predators, and pathogens. Single-species studies risk missing indirect effects of stressors, which are mediated through other members of the community, and may also ignore sublethal effects, mitigating factors, and species identity (Rohr et al. 2006). Investigating effects of stressors under a more complex community context allows for the detection of both direct and indirect effects on sensitive species. Furthermore, it may provide more general insights about effects of

stressors on entire communities. Acknowledging this context-dependency and testing species interactions experimentally within the context of an ecological community often reveals unexpected and counterintuitive results (e.g. Leibold and Wilbur 1992, Walls and Williams 2001, Mills and Semlitsch 2004, Relyea et al. 2005). The field of ecotoxicology has benefitted from recent efforts to study the effects of contaminants under realistic conditions (e.g. Rohr et al. 2006), but relatively few studies have investigated effects of other stressors under a community context.

Food webs provide a useful framework for investigating direct and indirect effects of anthropogenic and natural stressors on sensitive species and community dynamics. A food web is a depiction of a network of trophic relationships (Pimm 1982, Polis and Winemiller 1996, Pascual and Dunne 2006). Food webs illustrate predatory and competitive interactions, energy flow through a system, and some indirect effects. Stressors such as climate change, contaminants, invasive species, and emerging infectious diseases may alter species interactions, leading to trophic cascades or other community-level effects.

Traditionally, parasites have been excluded from food web analyses because they are cryptic and their abundance and impacts are difficult to quantify (Marcogliese and Cone 1997). However, parasites comprise half of all biodiversity (Toft 1986, deMeeus and Renaud 2002, Dobson et al. 2008), and parasitism is the most common consumer strategy (de Meeus and Renaud 2002). Though they are diminishingly small compared to other consumers, the total biomass of parasites in an ecosystem can

exceed that of top predators such as birds (Kuris et al. 2008). Impacts of parasites on host populations can be dramatic. Recent work has revealed that inclusion of parasites in food webs can lead to increases in species richness, number of links, food chain length, nestedness, and connectance (Lafferty et al. 2006), and may also affect food web stability, interaction strength, and energy flow (Lafferty et al. 2008). Moreover, consideration of parasites as functional members of ecological communities has led to the conclusion that biodiversity can profoundly affect the emergence and transmission of infectious diseases (Keesing et al. 2010).

Consumption of free-living stages of heminths, fungi, protists, and some ectoparasites by a variety of aquatic and terrestrial predators (reviewed by Johnson et al. 2010) can lead to important changes in community structure, ecosystem function, and disease risk. For example, zoospores of the chytrid fungus *Zygorhizidium planktonicum* are consumed by *Daphnia spp.*, thus protecting the diatom *Asterionella formosa* from infection (Kagami et al. 2004) and providing a nutritional benefit to the predatory zooplankton (Kagami et al. 2007).

Chytridiomycosis, a disease caused by the fungus *Batrachochytrium dendrobatidis* (hereafter Bd), is a major driver of ongoing loss of global amphibian biodiversity (Mendelson et al. 2006, Skerratt et al. 2007, Wake and Vredenburg 2008, Rohr et al. 2008, Fisher et al. 2009). Bd has been implicated in recent population declines and extinctions of several hundred amphibian species (Skerratt et al. 2007). The infective stage of the fungus, an aquatic flagellated zoospore, infects keratinized

tissues of amphibian hosts. In larvae, keratinized jaw sheaths and tooth rows may become infected, causing mortality in some species (e.g. Blaustein et al. 2005) or more likely, sublethal effects including reduced foraging efficiency (Venesky et al. 2009), reduced rates of growth and development (Parris and Cornelius 2004, Parris and Beaudoin 2004, Parris and Baud 2004), and behavioral changes (Parris et al. 2006, Han et al. 2008, Venesky et al. 2011). Effects of reduced growth and development during the larval stage may persist into adult stages. Larvae may also function as important reservoirs of the pathogen (Briggs et al. 2010). Infection of the keratinized epidermis of metamorphic and adult amphibians may lead to disruption of electrolyte transport and in severe cases, cardiac arrest (Voyles et al. 2009). After initial Bd introduction into a population, infections may rapidly approach a critical threshold of intensity, causing host mortality and population extinctions before density-dependence or host immunity can limit disease spread (Vredenburg et al. 2010). Control measures designed to prevent infection intensity from reaching this critical threshold may allow populations to persist with Bd in an endemic state, rather than experiencing epidemics (Blaustein and Johnson 2010, Briggs et al. 2010).

Zoospores of Bd are 3-5µm in diameter (Longcore et al. 1999), well within the preferred range of food particle size for zooplankton (Knisely and Geller 1986, Sanders and Porter 1990, Pace and Funke 1991, Kagami 2004, Gleason et al. 2008). Woodhams et al. 2011 and Hamilton et al. 2012 demonstrated that microcrustaceans reduce Bd zoospore densities in laboratory cultures, and Buck et al. (2011a) confirmed

the presence of Bd in the gut of *Daphnia pulex*. When consumed by zooplankton, chytrid zoospores should be digested in the gut, because they are rich in nutrients (polyunsaturated fatty acids and cholesterol, in particular) and lack thick cell walls and sheaths (Beakes et al. 1988, Kagami et al. 2007, Gleason et al. 2008). In support of this assertion, Kagami et al. (2007) found that zooplankton populations could benefit numerically from supplementation with a chytrid fungus, suggesting that some nutritional benefit was derived from consumption of zoospores. However, it is not known whether zooplankton consume Bd zoospores in the presence of alternative food sources, or what effects this might have on host-pathogen dynamics.

To investigate the dynamics of the Bd host-pathogen system within a community context, we conducted a 2 x 2 x 2 factorial experiment in which we eliminated members of the aquatic community (amphibian larvae, zooplankton, Bd). Based on our previous research (Buck et al. 2011a), we predicted that zooplankton would consume Bd zoospores which could 1) reduce Bd infection intensity of larval amphibians, or 2) induce a trophic cascade in which zooplankton populations, numerically bolstered by supplementation with Bd zoospores, reduce phytoplankton concentrations, which in turn increases resource availability, benefitting periphyton, a primary food resource of rasping tadpoles (Fig 1).

Methods

This experiment employed a completely randomized 2 x 2 x 2 factorial design. We manipulated the presence of larval *R. cascadae* (absent or present at a density of 20 larvae per mesocosm) with zooplankton (absent or present) and Bd (absent or present at an average dose of 1.16×10^4 zoospores L^{-1}). Each of the 8 treatments was replicated 6 times for a total of 48 experimental units. The experiment occurred at Oregon State University's Lewis-Brown Horticulture Research Farm near Corvallis, OR (elevation = 68 m), Benton County, USA, and ran from 16-Jun-2010 through 25-Aug-2010. Forty-eight plastic tanks (94 cm L x 70 cm W x 33 cm H) were filled with ~120 L of tap water (pH = 8.0) on 31 May and were covered with weighted screen lids. On 1 June we added 30 g of leaf litter to each tank, which provided habitat heterogeneity. On 3 June we inoculated all tanks with phytoplankton and periphyton collected from natural ponds in the area.

We chose to use *R. cascadae* because oral deformities induced by Bd infection are common in this species (Blaustein et al. 2005) and can impair grazing ability (Venesky et al. 2009, Venesky et al. 2010). Partial clutches of *R. cascadae* (4 masses) were collected on 6-May within 48 hours of oviposition from Site 1, a natural pond in the Cascade Mountains (elevation = 1140 m). Eggs hatched and larvae were reared in outdoor holding tanks near the experimental site. We removed remnants of egg masses including any unhatched eggs from the holding tanks on 19-May. Larval amphibians in holding tanks were fed rabbit chow ad libitum. On 10-Jun 20 larvae of Gosner

(1960) stage 25-27 were added to half of the experimental tanks. The initial mass of the larvae was 122 ± 7 mg (mean ± 1 SE).

On the same day, half of the experimental tanks were inoculated with zooplankton collected from natural ponds in the area. All tanks receiving this treatment were checked after 7 days to confirm the presence of zooplankton.

On day 1 of the experiment (16-Jun) and every 1 wk thereafter for a total of ten inoculations, Bd was added to tanks assigned to the Bd treatment. We grew the fungus in pure culture on plastic Petri plates (10 cm-diameter) with standard TGhL nutrient agar medium (16g tryptone, 4g gelatin hydrolysate, 2g lactose, 12g agar, 1L distilled water; Longcore et al. 1999). We inoculated plates with liquid culture of Bd isolate JEL 274, originally isolated from *Anaxyrus boreas* from Colorado, and incubated them at 22˚C for 8 d prior to use. A broth containing Bd scraped from 50 flooded plates was diluted to 800 mL using dechlorinated water and 30 mL of this broth was added to each tank in the Bd treatment. We examined a small sample of this broth in the laboratory with the use of a hemocytometer to determine zoospore concentration. Average zoospore concentration in mesocosms following Bd inoculation was 11,600 zoospores L^{-1}. A broth containing water from 50 flooded control plates was diluted to 800 mL and 30 mL of this broth was added to each tank in the Bd control treatment.

To determine how treatments affected the pond community, we sampled zooplankton, phytoplankton, and periphyton on 17 and 18-Jun and every week thereafter for ten weeks. To measure the abundance of zooplankton, we plunged a 1.5-

cm aquarium uplift tube holding approximately 30 mL of water vertically through the water column and sealed it near the bottom of the tank. Three samples were taken on opposite sides of each tank and in the center and combined. We repeated this procedure three times for each tank for a total of three samples of 90 mL each. Water samples were filtered through 150 μm mesh (Florida Aquatics), and zooplankton from each sample were combined and preserved in 30% ethanol for later quantification. Zooplankton were identified to the level of copepods and cladocerans.

To measure the concentration of phytoplankton, a 25 mL water sample from each of the three combined water samples described above was filtered through a Whatman GF/F filter. To minimize chlorophyll breakdown, filtering was conducted under full shade and filters were stored in 25 mL centrifuge tubes on ice. Samples were stored at -20˚C for 4 d before chlorophyll extraction. Following the Welschmeyer (1994) method, chlorophyll-*a* was extracted with 10 mL of 90% acetone, agitated, and incubated for 24 hrs at -20˚C. Fluorescence measurements were taken using a Turner Designs fluorometer (model TD-700, Sunnyvale, CA), and chlorophyll-a concentration was calculated as the mean value of the three replicates from each mesocosm.

To measure the biomass of periphyton, ten standard glass microscope slides were mounted vertically using silicone I clear rubber sealant (General Electric) on one side of each tank 2 wks before the tanks were filled. On each sampling occasion, one slide was removed and the periphyton on the outer side of the slide was scraped into a

Petri plate using a straight-edge razor blade. Contents of the Petri plate were filtered through a 25-mm Whatman GF/F filter that had been previously dried for 24 hrs at 60˚C and weighed. Filters were dried again for 24 hrs at 60˚C and reweighed to determine periphyton biomass.

We deployed iButton temperature probes (Maxim, Sunnyvale, CA) in twelve tanks on 12-Jun. Each probe logged temperature every hour over the course of the experiment. Dissolved oxygen and pH measurements were taken using digital meters (Oakton Instruments, Vernon Hills, IL) on days 12 and 58 of the experiment.

On day 19, three amphibian larvae from each tank containing amphibians were haphazardly chosen and tested for Bd infection. Their mouthparts were swabbed for Bd using a sterile fine-tip swab (Medical Wire and Equipment), and the individuals were immediately returned to their tank (Retallick et al. 2006). Swabs were placed in sterile vials and stored at -20ºC for later analysis via qPCR.

Amphibians were removed from tanks upon emergence at Gosner (1960) stages 45-46 (metamorphosis). The first newly metamorphosed amphibian (metamorph) was observed on day 34. Following this initial observation, tanks were checked daily for metamorphs until the end of the experiment on day 71. Individuals emerging on day 34 and every third day thereafter were transported to the laboratory where weight and SVL were measured. Following general methods of Searle et al. (2011), these individuals were kept in large Petri plates (140 x 30 mm) with air holes in the lid for two weeks to allow Bd infection to develop. The laboratory was

maintained at a temperature of 14-16ºC with a 14:10 light:dark photoperiod. Twenty-five mL of dechlorinated water was added to each Petri plate to cover the bottom of the dish, which kept the animals in constant contact with the water. Three d after metamorphosis and every 3 d subsequently, individuals were fed 3 pinhead crickets. Each individual's Petri plate was changed 7 d after metamorphosis. 14 d after metamorphosis, individuals were euthanized using an overdose of MS-222 and preserved in 90% ethanol. Infection status of a sample of these individuals was assessed via qPCR. All other emerging individuals were transported to the laboratory, weighed, measured, immediately euthanized using an overdose of MS-222, and preserved in 90% ethanol. At the end of the experiment, all remaining individuals (~18% of all individuals added to mesocosms) were preserved, regardless of Gosner stage; these individuals were excluded from statistical analyses.

While field experiments are often recognized as the standard for ensuring the realism of experimental work in ecology, this option is not usually ethically acceptable when manipulating pathogens. Therefore we used mesocosms as the next best option for community-level experimentation. Despite some caveats (Skelly 2002), experimental ponds are excellent venues for mimicking pond communities that approach the realism of natural communities (Wilbur 1997). We recognize the risk of releasing pathogens into the environment with this type of approach (Parris and Beaudoin 2004), and we took precautions to minimize the risk of pathogen escape. Each tank was covered with a tightly-fitting screen lid to prevent escape of

metamorphic amphibians, colonization by aquatic insects, and use of tanks as a water source by mammals and birds. Water levels were kept low to prevent overflow. All equipment was thoroughly disinfected with 10% bleach (6% sodium hypochlorite) solution during and after the experiment and all used Petri plates were autoclaved. After the conclusion of the experiment, bleach was added to tanks to yield a 10% solution, which is sufficient to kill Bd. After breakdown (4 wks), tanks were emptied and scrubbed.

