The ecology of chytridiomycosis in red-legged frog (*Rana aurora*) tadpoles

by

Phineas Hamilton
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Dr. Purnima P. Govindarajulu (School of Environmental Studies)
Outside Member

Abstract

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Chytridiomycosis is an emerging infectious disease of amphibians caused by the chytrid fungus *Batrachochytrium dendrobatidis* (Bd). Chytridiomycosis has caused declines and extinctions of amphibian species worldwide. Although the disease can be highly virulent, there are large differences both within and between amphibian species in response to Bd infection. Environmental factors are increasingly shown to be critical in the outcome of Bd-infection and emergence of the disease, although these factors remain poorly defined. Using a series of mesocosm experiments, I examine the influence of different environmental and ecological factors on the outcome of exposure to Bd in red-legged frog (*Rana aurora*) tadpoles, a species in decline in British Columbia.

First, I tested the hypothesis that *Daphnia,* a keystone genus of zooplankton in shallow freshwater ecosystems, consume Bd zoospores in the water column to decrease the transmission of Bd infection in tadpoles. Although *Daphnia* are nearly always included in amphibian mesocosm experiments, their effects in these systems are overlooked. As such, I also examined the effect of *Daphnia* on *R. aurora* in general. I found that

Daphnia had dramatic beneficial effects on tadpoles, that ostensibly herbivorous tadpoles consumed large numbers of *Daphnia*, and that *Daphnia* interacted with the presence of Bd to influence tadpole survival, with tadpole survival highest in the absence of Bd and presence of *Daphnia*. Although *Daphnia* consumed Bd zoospores in the laboratory, they had no discernible effect on transmission in mesocosms. These results have broad implications for the interpretation of mesocosm studies in general.

Climate change has been implicated as a trigger of outbreaks of chytridiomycosis, yet, paradoxically, high temperatures are lethal to Bd. Climate change has also impacted amphibian communities by uncoupling the phenology of interacting species. I manipulated the temperature in mesocosms to test the effects of small temperature changes on the outcome of Bd-exposure in *R. aurora.* I also tested the effect of the presence of the sympatric Boreal chorus frog (*Pseudacris regilla*) on *R. aurora* at different temperatures, and in the presence and absence of Bd. I found that negative effects of Bd on tadpole body condition increased with temperature, although when Bd was absent tadpoles benefitted at higher temperatures. Furthermore, both Bd and temperature increased the development rates of *P. regilla* but not *R. aurora*, uncoupling the phenology of the species. Increased temperatures thus favoured P. *regilla* at the expense of *R. aurora*. In general, slightly higher and more variable temperatures shifted the host-pathogen balance to the detriment of the *R. aurora*, helping to explain a mechanism by which increasing temperatures may trigger chytridiomycosis outbreaks in susceptible. Together, these experiments clearly demonstrated the importance of ecological context in the outcome of Bd exposure in tadpoles.

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Dedication

to the frogs

Chapter 1: The ecology of chytridiomycosis in anuran communities: An introduction

The worldwide decline of amphibians represents a crisis in conservation biology (Wake 1991, Houlahan et al. 2000, Stuart et al. 2004) and has been called the sixth mass extinction event in Earth's history (Wake and Vredenburg 2008). Currently, over 40% of known amphibian species are in decline, and a third of species are threatened with extinction (Stuart et al. 2004). There has only been widespread awareness of the threat faced by amphibians for the last two decades (Wake 1991). Declines have variously been attributed to factors including destruction of habitat, alien species and over-exploitation that are well known to impact biodiversity in general (reviewed by Collins and Storfer 2003). However, striking and catastrophic amphibian declines and extinctions have occurred in protected, ostensibly pristine ecosystems. For example, the 1980's saw the collapse of entire amphibian communities in protected areas in upland Costa Rica (Pounds and Crump 1994) and eastern Australia (Laurance et al. 1996). Different hypotheses have been proposed to explain these 'enigmatic' declines, and include global climate change, increasing anthropogenic pollutants in ecosystems, and the spread of infectious disease (Collins and Storfer 2003).

Chytridiomycosis is a cutaneous disease of amphibians, caused by infection with the chytrid fungus *Batrachochytrium dendrobatidis* (Bd) (Berger et al. 1998, Longcore et al. 1999). Since its discovery (Berger et al. 1998), chytridiomycosis has been implicated in amphibian mass-mortalities in North and Central America (Lips et al. 2006, Rachowicz et

al. 2006, Lips et al. 2008), Europe (Bosch et al. 2001) and Australia (Skerratt et al. 2007) and is increasingly linked to enigmatic declines. Chytridiomycosis is unusual in its ability to drive species to extinction (De Castro and Bolker 2005) and has been involved in the extinction of multiple amphibian species (reviewed by Skerratt et al. 2007, Wake and Vredenburg 2008). The IUCN Amphibian Conservation Action Plan (http://amphibiaweb.org/declines/acap.pdf) calls chytridiomycosis 'the worst infectious disease ever recorded among vertebrates'. Although the origin of the disease is unclear (Weldon and Du Preez 2005, James et al. 2009, Goka et al. 2009) it is likely that Bd is spreading, and mass-mortality from infection is related to host naiveté (Skerratt et al. 2007, but see Rachowicz et al. 2005).