Measures of performance for metamorphs from each tank were mean daily growth rate (mass and snout-vent length at metamorphosis divided by the number of days from the addition of individuals to the mesocosms until metamorphosis), larval period, survival to metamorphosis (the proportion of larvae surviving to metamorphosis from those initially added to each tank), and survival past metamorphosis (the proportion of metamorphs surviving two weeks past metamorphosis). We used real-time quantitative polymerase chain reaction (qPCR) to quantify the infection status of the 3 larvae swabbed from each tank containing amphibians (72 individuals) and a randomly selected sample of 14 of the metamorphs that were kept in the laboratory from each treatment (56 individuals). The left ventral surface of each metamorph extending from the drink patch to the toe tips was swabbed 10 times, and the swabs were stored in sterile vials. We added 60 µL Prepman Ultra (Applied Biosystems) to each vial containing a swab. Vials were heated to 100ºC for 10 minutes and then cooled for 2 minutes. Supernatant was collected, diluted, and

qPCR analysis was conducted on an Applied Biosystems StepOne Plus real-time PCR machine (Applied Biosystems, Inc., CA, USA) following the methods of Boyle et al. (2004). Each sample was run in triplicate against a Bd standard titration from 10^{-1} to 10^{2} zoospores. The experimenter was unaware of the treatment from which each sample originated at the time of qPCR analysis. An individual was considered infected if two of three replicates tested positive, and replicates were averaged for each sample.

Statistical analyses

Amphibians

Statistical analyses were conducted using R 2.13.0 (2011). Response variables for amphibians included daily growth rate (mass), daily growth rate (snout-vent length), larval period, survival to metamorphosis, and survival past metamorphosis. Both measures of survival were arcsine transformed to meet parametric assumptions. We used linear mixed effects models to determine the effects of zooplankton, Bd, and their interaction on daily growth rate (mass), daily growth rate (snout-vent length), and larval period of amphibians. To avoid pseudoreplication, individuals were nested by tank (i.e. tank was treated as a random effect). A logit link function was used to analyze survival of larvae and metamorphs.

We also conducted qPCR analysis on 3 randomly chosen animals from each tank that were swabbed on day 19 of the experiment (n=72), and 14 animals from each treatment that were held for two weeks past metamorphosis in the laboratory (n=56). We used ANOVAs to test for effects of treatment on infection status and intensity.

Community

Abundance of cladocerans and copepods, phytoplankton concentration, and periphyton biomass were log transformed to meet parametric assumptions. Copepods were not abundant enough to detect until week 3, so the first two sampling occasions were excluded for this response variable only. For community response variables, we performed a series of repeated measures ANOVAs to determine effects of zooplankton, Bd, and their interactions. We used Mauchly's test for sphericity to test the assumption that the variances of the differences between the repeated measurements were similar, and we report Greenhouse-Geisser-corrected p-values.

Results

Amphibians

Daily growth rate (mass) of amphibians was unaffected by the presence of zooplankton (P=0.20), Bd (P=0.69), and their interaction (P=0.33) (Table 1, Fig. 4.2). Daily growth rate (SVL) of amphibians was marginally reduced in the presence of zooplankton (P=0.08), and by the interaction between zooplankton and Bd (P=0.09), but was unaffected by Bd alone (P=0.12) (Table 1, Fig. 4.3). The larval period of amphibians was marginally extended in the presence of zooplankton (P=0.16), but it was unaffected by Bd (P=0.24), and their interaction (P=0.25) (Table 1, Fig. 4.4). Arcsine survival of amphibians was 92.5% overall and was reduced by 12% in the presence of zooplankton (P=0.044), but was unaffected by Bd (P=0.24) or the

interaction of zooplankton and Bd (P=0.14) (Table 1, Fig. 4.5). Survival two weeks past metamorphosis was nearly 100% and was unaffected by the presence of zooplankton (P=0.96), Bd (P=0.96), or their interaction (P=0.94).

qPCR analysis indicated that 26 of 36 Bd-exposed larvae that were swabbed on day 19 were infected, however infection intensity was low (0.6 – 1.7 Bd genome equivalents) compared to exposed animals in many laboratory studies. Two weeks past metamorphosis, 14 of 28 exposed individuals harbored infections, and infection intensity was similarly low (0.9 – 2.0 Bd genome equivalents). All unexposed larvae and metamorphs that were tested (n=36 and n=28 respectively) were uninfected. Zooplankton presence during the larval period was not a significant predictor of infection status or intensity for larvae or metamorphs.

Community

The abundance of cladocerans fluctuated over time (P<0.0001) and was reduced by 14% in the presence of amphibians (P=0.0001), but was unaffected by Bd (P=0.72) (Table 2, Fig. 4.6). In contrast, copepod abundance depended only on the sampling occasion (time) (P<0.0001) and not on the presence of amphibians (P=0.68) or Bd (P=0.70) (Table 2).

Phytoplankton concentration fluctuated over time (P<0.0001) and was reduced by 22% in the presence of zooplankton (P<0.0001), but was unaffected by the presence of amphibians (P=0.20) and Bd (P=0.11) (Table 3, Fig. 4.7). However, the interaction between zooplankton and amphibians reduced phytoplankton concentration

(P=0.02) (Table 3). Periphyton biomass fluctuated over time (P<0.0001) and was reduced by 48% in the presence of amphibians (P<0.0001), but unaffected by the presence of zooplankton (P=0.084) and Bd (P=0.97) (Table 3, Fig. 4.8).

Discussion

Amphibian survival was reduced in the presence of zooplankton (Fig 4.5), and multiple analyses suggested that the presence of zooplankton may have negatively affected the growth and development of amphibians (Figs. 4.2-4.4). These results suggest a competitive interaction between larval amphibians and zooplankton for phytoplankton, a shared resource. Negative effects between tadpoles and zooplankton have been reported from mesocosm studies (e.g. Boone and Semlitsch 2001, 2002, 2003) and from a study in a permanent pond (Seale 1980), but more commonly, tadpoles and filter-feeding zooplankton are assumed to use different resources (periphyton vs. phytoplankton), which share nutrient and light resources (Leibold and Wilbur 1992, Mills and Semlitsch 2004, Relyea et al. 2005, Relyea and Diecks 2008). However, rasping tadpoles such as *R. cascadae* are facultative suspension feeders (Seale 1982) and phytoplankton (including diatoms, dinoflagellates, and cryptophytes) may be an important food source for them (Altig 2007, Whiles et al. 2010, Buck et al. 2012), suggesting the potential for competition with filter-feeding zooplankton.

Previous studies have shown that amphibian larvae may benefit from pesticide-induced mortality of filter-feeding zooplankton (Boone and Semlitsch 2001, 2002,

2003), which suggests interspecific competition for shared algal resources. However, other studies have shown that pesticide-induced reduction of zooplankton populations may negatively affect amphibians through a trophic cascade (Relyea et al. 2005, Rohr and Crumrine 2005, Relyea and Diecks 2008); cladocerans are suspension feeders, consuming phytoplankton in the water column, thus increasing water clarity, allowing sunlight to reach periphyton, a primary food resource of rasping tadpoles (Fig. 4.1A; Leibold and Wilbur 1992). The addition of Bd zoospores to this system could benefit larval amphibians by enhancing zooplankton populations through resource supplementation (Kagami et al. 2007), thus inducing a trophic cascade (Fig. 4.1B). Another mechanism by which amphibians could benefit from zooplankton is through direct consumption (Altig et al. 2007, Hamilton et al. 2012).

Because the infection is limited to the keratinized mouthparts in tadpoles (Garner et al. 2009), sublethal effects of Bd are more common than lethal effects among larvae, including those of *R. cascadae* (Blaustein et al. 2005, Gervasi et al. 2013). However, in the presence of other stressors, sublethal costs of infection could lead to mortality. Infected larval amphibians may exhibit reduced rates of resource consumption due to reduced feeding efficiency (Venesky et al. 2009, Venesky et al. 2010), or they may switch from a diet composed primarily of periphyton to one rich in phytoplankton (Buck et al. 2012). In our experiment, resource limitation due to competition with zooplankton could have exacerbated these effects, and both stressors might be required to induce mortality. Interestingly, we found that the negative effects

of zooplankton on growth and development of larval amphibians were minimized in the presence of Bd (Figs. 4.2-4.5), but this may have been due to competitive release of conspecifics after increased mortality of larval amphibians in the presence of Bd. Moreover, zooplankton may have partially substituted Bd zoospores for phytoplankton in their diet (Woodhams et al. 2011, Buck et al. 2011, Hamilton et al. 2012, Searle et al. unpublished data), thus stimulating competitive release.

As further evidence of competition between larval amphibians and zooplankton for phytoplankton resources, in our experiment, phytoplankton concentration and zooplankton abundance were reduced, rather than increased, in the presence of larval amphibians. A significant interaction between zooplankton and amphibians suggests that together they reduced phytoplankton resources within experimental tanks (Table 3, Fig. 4.7). Previous studies have shown that zooplankton populations benefit from the presence of larval amphibians; phytoplankton populations, a primary food source of zooplankton, benefit from the nutrients that are released when amphibians scrape surfaces free of periphyton (Leibold and Wilbur 1992). This is thought to offset negative effects of competition between larval amphibians and zooplankton for phytoplankton resources (Wilbur 1997). Alternatively, zooplankton populations may be reduced by larval amphibians through direct consumption (Altig et al. 2007, Hamilton et al. 2012). Our results indicate a competitive effect between zooplankton and larval amphibians, rather than beneficial or harmful effects to zooplankton populations due to amphibian-induced release of

nutrients or direct consumption. Interphyletic competition may be important in shaping community structure (Brown and Davidson 1977, Woodin and Jackson 1979, Schoener and Spiller 1987, Morin et al. 1988, Mokany and Shine 2003, Jennings et al. 2010), and our study provides further evidence of competition between vertebrates and invertebrates in an aquatic system.

Kagami et al. (2007) showed that the addition of zoospores of a chytrid fungus numerically benefitted *Daphnia* populations through resource supplementation. We found no such result; cladoceran abundance was not affected by Bd (Table 2, Fig. 4.6). It is possible that in our experiment, Bd zoospores were partially substituted for phytoplankton, rather than supplementing phytoplankton in the diet of cladocerans.

Our results reveal competitive effects between larval amphibians and zooplankton for a shared resource (phytoplankton). This negative effect seems to overshadow indirect benefits for amphibians of zooplankton predation on Bd zoospores. This finding represents a complex interaction between competition and predation, and provides experimental evidence that interphyletic competition can be important in structuring aquatic communities.

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Table 4.1. Output of linear mixed effects models on daily growth rate (mass) (A), daily growth rate (snout-vent length) (B), larval period (C), and survival to metamorphosis (D) of amphibians.

A. DGRmass	Value	Std error	df	P-value
Zooplankton	-0.000814	0.000609	20	0.196
Bd	-0.000243	0.000607	20	0.693
Zooplankton * Bd	0.000863	0.000866	20	0.331
B. DGRSVL				
Zooplankton	-0.015373	0.008347	20	0.080
Bd	-0.013444	0.008259	20	0.119
Zooplankton * Bd	0.021434	0.012040	20	0.090
C. Larval period				
Zooplankton	1.91489	1.302108	20	0.157
Bd	1.55031	1.288326	20	0.243
Zooplankton * Bd	-2.23385	1.874700	20	0.247

D. Survival to metamorphosis	df	Deviance	Residual df	Residual deviance	P-value
Zooplankton	1	4.0586	477	532.22	0.044
Bd	1	1.3535	478	536.28	0.245
Zooplankton * Bd	1	2.1893	476	530.03	0.139

Table 4.2. Output of repeated measures ANOVAs on cladoceran abundance and copepod abundance. Included are all main effects and any significant interactions.

Cladoceran abundance	DFn	DFd	F	P-value
Bd	1	20	0.13	0.725
Amphibians	1	20	23.39	<0.001
Time	9	180	112.88	<0.001
Amphibians * Time	9	180	5.61	<0.001
Copepod abundance				
Bd	1	20	0.15	0.699
Amphibians	1	20	0.18	0.678
Time	7	140	169.44	<0.001
Amphibians * Bd	7	140	3.91	<0.001

Table 4.3. Output of repeated measures ANOVAs on phytoplankton concentration and periphyton biomass. Included are all main effects and any significant interactions.

Phytoplankton concentration	DFn	DFd	F	P-value
Bd	1	40	2.60	0.115
Amphibians	1	40	1.70	0.200
Zooplankton	1	40	43.60	<0.001
Time	9	360	59.11	<0.001
Amphibians * Time	9	360	5.56	<0.001
Zooplankton * Time	9	360	10.64	<0.001
Periphyton biomass				
Bd	1	40	0.001	0.970
Amphibians	1	40	230.37	<0.001
Zooplankton	1	40	3.15	0.084
Time	9	360	4.81	<0.001
Amphibians * Time	9	360	9.05	<0.001

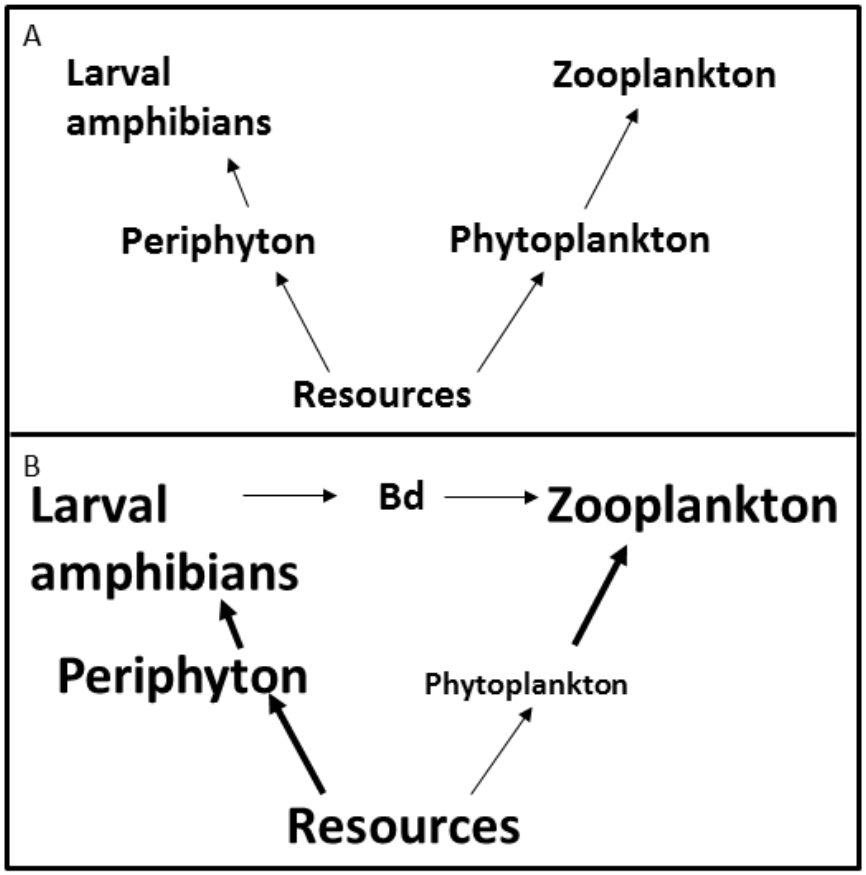

Figure 4.1. Using the aquatic interaction web in panel A as a comparison, panel B shows the hypothesized trophic cascade when Bd zoospores are added to the system. Zooplankton populations, numerically bolstered by supplementation with Bd zoospores, reduce phytoplankton concentrations, which in turn increases resource availability, benefitting periphyton, a primary food resource of rasping tadpoles. The direction of arrows indicates energy transfer. Modified from Relyea et al. 2005.

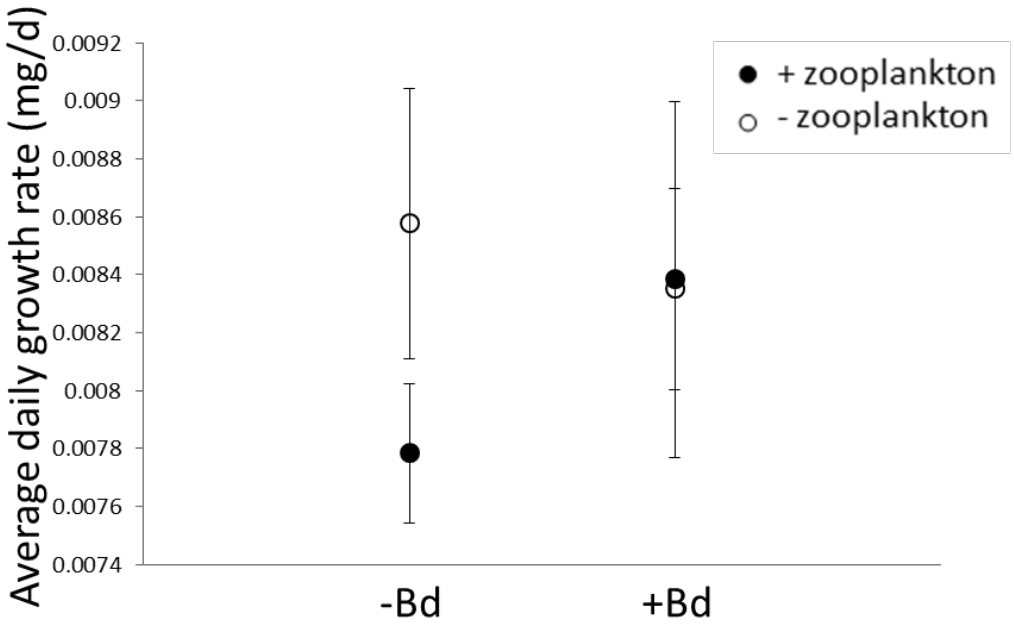

Figure 4.2. Daily growth rate (mass) of larval *R. cascadae* in the absence (open circles) and presence (darkened circles) of zooplankton and not exposed (left) and exposed (right) to Bd.

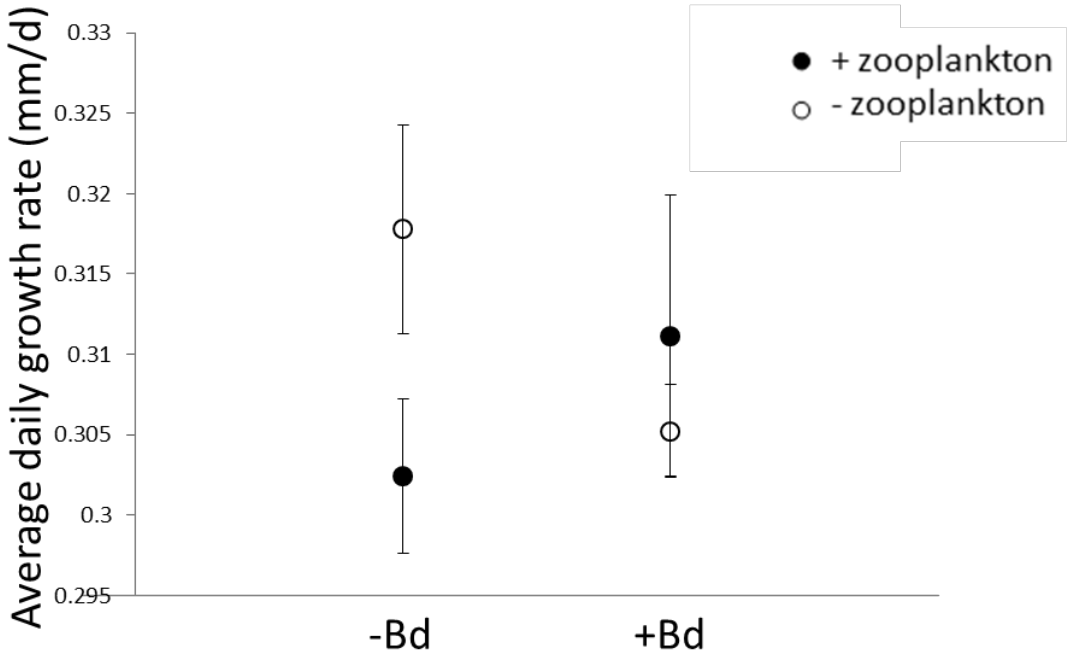

Figure 4.3. Daily growth rate (snout-vent length) of larval *R. cascadae* in the absence (open circles) and presence (darkened circles) of zooplankton and not exposed (left) and exposed (right) to Bd.

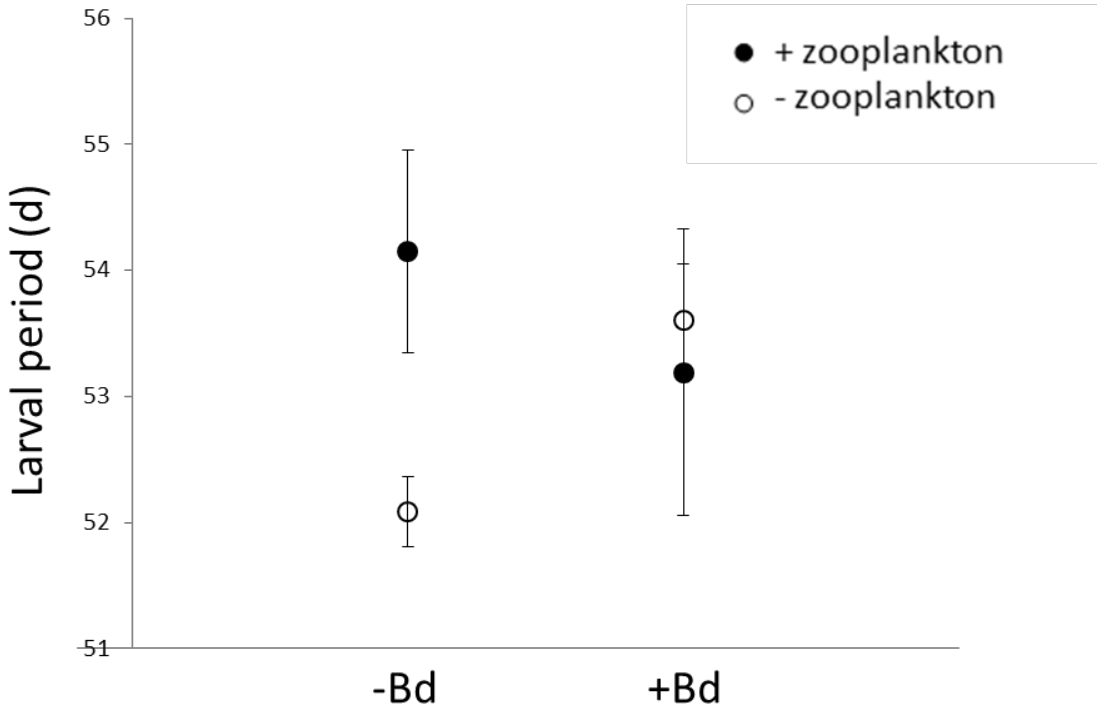

Figure 4.4. Larval period of larval *R. cascadae* in the absence (open circles) and presence (darkened circles) of zooplankton and not exposed (left) and exposed (right) to Bd.

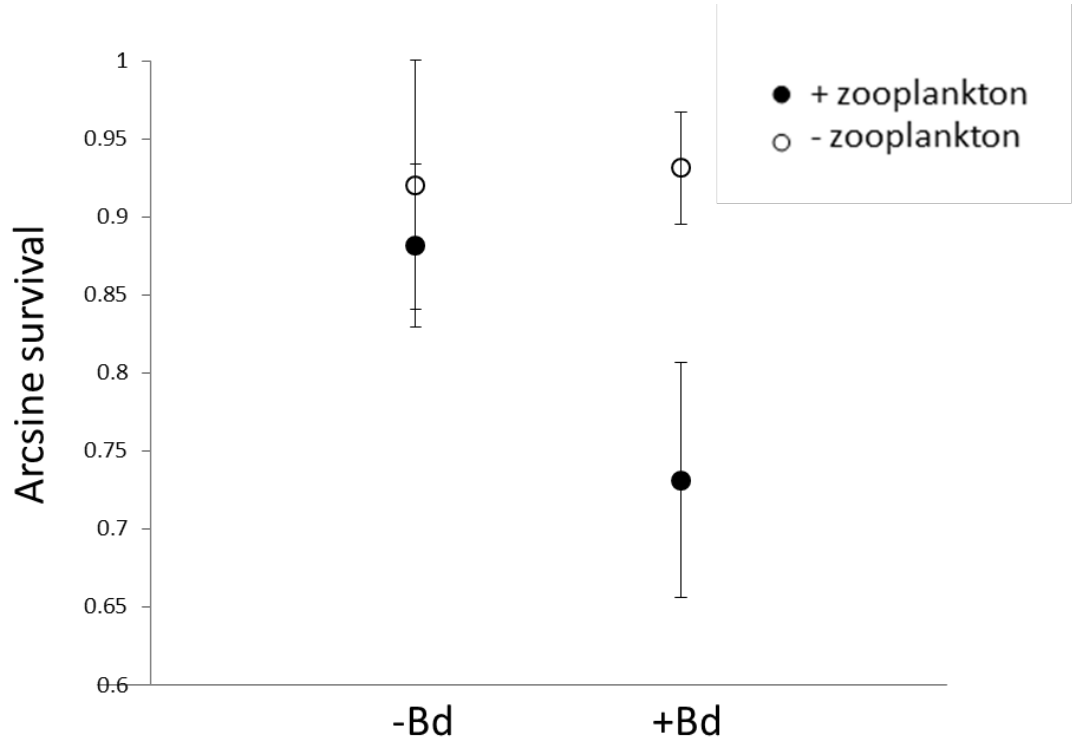

Figure 4.5. Arcsine survival of larval *R. cascadae* in the absence (open circles) and presence (darkened circles) of zooplankton and not exposed (left) and exposed (right) to Bd.

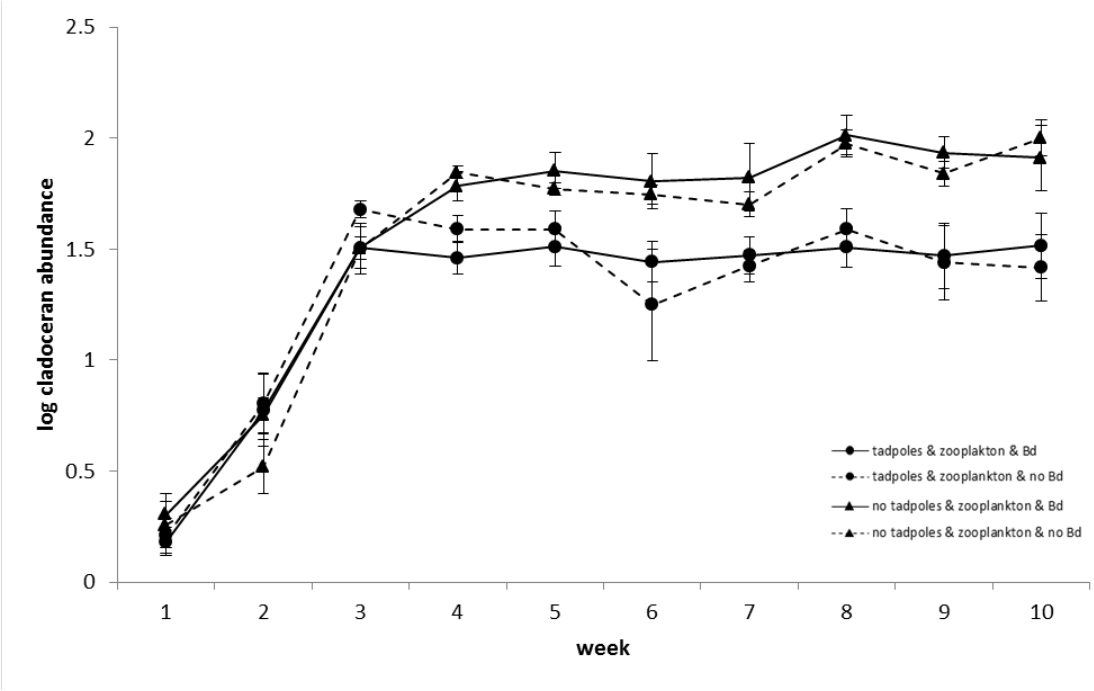

Figure 4.6. Log cladoceran abundance over the 10 week experiment.

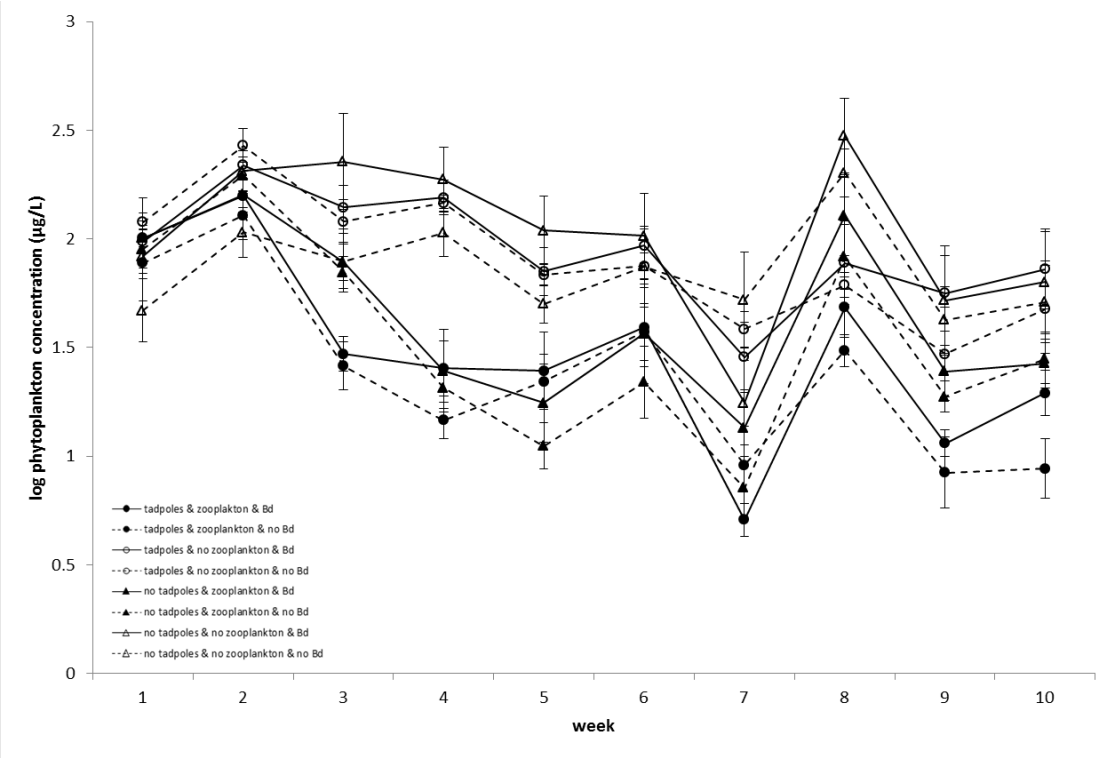

Figure 4.7. Log phytoplankton concentration over the 10 week experiment.

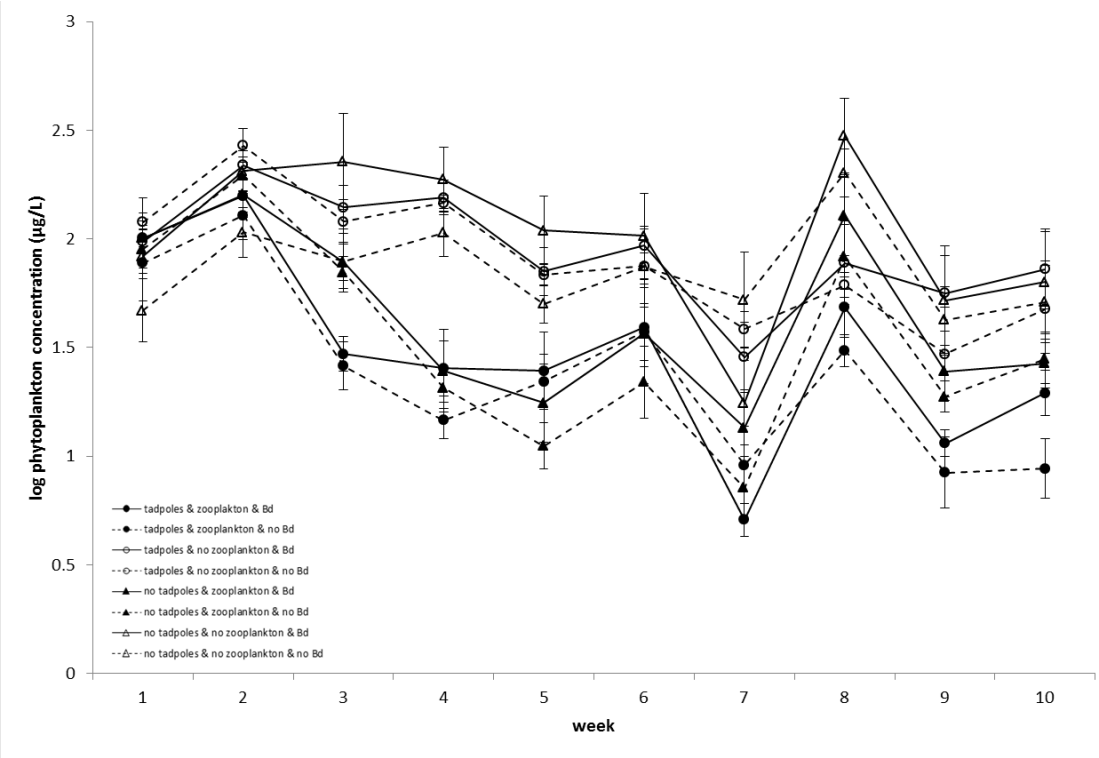

Figure 4.8. Log periphyton biomass over the 10 week experiment.

Chapter 5

Effects of nutrient supplementation on host-pathogen dynamics of the amphibian chytrid fungus: a community approach

Julia C. Buck and Andrew R. Blaustein

In review: Ecological Applications

Abstract

Anthropogenic modification of the environment threatens biodiversity globally. Nutrient loading can cause eutrophication in aquatic ecosystems, profoundly changing community dynamics, including interactions between hosts and their pathogens. Effects may occur through direct or indirect mechanisms, and eutrophication is usually associated with increased disease risk at moderate levels. We investigated effects of resource supplementation on an aquatic community that included a fungal pathogen, Batrachochytrium dendrobatidis (Bd) and a larval amphibian host. In outdoor mesocosms, we experimentally manipulated the presence/absence of nutrients (nitrogen and phosphorus) and the presence of Bd-exposed or unexposed larval amphibians. We predicted that resource supplementation would alleviate competition between tadpoles and zooplankton for shared algal resources, and minimize negative effects of infection.

Nutrient additions caused an initial bloom of phytoplankton, which then decayed. In contrast, nutrient additions did not cause a bloom of periphyton until the end of the experiment. Cladoceran populations, which are dependent on phytoplankton resources, did not benefit from nutrient supplementation. Larval amphibians benefitted from nutrient-induced algal growth, exhibiting increased rates of growth and development, and enhanced survival to metamorphosis. Although this pattern was not statistically significant, Bd-exposed amphibians may have consumed more phytoplankton than unexposed individuals. Bd-exposed amphibians metamorphosed

earlier and allocated resources differently than unexposed individuals, which may be due to the diet shift or modification of developmental cues by Bd infection.

Introduction

Anthropogenic modifications of the environment, including habitat alteration, invasive species, climate change, and contaminants, are causing population declines and extinctions of wildlife and are threatening biodiversity globally (Pimm et al. 1995, May 2010, Barnosky et al. 2011). Degradation of ecosystems can lead to loss of biological diversity at all levels of organization, and changes to ecosystems may be irreversible (Mooney 2010). Such modifications can have profound effects on community dynamics and ecosystem function.

Alteration of global nutrient cycles represents a significant threat to global biodiversity. Nonpoint inputs of nutrients from sources such as agricultural runoff, animal feeding operations, urban areas, and wastewater treatment plants represent the dominant source of nitrogen and phosphorus to surface waters in the United States (Carpenter et al. 1998, National Research Council, 2000). Nutrient loading can cause eutrophication, which generally degrades aquatic ecosystems (Carpenter et al. 1998), leading to changes to water quality parameters and biological productivity. Resulting algal blooms and shifts in algal species composition often deplete oxygen as they decay (Carpenter et al. 1998), the effects of which can reach higher trophic levels. Responses of producers can also be strongly modified by consumer communities, and

can trigger trophic cascades (Schindler 2006), profoundly affecting entire ecosystems (Smith et al. 2006). Technology, land use practices, and conservation measures aimed at decreasing nutrient inputs may mitigate impacts, but the eutrophic state can be persistent and recovery is often slow (Carpenter et al. 1998).

Another factor threatening global biodiversity is the recent emergence of infectious diseases in wildlife (Daszak et al. 2000, Jones et al. 2007, Smith et al. 2009). Epidemiological theory suggests that infectious diseases are unlikely to cause species extinctions in isolation (Anderson and May 1979, deCastro and Bolker 2005, Smith et al. 2006), but may contribute to extinctions through their interactions with other factors such as habitat loss, climate change, and contamination (deCastro and Bolker 2005, Smith et al. 2009). Environmental change may affect host and pathogen physiology, or may influence disease emergence through indirect effects such as interactions with other species (Daszak et al. 2000, Harvell et al. 2002, Lafferty and Gerber 2002, Smith et al. 2009). Freshwater habitats are particularly susceptible to emerging infectious diseases (EIDs) because (i) desiccation and dispersal, challenges commonly faced by infective stages, may be minimized in freshwater habitats, (ii) many vectors and intermediate hosts are aquatic or have aquatic life history stages, (iii) terrestrial and aquatic host species congregate around freshwater habitats, thereby facilitating transmission, and (iv) anthropogenic environmental changes affecting freshwater habitats may facilitate disease emergence (Johnson and Paull 2011).

Human-induced modifications of the environment such as eutrophication can have profound effects on community dynamics including interactions between hosts and their pathogens (Johnson et al. 2010). At low to moderate levels, nutrient loading is generally positively associated with disease risk (Johnson and Carpenter 2008). Nutrient supplementation may promote disease by directly influencing abundance, virulence, and survival of disease agents (Smith and Schindler 2009). For example, phosphate loading can enhance the replication rate of aquatic viruses (Wilson et al. 1996). Eutrophication may also promote infection through effects on host abundance and susceptibility. By definition, hosts and parasites share common resources, and parasites must compete effectively to obtain the resources needed to sustain themselves, complete their life cycle, and avoid host immune responses (Bush et al. 2001, Smith 2007). The availability of nutrients can have considerable effects on the host's ability to mount these costly responses (Smith et al. 2005). In addition to direct effects on pathogens and hosts, eutrophication may also affect disease risk through indirect pathways, such as intermediate or vector hosts, or trophic interactions (Lafferty and Holt 2003, McKenzie and Townsend 2007). For example, eutrophication can provide increased resources for growth and reproduction of snails, the first intermediate hosts of a trematode parasite, leading to increased density of infected snails and increased per-capita rate of production of free-swimming stages which infect amphibians (Johnson et al. 2007). Manipulative experiments are needed to elucidate such complex mechanisms (McKenzie and Townsend 2007).

Biodiversity is being lost at unprecedented rates (May 2010) and there is an urgent need to increase our understanding of factors threatening sensitive taxa. For example, recent estimates suggest that more than 40% of amphibian species have experienced population declines and extinctions (Stuart et al. 2004). Thus, amphibians are at the forefront of the biodiversity crisis (Stuart et al. 2004). The causes of amphibian population declines are varied (Stuart et al. 2004, Blaustein and Kiesecker 2002, Wake and Vredenburg 2008) and include environmental contamination and disease.

The emerging infectious disease chytridiomycosis, caused by the fungal pathogen Batrachochytrium dendrobatidis (hereafter Bd), is a major driver of ongoing loss of amphibian biodiversity globally (Skerratt et al. 2007, Wake and Vredenburg 2008, Rohr et al. 2008, Olson et al. 2013). The infective stage of the fungus, an aquatic flagellated zoospore, infects keratinized tissues of amphibian hosts, including mouthparts of larval amphibians. This hinders the efficiency at which infected larvae feed on attached algae (periphyton) (Venesky et al. 2009, 2010) and decreases the length of their intestines (Venesky et al. 2013), which can lead to reduced growth and slower development rates (Parris 2004, Parris and Baud 2004, Parris and Cornelius 2004). Buck et al. 2012 suggested that larval amphibians compete with zooplankton for suspended algal particles (phytoplankton), and this pattern may be augmented by Bd-induced mouthpart abnormalities. Sublethal effects of Bd on larval amphibians

suggest the potential for direct and indirect interactions between Bd and other members of the aquatic community.

Nutrient supplementation may mitigate or exacerbate effects of Bd on amphibians. If algal resources are plentiful due to nutrient supplementation, negative effects of Bd infection on larval growth and development might be overcome, and competitive effects between tadpoles and zooplankton could be minimized. Alternatively, nutrient supplementation might augment pathological effects on amphibians. Direct or indirect benefits to the pathogen, or an increase in host survival could increase the duration of infectivity and/or transmission rate (Pulkkinen and Ebert 2004, Johnson et al. 2010). Bruning (1991) investigated the consequences of phosphorus limitation on the diatom *Asterionella* and its chytrid parasite Rhizophydium planktonicum, and found that phosphorus limitation resulted in a lower growth rate of the fungus at a given host density.

In this study, we investigated the effects of resource supplementation on the dynamics of a larval amphibian host and its fungal pathogen, Bd, within a community context. In outdoor mesocosms, we manipulated the presence/absence of nutrients (nitrogen and phosphorus) with the presence of Bd-exposed or unexposed larval amphibians. We predicted that resource supplementation would increase algal growth, thus minimizing competition between larval amphibians and zooplankton, and allowing infected larval amphibians to overcome negative effects of infection.

Methods

We manipulated the presence of nutrients (nitrogen and phosphorus) and Bd in a completely randomized 2 x 2 factorial design in artificial ponds (mesocosms). While field experiments are often recognized as the standard for ensuring the realism of experimental work in ecology, this option is not usually ethically acceptable when manipulating pathogens. We therefore used mesocosms as the next best option for community-level experimentation. Despite some caveats (Skelly 2002), experimental ponds are excellent venues for mimicking pond communities that approach the realism of natural communities (Wilbur 1997). We recognize the risk of releasing pathogens into the environment with this type of approach (Parris and Beaudoin 2004), and we took precautions to minimize the risk of pathogen escape. Each tank was covered with a tightly-fitting screen lid to prevent escape of metamorphic amphibians, colonization by aquatic insects, and use of tanks as a water source by mammals and birds. Water levels were kept low to prevent overflow. All equipment was thoroughly disinfected with 10% bleach (6% sodium hypochlorite) solution during and after the experiment and all used Petri plates were autoclaved. After the conclusion of the experiment, bleach was added to tanks to yield a 10% solution, which is sufficient to kill Bd. After breakdown (4 wks), tanks were emptied and scrubbed.

We crossed nutrient additions (absent or added nitrogen and phosphorus) with unexposed larval amphibians or Bd-exposed larval amphibians. Each of the 4 treatments was replicated 8 times for a total of 32 experimental units. The experiment

was conducted at the Oregon State University Lewis-Brown Horticulture Research Farm near Corvallis, OR (68m, Benton County, USA) and ran from 22-Jul-2011 through 30-Sept-2011. 32 plastic tanks (94 cm L x 70 cm W x 33 cm H) were filled with ~120 L of tap water (pH = 8.0) on 1-Jul and were covered with weighted screen lids. On 5-Jul we added 2 g of leaf litter to each tank, which provided habitat heterogeneity. On the same day, we dosed half of the tanks with nitrogen (NH_4NO_3) and phosphorus (H_3PO_4) to achieve concentrations of 1,800 µg/L and 200µg/L respectively (Johnson et al. 2007). At the end of the experiment, a water sample of 10mL was collected from each tank and pooled by nutrient treatment. Samples were frozen in pre-cleaned amber glass jars and shipped to Mississippi State Chemical Laboratory (Mississippi State, MS, U.S.A.) for independent analysis of nutrient concentrations. Results of these analyses indicated that nutrient-control pools had average concentrations of 510µg/L N and 90µg/L P and nutrient-treated pools had average concentrations of 540µg/L N and 180µg/L P. These levels are ecologically relevant (Johnson et al. 2002, Johnson and Chase 2004, Johnson et al. 2007). On 12-Jul we inoculated all tanks with zooplankton, phytoplankton and periphyton collected from natural ponds in the area. Tanks were checked after 7 days to confirm the presence of zooplankton.

Clutches of *Pseudacris regilla* eggs (23 masses) were collected on 23-Jun within 48 hours of oviposition from a natural pond in the Cascade Mountains (Linn county, elevation = 1140 m). Eggs were hatched and larval amphibians were reared in

outdoor holding tanks at the experimental site. Larval amphibians in holding tanks were fed rabbit chow ad libitum. On 6-Jul, larval amphibians were split into two groups for Bd-exposure. The Bd exposure group was exposed to Bd inoculate (2.0 x 10^5 zoospores L^{-1}) three times, six days apart (on 7-Jul, 13-Jul, and 19-Jul; see culture methods below). This dose is within the range of doses normally used to infect larvae in mesocosm and laboratory experiments (e.g. Searle et al. 2011a, Buck et al. 2012, Hamilton et al. 2012, Gervasi et al. 2013). The Bd-control group was treated as the Bd-exposure group, except that they were exposed to sham inoculations scraped from Bd-control plates on the same schedule as the Bd-exposure group. On 22-Jul (day 1 of the experiment), 40 larvae from either the Bd-exposure or the Bd-control group were added to each experimental tank. The initial mass of the larvae (mean ± 1 SE) was 82.3 ± 8 mg and their developmental stage (Gosner 1960) was 25-27 (hind limb buds beginning to develop).

Bd was grown in pure culture on plastic Petri plates (10 cm-diameter) with standard TGhL nutrient agar medium (16g tryptone, 4g gelatin hydrolysate, 2g lactose, 12g agar, 1L distilled water; Longcore et al. 1999). We inoculated plates with liquid culture of Bd isolate JEL 274, originally isolated from *Anaxyrus boreas* from Colorado, and incubated them at 22˚C for 8 d prior to use. A broth containing Bd scraped from flooded plates was diluted using dechlorinated water and this broth was added to each tank in the Bd treatment. We examined a small sample of this broth in the laboratory with the use of a hemocytometer to determine zoospore concentration.

Average zoospore concentration in the Bd-exposed holding tank following Bd inoculation was 2.0×10^5 zoospores L^{-1}. A broth containing water from flooded control plates was diluted and added to the Bd control holding tank.

To determine how treatments affected the pond community, we sampled zooplankton, phytoplankton, and periphyton on 28 and 29-Jul and every two weeks thereafter (4 times total). To measure the abundance of zooplankton (cladocerans), we plunged a 1.5-cm tube sampler holding approximately 30 mL of water vertically through the water column and sealed it near the bottom of the tank. Three samples were taken on opposite sides of each tank and in the center and combined. We repeated this procedure three times for each tank for a total of three samples of 90 mL each. Water samples were filtered through 150 μm mesh (Florida Aquatics), and zooplankton from each sample were combined and preserved in 30% ethanol for later quantification.

To measure the concentration of phytoplankton, a 25 mL water sample from each of the three combined water samples described above was filtered through a Whatman GF/F filter. To minimize chlorophyll breakdown, filtering was conducted under full shade and filters were stored in 25 mL centrifuge tubes on ice. Samples were stored at -20˚C for 4 d before chlorophyll extraction. Following the Welschmeyer (1994) method, chlorophyll-*a* was extracted with 10 mL of 90% acetone, agitated, and incubated for 24 hrs at -20˚C. Fluorescence measurements were taken using a Turner Designs fluorometer (model TD-700, Sunnyvale, CA), and

chlorophyll-a concentration was calculated as the mean value of the three replicates from each mesocosm.

To measure the biomass of periphyton, four standard glass microscope slides were mounted vertically using silicone I clear rubber sealant (General Electric) on one side of each tank 2 wks before the tanks were filled. On each sampling occasion, one slide was removed and the periphyton on the outer side of the slide was scraped into a Petri plate using a straight-edge razor blade. Contents of the Petri plate were filtered through a 25-mm Whatman GF/F filter that had been previously dried for 24 hrs at 60˚C and weighed. Filters were dried again for 24 hrs at 60˚C and reweighed to determine periphyton biomass.

We deployed iButton temperature probes (Maxim, Sunnyvale, CA) in 16 tanks at the beginning of the experiment. Each probe logged temperature every hour over the course of the experiment. Dissolved oxygen and pH measurements were taken using digital meters (Oakton Instruments, Vernon Hills, IL) on day 14 of the experiment.

On day 20, ten larval amphibians from each tank were haphazardly chosen, euthanized using an overdose of MS-222, and preserved in 90% ethanol. The mass, snout-vent length (SVL), and Gosner (1960) stage of these individuals was later measured. We used real-time quantitative polymerase chain reaction (qPCR) to quantify the infection status of all Bd-exposed individuals (n=160) and two randomly-selected individuals not exposed to Bd (32 individuals). Their mouthparts were

dissected and placed in sterile vials. We added 60 µL Prepman Ultra (Applied Biosystems) to each vial and heated it to 100ºC for 10 minutes and then cooled it for 2 minutes. Supernatant was collected, diluted, and qPCR analysis was conducted on an Applied BiosystemsStepOne Plus real-time PCR machine (Applied Biosystems, Inc., CA, USA) following the methods of Boyle et al. (2004). Each sample was run in triplicate against a Bd standard titration from 10^{-1} to 10^{2} zoospores. The experimenter was unaware of the treatment from which each sample originated at the time of qPCR analysis. An individual was considered infected if two of three replicates tested positive, and replicates were averaged for each sample.

Amphibians were removed from tanks upon emergence at Gosner (1960) stages 45-46 (metamorphosis). The first newly metamorphosed amphibian (metamorph) was observed on day 26. Following this initial observation, tanks were checked daily for metamorphs until the end of the experiment on day 71. Metamorphosed individuals were euthanized using an overdose of MS-222 and preserved in 90% ethanol for later measurement of mass and SVL. At the end of the experiment, all remaining individuals (~9% of all individuals added to mesocosms) were preserved, regardless of Gosner stage; these individuals were excluded from statistical analyses.

Statistical analyses

Amphibians

Statistical analyses were conducted using R 2.15.2 (2012). Response variables for tadpoles included daily growth rate (mass), daily growth rate (snout-vent length), and developmental stage (Gosner 1960). Response variables for metamorphs included larval period, daily growth rate (mass), daily growth rate (snout-vent length), and survival to metamorphosis. Survival was arcsine transformed to meet parametric assumptions. We used linear mixed effects models to determine the effects of nutrients, Bd exposure, and their interaction on larval period, daily growth rate (mass), daily growth rate (snout-vent length), and Gosner (1960) stage of amphibians. To avoid pseudoreplication, individuals were nested by tank (i.e. tank was treated as a random effect). A logit link function was used to analyze survival of tadpoles to metamorphosis.

We also conducted qPCR analysis on all 10 Bd-exposed animals from each tank that were preserved on day 20 of the experiment (n=160), and 2 randomly-selected unexposed animals from each tank (n=32). We used ANOVAs to test for effects of treatment on infection status and intensity.

Community

Abundance of zooplankton, phytoplankton concentration, and periphyton biomass were log transformed to meet parametric assumptions. Copepods were not abundant enough for analysis, so we report results for cladocerans only. For community response variables, we performed a series of repeated measures ANOVAs to determine effects of nutrient supplementation, Bd, and their interaction. We used

Mauchly's test for sphericity to test the assumption that the variances of the differences between the repeated measurements were approximately the same, and we report Greenhouse-Geisser-corrected p-values.

Results

Amphibians

For the tadpoles preserved on day 20 of the experiment, we measured the daily growth rate in mass and snout-vent length (SVL), and quantified the Gosner (1960) stage. Daily growth rate (mass) of tadpoles was increased by nutrient supplementation ($P<0.001$), but was unaffected by Bd exposure ($P=0.972$) and the interaction between nutrients and Bd exposure ($P=0.597$) (Table 5.1A, Fig. 5.1A). Daily growth rate (SVL) of tadpoles was unaffected by nutrient additions ($P=0.103$), Bd exposure ($P=0.101$), and their interaction ($P=0.436$) (Table 5.2A, Fig. 5.2A). Gosner (1960) stage of tadpoles was positively affected by nutrients ($P=0.008$), but unaffected by Bd exposure ($P=0.299$) and the interaction between nutrients and Bd exposure ($P=0.548$) (Table 5.3, Fig. 5.3).

For the individuals preserved at metamorphosis, we measured the length of the larval period, daily growth rate in mass and snout-vent length, and quantified percent survival to metamorphosis. The larval period of amphibians was reduced by nutrient supplementation ($P<0.001$) and Bd exposure ($P=0.014$), but was unaffected by their interaction ($P=0.932$) (Table 5.4, Fig. 5.4). Daily growth rate (mass) of metamorphs

was increased by nutrient supplementation (P<0.001), but was unaffected by Bd exposure (P=0.279) and the interaction between nutrients and Bd exposure (P=0.151) (Table 5.1B, Fig. 5.1B). Daily growth rate (SVL) of metamorphs was increased by nutrient supplementation (P<0.001) and Bd exposure (P=0.020), but was unaffected by the interaction between nutrients and Bd exposure (P=0.475) (Table 5.2B, Fig. 5.2B). Survival to metamorphosis was 86% overall and was increased by nutrient additions (P<0.001), but was unaffected by Bd exposure (P=0.3049) and the interaction between nutrients and Bd exposure (P=0.365) (Table 5.5, Fig. 5.5).

qPCR analysis indicated that 78 of 160 (49%) Bd-exposed larvae were infected, and average infection intensity was 0.1 – 2.2 Bd genome equivalents. This infection intensity is low compared to infection loads detected in many laboratory studies (e.g. Searle et al. 2011a, Searle et al. 2011b, Gervasi et al. 2013), but is comparable to infection intensities observed in mesocosm experiments (Buck et al. 2012, Hamilton et al. 2012). All unexposed individuals that were tested (n=32) were uninfected. Nutrient supplementation was not a significant predictor of infection status (P=0.407) or infection intensity (P=0.530) of amphibians.

Community

The abundance of cladocerans fluctuated over time (P<0.001), but was not affected by nutrient additions (P=0.587), Bd exposure status of amphibians (P=0.366), or the interaction between nutrients and Bd exposure (P= 0.440) (Table 5.6A, Fig. 5.6). Phytoplankton concentration fluctuated over time (P<0.001) and was affected by

the interaction of time and nutrient additions ($P<0.001$). Although this result was not statistically significant, phytoplankton concentration was also reduced in the presence of Bd-exposed amphibians ($P=0.069$) (Table 5.6B, Fig. 5.7). Periphyton biomass fluctuated over time ($P<0.001$) and was positively affected by nutrient additions ($P=0.018$), but unaffected by the Bd exposure status of amphibians ($P=0.819$) and the interaction between nutrients and Bd exposure ($P=0.167$) (Table 5.6C, Fig. 5.8).

Discussion

The addition of nitrogen and phosphorus to experimental mesocosms caused changes in algal growth (Fig. 5.7 and 5.8). A bloom of phytoplankton (suspended algal particles) occurred within two weeks of nutrient additions, but then decayed (Fig 5.7). By week 8, phytoplankton concentrations in tanks receiving the nutrient-enrichment treatment were lower than in tanks that had received no supplemental nutrients. Short term phytoplankton blooms followed by decay events are common, and may affect other members of the aquatic community including amphibians (Mills and Semlitsch 2004, Distel and Boone 2009). In contrast to phytoplankton, the biomass of periphyton (attached algal particles, detritus, and other organic matter) was not affected by nutrient additions until the end of the experiment, when higher periphyton biomass was detected in tanks receiving the nutrient-enrichment treatment (Fig 5.8). Previous studies have shown that phytoplankton and periphyton compete for resources including nutrients and light (Leibold and Wilbur 1992, Mills and Semlitsch 2004,

Distel and Boone 2009). In this experiment, it seems that nutrient supplementation initially favored phytoplankton, but after the bloom, resources became sequestered in periphyton.

Larval amphibians benefitted from nutrient-induced algal growth. In the presence of nutrient additions, larval amphibians experienced increased rates of growth and development, and enhanced survival to metamorphosis (Figs. 5.1-5.5). Previous studies have shown that nutrient supplementation benefits larval amphibians by increasing the growth of periphyton (Leibold and Wilbur 1992, Kiffney and Richardson 2001). However, in our experiment, nutrient additions did not change periphyton biomass until near the end of the experiment (Fig 5.8). Instead, larval amphibians likely benefitted from consumption of phytoplankton, which bloomed soon after the start of the experiment (Fig 5.7). Many species of rasping tadpoles are facultative suspension feeders (Seale 1982) and they may incorporate significant amounts of phytoplankton into their diet, especially when periphyton resources are limited (Altig et al. 2007, Whiles et al. 2010, Buck et al. 2012).

In contrast to larval amphibians, cladocerans generally do not exhibit dietary plasticity when their primary resource, phytoplankton, is scarce (but see Siehoff et al. 2009). Limited by the supply of phytoplankton resources for the majority of the experiment, cladoceran populations did not benefit from nutrient supplementation (Fig. 5.6). This result counters findings from previous studies (Vanni 1987, Carpenter et al. 1996, Leibold and Wilbur 1992, Brett and Goldman 1997), but a long term

response of cladoceran populations should not be expected since the response of phytoplankton to nutrient supplementation was transient in this study. It is possible that larval amphibians suppressed cladoceran populations indirectly through competition for shared phytoplankton resources, as previously suggested by Buck et al. (2012).

Of the amphibians preserved on day 20, about half of the Bd-exposed individuals tested positive for Bd infection. Despite their positive infection status, the infection had not yet progressed far enough to cause changes in growth and development (Figs. 5.1-5.3). However, Bd-exposed amphibians metamorphosed earlier than unexposed individuals (Fig 5.4). Unfavorable conditions or high mortality risk in the embryonic or larval environment often induce developmental plasticity in amphibians, including earlier metamorphosis (Wilbur and Collins 1973, Werner and Gilliam 1984, Werner 1986, Benard 2004, Pechenik 2006, Warkentin 2011). Plasticity of amphibians in response to pathogen exposure has been documented for the water mold *Saprolegnia* (Touchon et al. 2006, Uller et al. 2009). In response to Bd infection, larval amphibians generally delay metamorphosis (Parris and Cornelius 2004, Parris and Baud 2004). The mechanism proposed for such a delay is that Bd causes mouthpart deformities that reduce larval feeding efficiency (Venesky et al. 2009, 2010) and decrease the length of the intestine (Venesky et al. 2013). However, reduced feeding efficiency does not necessarily lead to delayed metamorphosis in amphibians. Wassersug (1986) proposed that a hormone in the prostaglandin E group

(PGE2) is secreted into oral mucus where it is ingested with food particles, inhibiting development of adult stomach features and thereby delaying metamorphosis. Thus, individuals that consume large quantities of food may develop more slowly than individuals that consume less. If Bd infection induces mouthpart abnormalities that reduced feeding efficiency of larval amphibians (Venesky et al. 2009, 2010), lower quantities of PGE2 might be ingested, which could increase developmental rate.

In addition to faster development rates, by metamorphosis, Bd-exposed amphibians in this experiment exhibited greater daily growth rate in body length than unexposed individuals (Fig. 5.2B), but this pattern was not mirrored in their daily growth rate in mass (Fig. 5.1B). Contrary to suggestions in the literature (Venesky et al. 2009, 2010) periphyton resources were not affected by the Bd-exposure status of amphibians. Instead, a pattern suggested that phytoplankton concentrations were reduced in the presence of Bd-exposed amphibians. This result supports our previous hypothesis that Bd-exposed amphibians with mouthpart abnormalities may substitute phytoplankton for periphyton in their diet (Buck et al. 2012). Differences in body condition between Bd-exposed and unexposed individuals in this experiment suggest that infected amphibians may have allocated resources toward growth in length, at the expense of growth in mass, possibly due to this diet shift. Previous studies show that consequences of differential development during the larval stage may persist into adult stages (Smithgill and Berven 1979, Alford and Harris 1988, Metcalfe and Monaghan 2001, Altwegg and Reyer 2003).

Our results show that nutrient supplementation increased algal growth, induced higher rates of growth and development in larval amphibians, but did not affect cladoceran populations. Exposure to Bd increased the development rate and snout-vent length, but not the mass at metamorphosis of amphibians, and may have caused a shift in their diet. We hypothesized that resource supplementation would minimize negative effects of Bd on amphibians, but we did not find interactive effects of nutrient supplementation and Bd in this experiment. Instead, resource supplementation may alter effects of the pathogen in subtle ways.

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Table 5.1. Output of linear mixed effects models on daily growth rate (mass) of tadpoles (A) and metamorphs (B).

A. Tadpoles	Value	Std error	df	P-value
Nutrients	0.00566	0.00144	28	<0.001
Bd	0.00005	0.00144	28	0.972
Nutrients * Bd	-0.00109	0.00204	28	0.597
B. Metamorphs				
Nutrients	0.00192	0.00033	28	<0.001
Bd	0.0037	0.00034	28	0.279
Nutrients * Bd	-0.00068	0.00046	28	0.151

Table 5.2. Output of linear mixed effects models on daily growth rate (snout-vent length) of tadpoles (A) and metamorphs (B).

A. Tadpoles				
Nutrients	0.03863	0.00229	28	0.1033
Bd	0.03894	0.02294	28	0.1007
Nutrients * Bd	-0.02563	0.03244	28	0.4362
B. Metamorphs				
Nutrients	0.006356	0.01190	28	<0.001
Bd	0.03001	0.01220	28	0.020
Nutrients * Bd	-0.01214	0.01676	28	0.475

Table 5.3. Output of a linear mixed effects model on Gosner (1960) stage of tadpoles.

Tadpoles				
Nutrients	2.8500	1.00324	28	0.008
Bd	1.0625	1.00324	28	0.299
Nutrients * Bd	0.8625	1.41879	28	0.608

Table 5.4. Output of a linear mixed effects model on larval period of metamorphs.

Metamorphs	Value	Std error	df	P-value
Nutrients	-5.65469	1.36881	28	<0.001
Bd	-3.72866	1.42216	28	0.014
Nutrients * Bd	0.16654	1.92281	28	0.932

Table 5.5. Output of a linear mixed effects model on survival to metamorphosis.

Metamorphs	Sum of squares	df	Mean square	F value	P-value
Nutrients	1.02570	1	1.02570	18.56444	<0.001
Bd	0.06034	1	0.06034	1.09217	0.305
Nutrients * Bd	0.04696	1	0.04696	0.84992	0.364

Table 5.6. Output of repeated measures ANOVAs on cladoceran abundance (A), phytoplankton concentration (B), and periphyton biomass (C). Included are all main effects and any significant interactions.

A. Cladoceran abundance	DFn	DFd	F	P-value
Nutrients	1	28	0.30166	0.587
Bd	1	28	0.84392	0.366
Time	3	84	11.67942	<0.001
B. Phytoplankton concentration				
Nutrients	1	28	0.33046	0.570
Bd	1	28	3.58472	0.069
Time	3	84	118.92037	<0.001
Nutrients * Time	3	84	15.83971	<0.001
C. Periphyton biomass				
Nutrients	1	28	6.27141	0.018
Bd	1	28	0.05295	0.820
Time	3	84	11.64643	<0.001
Nutrients * Time	3	84	5.75020	<0.001

Figure 5.1. Average daily growth rate (mass) for *P. regilla* tadpoles (A) and metamorphs (B) in the absence (open circles) and presence (darkened circles) of added nutrients and not exposed (left) and exposed (right) to Bd.

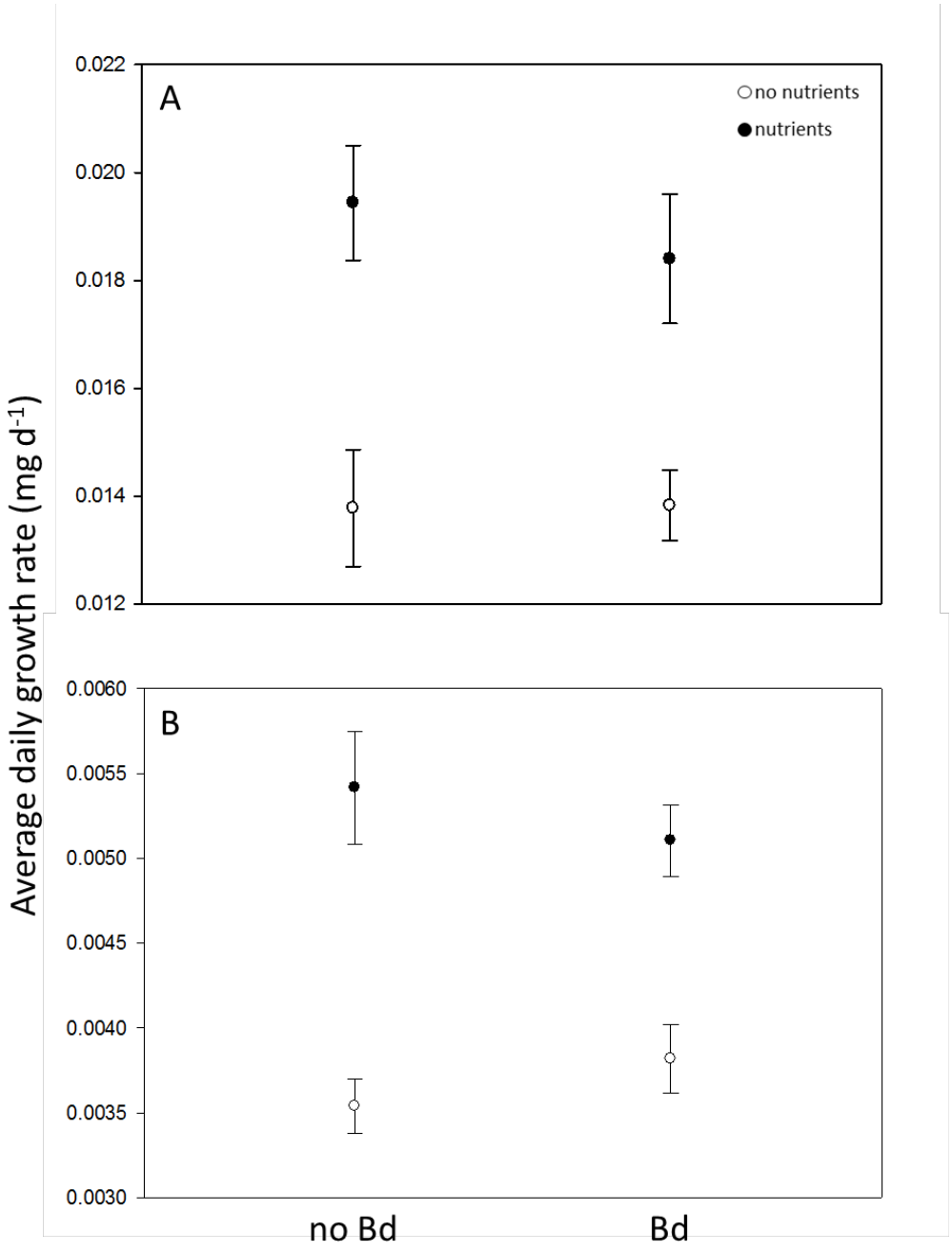


Figure 5.2. Average daily growth rate (snout-vent length) for *P. regilla* tadpoles (A) and metamorphs (B) in the absence (open circles) and presence (darkened circles) of added nutrients and not exposed (left) and exposed (right) to Bd.

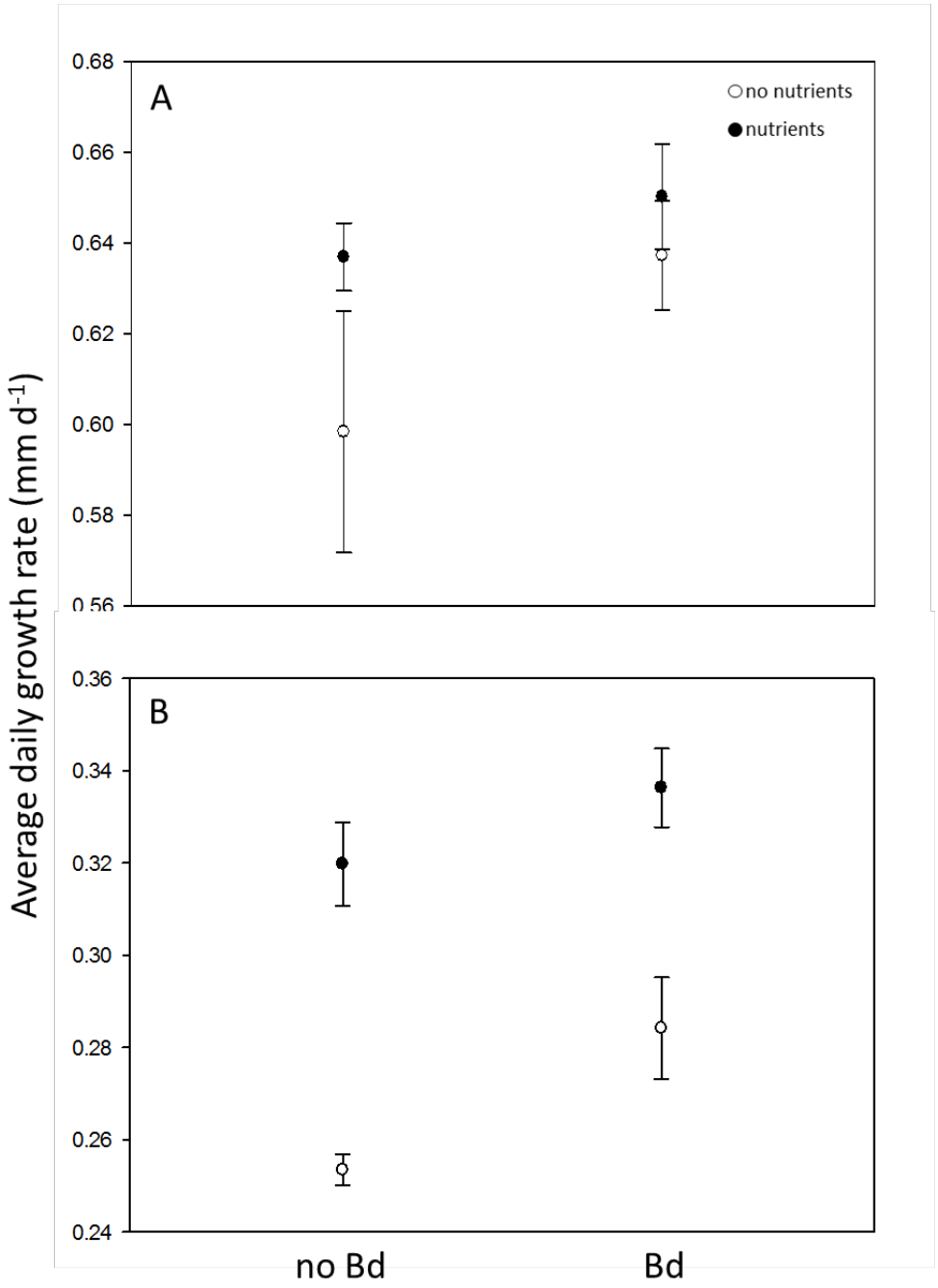


Figure 5.3. Average Gosner stage for *P. regilla* tadpoles in the absence (open circles) and presence (darkened circles) of added nutrients and not exposed (left) and exposed (right) to Bd.

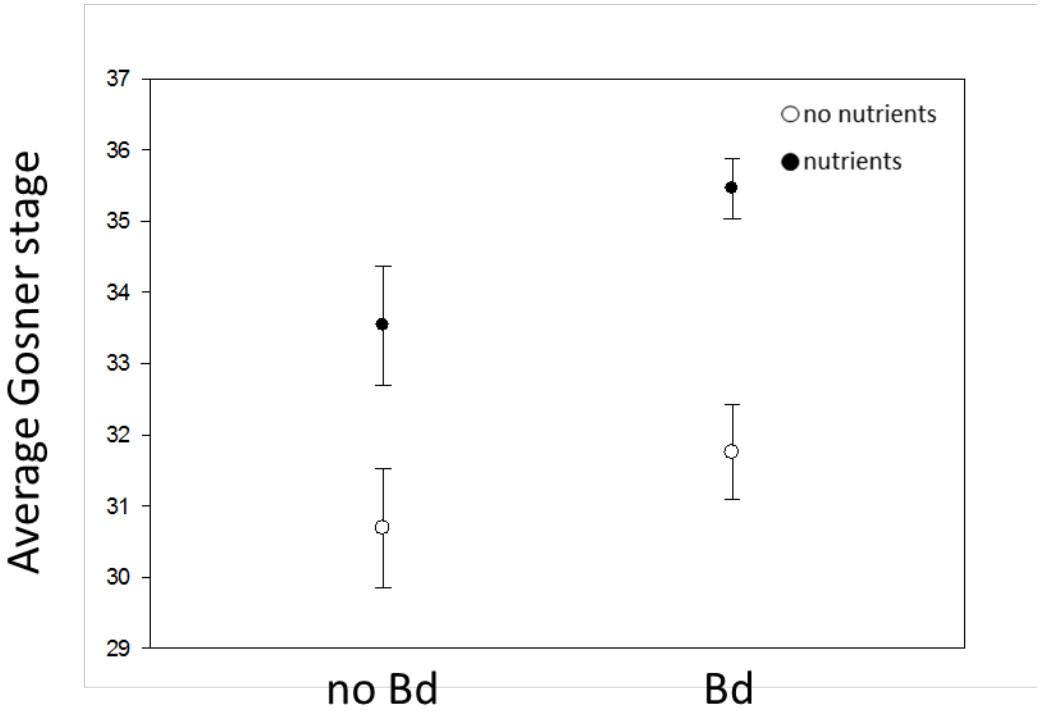


Figure 5.4. Average larval period for *P. regilla* metamorphs in the absence (open circles) and presence (darkened circles) of added nutrients and not exposed (left) and exposed (right) to Bd.

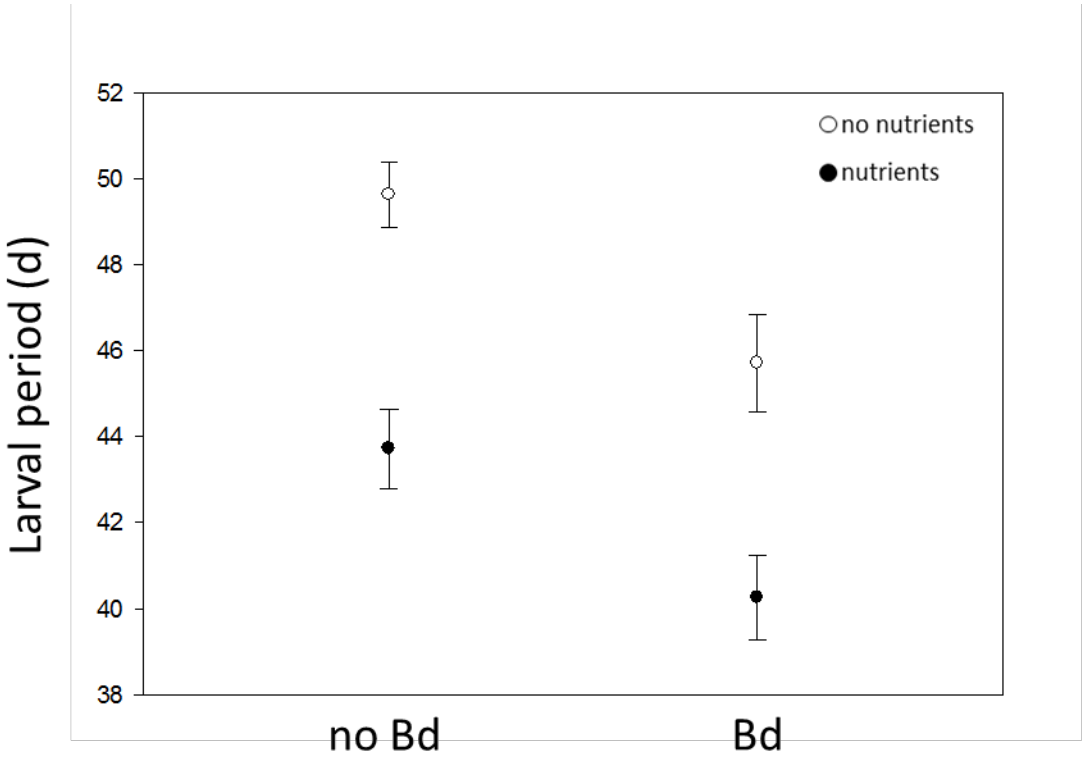


Figure 5.5. Average survival to metamorphosis for *P. regilla* metamorphs in the absence (open circles) and presence (darkened circles) of added nutrients and not exposed (left) and exposed (right) to Bd.

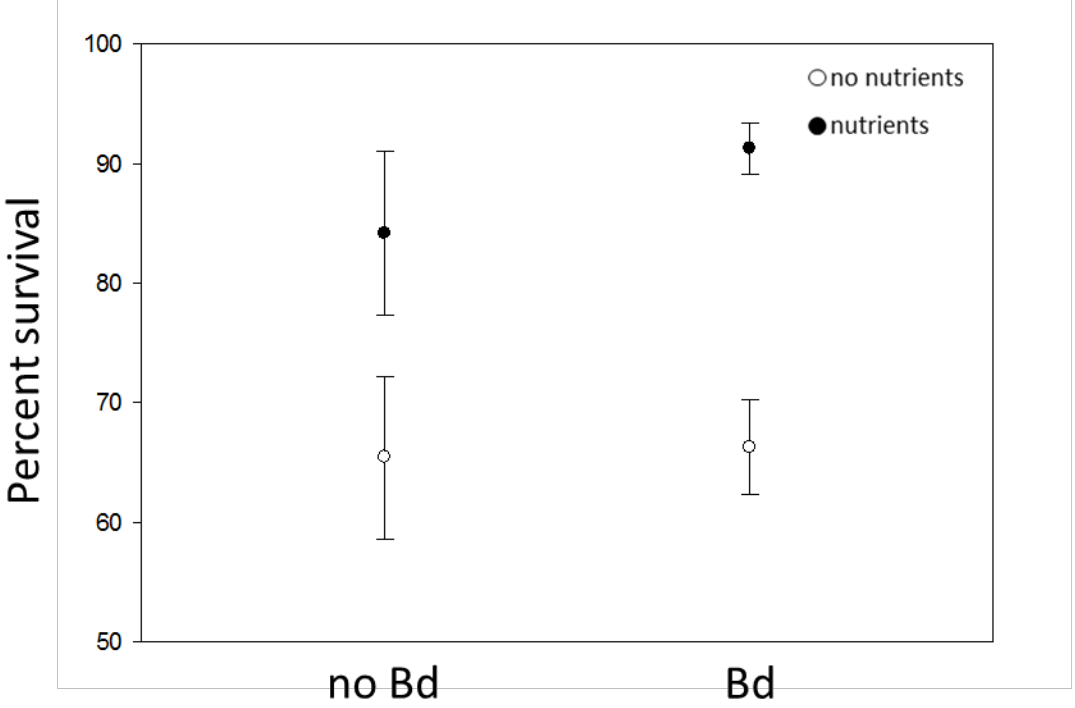


Figure 5.6. Log cladoceran abundance in the presence/absence of nutrient additions (darkened vs. open symbols) and the presence/absence of Bd (circles vs. triangles) over the four sampling periods.

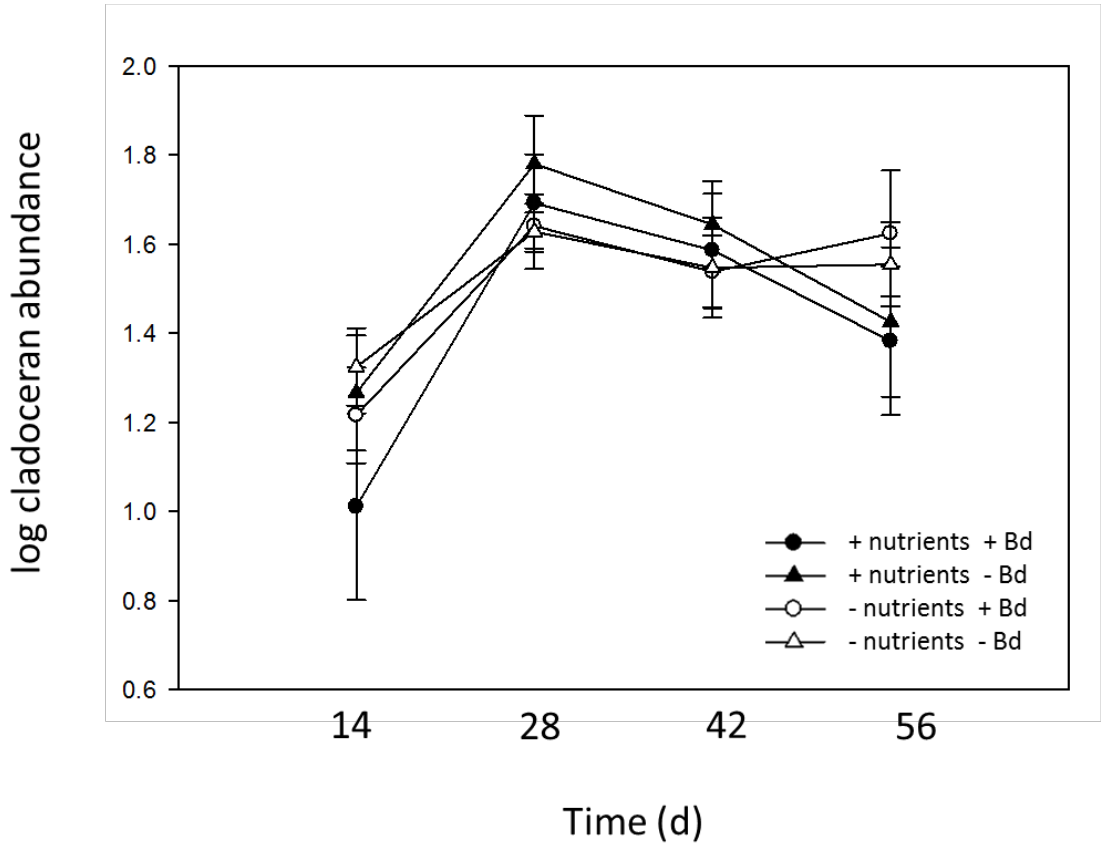


Figure 5.7. Log phytoplankton concentration in the presence/absence of nutrient additions (darkened vs. open symbols) and the presence/absence of Bd (circles vs. triangles) over the four sampling periods.

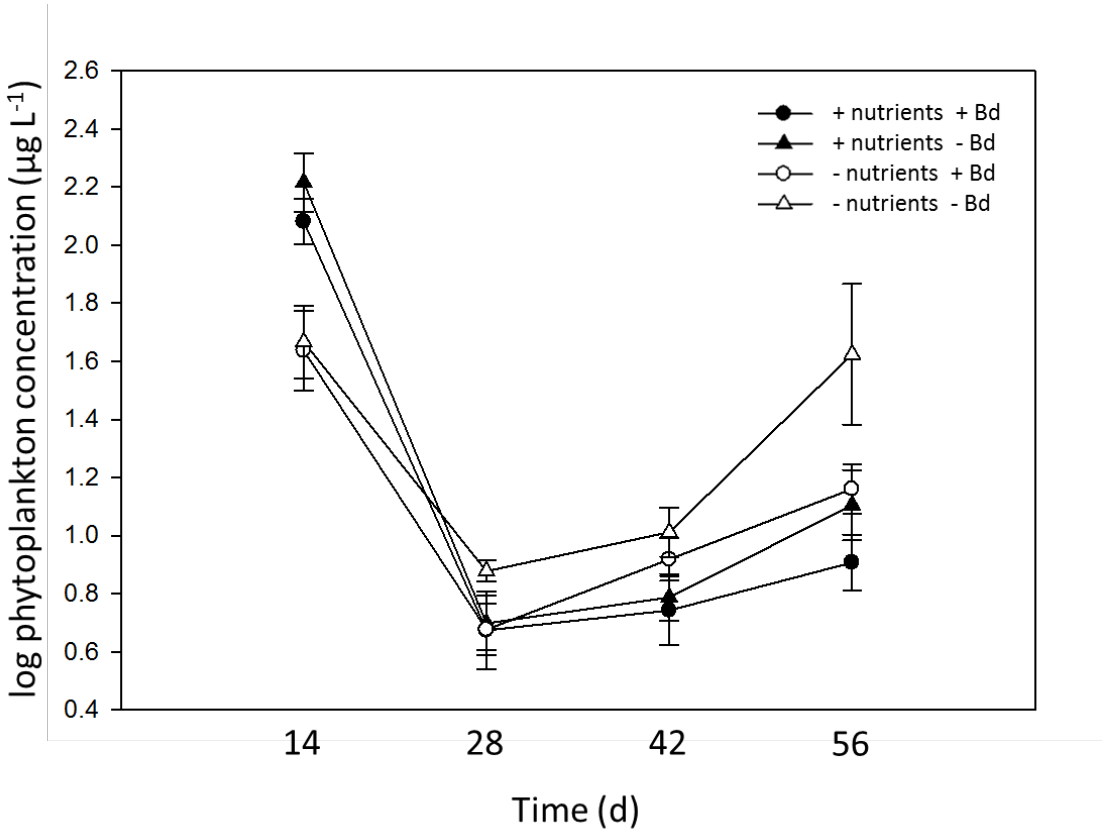
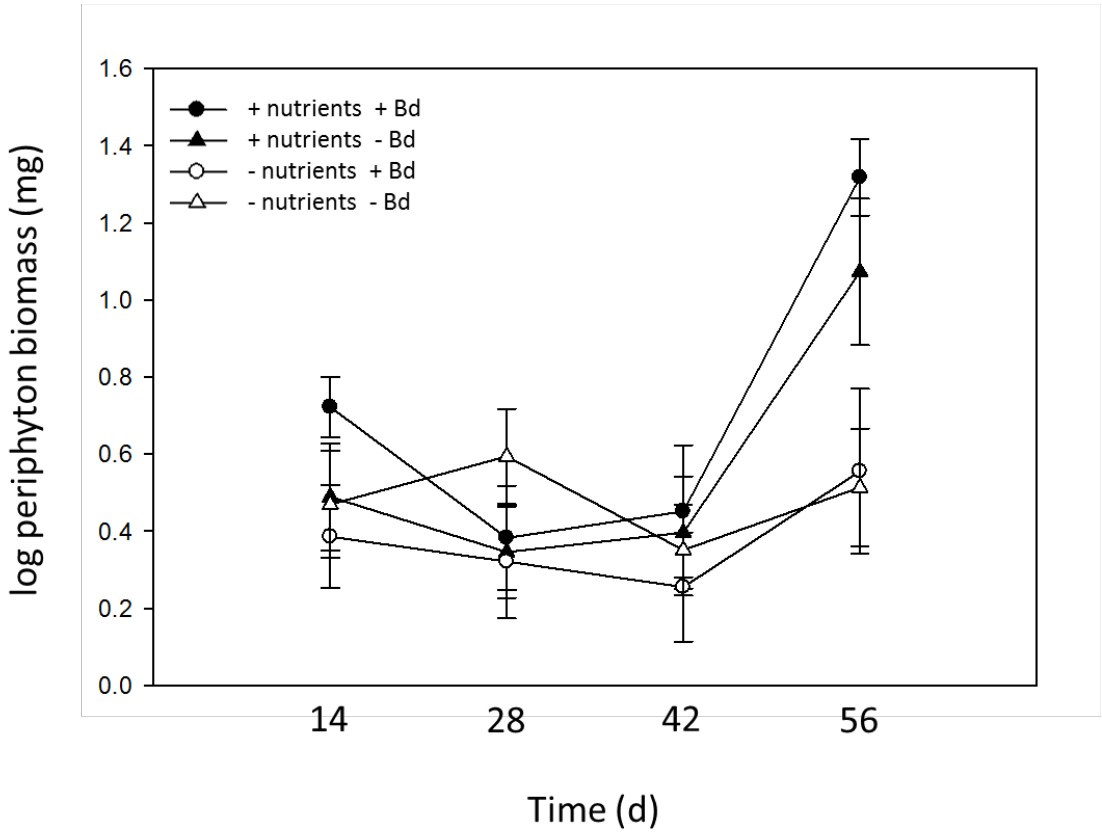


Figure 5.8. Log periphyton biomass in the presence/absence of nutrient additions (darkened vs. open symbols) and the presence/absence of Bd (circles vs. triangles) over the four sampling periods.



Chapter 6

Conclusion

Julia C. Buck and Andrew R. Blaustein

Hosts and their pathogens are functional members of ecological communities (Lafferty et al. 2006, Lafferty et al. 2008). As such, they are influenced by abiotic and biotic components of the environment via direct and indirect mechanisms. For example, when exposed to stressful conditions, hosts may become more susceptible to infection (Luebke et al. 1997, Carey et al. 1999, Lafferty and Gerber 2002, Lafferty and Holt 2003, Marcogliese and Pietrock 2011, Kiesecker 2011). Alternatively, a stressor may affect a pathogen more than its host, another direct mechanism (Lafferty 1997, Sures 2004, Vidal-Martinez et al. 2010). Indirect effects on host-pathogen dynamics may occur through trophic interactions (Keesing et al. 2006, Thieltges et al. 2008, Lafferty 2012).

Emerging infectious diseases (EID's) are increasingly recognized as representing a significant threat to populations of animals and plants (Daszak et al. 2000, Smith et al. 2009, Fisher et al. 2012). For example, the emerging infectious disease chytridiomycosis, caused by the fungal pathogen *Batrachochytrium dendrobatids* (Bd), is found on every continent inhabited by amphibians (Hyatt et al. 2010). First described in 1999 (Longcore et al. 1999), this pathogen is a major driver of amphibian population declines and extinctions (Skerratt et al. 2007, Wake and Vredenburg 2008, Rohr et al. 2008). The recent emergence of chytridiomycosis affords a unique opportunity to study disease dynamics of amphibians within a community context. My dissertation explored the influence of abiotic and biotic

components of the environment on this pathogen, its amphibian hosts, and their interactions.

Chapter 1 reviewed the influence of abiotic and biotic components of the environment, including climate and atmospheric changes, contaminants, competition, predation, reservoir hosts, other pathogens, skin microbiota, and other species, on host-pathogen dynamics of Bd. These environmental factors may mitigate or exacerbate effects of the pathogen on its hosts through direct or indirect mechanisms, resulting in changes to the dynamics of Bd transmission and spread. It is clear that the effects of abiotic and biotic components of the environment are complex, context-dependent, and in need of further examination.

Chapter 2 examined the influence of an abiotic factor, the insecticide carbaryl, and three different assemblages of larval Pacific treefrogs (*Pseudacris regilla*) and Cascades frogs (*Rana cascadae*) on host-pathogen dynamics of Bd. I conducted a fully factorial experiment in outdoor mesocosms which simulated natural aquatic habitats of amphibians. This allowed for the investigation of the influence of a contaminant and amphibian assemblage on host-pathogen dynamics within a community context. I found separate effects of carbaryl, and amphibian assemblage, and minimal effects of Bd on the growth and development of larval amphibians, but detected no interactive effects among the treatments. However, Bd appeared to reduce phytoplankton abundance and increase periphyton biomass, an unexpected result that merited further investigation.

One possible explanation for the results described in chapter 2 is that zooplankton (a biotic influence) might consume Bd zoospores, the infective stage of the pathogen. I examined this hypothesis in chapter 3. I conducted laboratory experiments in which I exposed the zooplankter *Daphnia pulex* to Bd zoospores. Through quantitative PCR and visual inspection I confirmed the presence of Bd zoospores in the gut of *Daphnia*. This finding has implications for mitigation of the effects of Bd on amphibians.

Chapter 4 examined the consequences of consumption of Bd zoospores by *Daphnia*. I conducted a fully factorial mesocosm experiment investigating trophic dynamics of an aquatic community containing primary producers (phytoplankton, periphyton), grazers (amphibian larvae, zooplankton), and Bd. I measured growth, development, and survival of amphibians, as well as growth of other members of the community. I found complex effects on species interactions. Competition between larval Cascades frogs and zooplankton for phytoplankton resources reduced phytoplankton concentration, zooplankton abundance, and survival of amphibians. These effects were diminished in the presence of Bd, suggesting that zooplankton may have at least partially substituted Bd zoospores for phytoplankton in their diet, thus stimulating competitive release. However, competitive effects between zooplankton and larval amphibians overshadowed indirect positive benefits of zooplankton predation on Bd zoospores.

Results of chapter 4 suggested that host-pathogen dynamics of Bd might be affected by competition (a biotic influence) between larval amphibians and zooplankton for shared resources. Chapter 5 examined whether resource supplementation could overcome this effect. I conducted a fully factorial mesocosm experiment to examine how eutrophication (an abiotic influence) might affect host-pathogen dynamics of Bd and Pacific treefrogs within a community context. Nutrient additions caused increased growth of phytoplankton, which benefitted herbivorous larval amphibians, but not herbivorous cladocerans. Larval amphibians exposed to Bd altered their growth, development, and diet, and allocated resources differently than unexposed individuals. However, I did not find evidence to suggest that nutrient supplementation altered the response of larval amphibians to Bd.

As detailed in chapter 1, previous work has examined host-pathogen dynamics of Bd from the molecular level to the level of the species assemblage, but relatively few studies have examined disease dynamics within a community context. Doing so has provided some important insights. First, the interaction web shown in Figure 4.1A is commonly used to examine community dynamics of aquatic systems (e.g. Relyea and Diecks 2008). In chapters 2, 4, and 5, I found that tadpoles consumed significant amounts of phytoplankton, which set up a competitive interaction between zooplankton and tadpoles for this shared resource. Hamilton et al. (2012) showed another unexpected result in a mesocosm experiment: tadpoles reduced zooplankton abundance directly through predation. Together, these studies suggest that this

commonly-used interaction web needs to be reexamined. It was suggested by Altig et al. (2007) and Whiles et al. (2010) that the assumption that tadpoles with scraping mouthparts only (or even primarily) consume periphyton may be invalid. Here I show that this dietary plasticity may have consequences for community dynamics and host-pathogen dynamics.

Another important insight from my research involves the effects of Bd infection on larval amphibians. Most information on the effects of chytridiomycosis comes from studies on post-metamorphic stages of amphibians, but chytridiomycosis can also have lethal and sublethal effects on tadpoles. Venesky and Parris (2009) and Venesky et al. (2009, 2010) suggest that infection changes the feeding kinematics of larval amphibians and reduces their foraging efficiency. This may lead to reduced growth and delayed metamorphosis (Parris and Cornelius 2004, Parris and Beaudoin 2004, Parris and Baud 2004). In chapters 2 and 4, I found minimal effects of Bd exposure on larval amphibians. However, in chapter 5, I found unexpected effects of Bd exposure on growth and development of larval amphibians: tadpoles exposed to Bd metamorphosed earlier and allocated resources differently than unexposed individuals, which may have occurred due to a shift from a diet rich in periphyton to a diet rich in phytoplankton. The effects of differential development may carry over to later life stages.

Lastly, I found in chapter 3 that in a simple system under laboratory conditions, zooplankton ingest Bd zoospores. But in chapters 4 and 5, under a more

complex community context, I found more complex interactions taking place. This emphasizes the context-dependency of host-pathogen dynamics of Bd and highlights the importance of considering hosts and their pathogens not in isolation, but as functional members of the ecosystems in which they exist.

Consideration of hosts and their pathogens as functional members of ecological communities can contribute to our understanding of emerging infectious diseases and provide insights for conservation of sensitive species such as amphibians. For example, Vredenburg et al. (2010) found that chytridiomycosis infection may escalate rapidly, causing host mortality and population crashes before density-dependence or host immunity can limit disease spread. Environmental influences that restrict or promote the ability of Bd to reach this critical threshold of infection intensity could affect disease dynamics (Briggs et al. 2010). Bd is already endemic in many areas (Vredenburg et al. 2010, Briggs et al. 2010), and mitigating its effects on native amphibian species is an important conservation goal. Results from my dissertation research may inform amphibian conservation efforts. I recommend future research investigating disease dynamics within a community context.

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