Despite the high virulence of Bd to many amphibians, the effects of Bd infection are highly variable among species. For instance, although laboratory trial infections with Bd can result in 100% mortality of some species (Berger et al. 1998), many species show no clinical symptoms when infected (e.g., the African clawed frog, *Xenopus laevis* and the American bullfrog, *Lithobates catesbeianus;* Weldon et al. 2005, Daszak et al. 2003). Similarly, among and within susceptible species, surveys have found heterogeneity in both time and space in the prevalence and effects of infection (Berger et al. 1998, Berger et al. 2004, Puschendorf et al. 2006, Woodhams et al. 2007, Kriger and Hero 2007, 2008). Interactions between environmental factors and the host-pathogen relationship may be critical to the outcome of Bd infection in an individual or population, but remain poorly understood (reviewed by Kilpatrick et al. 2010).

The effects of Bd infection also depend on the life history stage of the host. Bd apparears to grow obligately on keratinized tissue in amphibians (Berger et al. 1998) and larvae (tadpoles) do not develop keratinized skin until after metamorphosis (Alford 1999). Although infection may result in decreased measures of fitness (e.g. smaller mass at metamorphosis) it is not generally lethal to tadpoles (Berger et al. 1998, Rachowicz and Vredenburg 2004, Rachowicz et al. 2006, but see Blaustein 2005). As such, it is important to distinguish between Bd infection and chytridiomycosis (Smith 2007, Garner et al. 2009). Although some researchers use the concepts interchangeably, not all animals infected with Bd develop chytridiomycosis, which, in post-metamorphic amphibians, is characterized by epidermal hyperkeratosis (thickening of the *stratum corneum*), sloughing of the skin, focal epidermal lesions and lethargy preceding the death of the host (Berger et al. 1998). Chytridiomycosis may therefore be said to mainly affect post-metamorphic animals. At the tadpole stages, Bd infection may cause degradation of keratinized mouthparts (Berger et al. 1998, Rachowicz and Vredenburg 2004, Rachowicz et al. 2007) that may incur fitness costs, but rarely cause mortality (Berger et al. 1998, Parris and Baud 2004, but see Blaustein et al. 2005). At the tadpole stage, mortality from Bd infection may therefore more likely be a result of accumulated stress rather than acutely pathophysiological (Garner et al. 2009).

Mesocosm experiments on amphibian larvae are an established and widespread means of testing ecological theory (e.g. Wilbur and Alford 1985, Werner and Anholt 1996, Altwegg 2002, Govindarajulu 2004) and more recently of investigating causes of amphibian declines (e.g. Boone and Semlitsch 2001, 2003, Parris and Beaudoin 2004,

Parris and Cornelius 2004, Rohr and Crumrine 2005, Boone et al. 2007, Relyea and Diecks 2008, Rohr 2008, Relyea 2009). Mesocosms are presumed to represent a trade-off between small-scale laboratory experiments and larger scale field studies (Skelly and Kiesecker 2001). In the study of chytridiomycosis, they have been only rarely employed (Parris and Beaudoin 2004, Parris and Cornelius 2004), likely because they focus on larval life stages that are less affected by Bd, and are laborious to conduct. Still, researchers increasingly recognize a need for mesocosm studies to experiemtally test the impacts of Bd in more natural systems (Kilpatrick et al. 2010). Mesocosm studies generally attempt to simulate realistic ecological communities by including community components found in wild systems, not the least of which are zooplankton.

The cladoceran zooplankton *Daphnia* is a keystone genus in freshwater ecosystems (Sarnelle 2005). *Daphnia* are highly effective filter feeders and can change the dynamics of bacterial and fungal disease outbreaks by removing bacteria and fungal spores from the water column (Kagami et al. 2004, Sarnelle 2005, Kagami et al. 2007) They are nearly always included in amphibian mesocosm experiments and are understood to be important to mesocosm function, but we have little understanding of how their presence affects the outcome of amphibian experiments. In Chapter 2, I test the hypothesis that *Daphnia* affect the outcome and transmission of Bd infection in red-legged frog (*Rana aurora*) tadpoles, directly by consuming infectious Bd zoospores, as well as indirectly by altering the structure of the aquatic landscape.

Chapter 3 focuses on interactions between the presence of Bd, temperature manipulation and the presence of a Bd-resistant heterospecific, Boreal chorus frog tadpoles (*Pseudacris regilla*) (Blaustein et al. 2005). Global warming has been suggested to trigger amphibian declines involving chytridiomycosis (Pounds et al. 2006, Bosch et al. 2007, Laurance et al. 2008), yet evidence of this is controversial (Lips et al. 2008, Rohr et al. 2008). Laboratory studies and field surveys clearly show increased pathogenicity and growth of Bd at lower temperatures (17-25°C growth optimum; Woodhams et al. 2003 Berger et al. 2004, Piotrowski et al. 2004, Woodhams et al. 2008), making it unclear how increased temperatures trigger disease outbreaks. Similarly, climate change may affect species by uncoupling the phenology of interacting species (reviewed in Parmesan 2006). Disease threats are embedded in an ecological context (Harvell et al. 2002, Ostfeld 2009), and understanding how climate affects interactions between species and in turn disease will be critical in coming years. In chapter 3, I use mesocosm experiments to examine potential host-pathogen tradeoffs that occur at different temperatures and influence the outcome of Bd exposure.

In Chapter 4, I synthesize the results of the preceding chapters, and discuss how they relate to a broader understanding of the impacts of chytridiomycosis on tadpoles and amphibian as a whole.

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Daphnia influence the outcome of exposure to *Batrachochytrium denrobatidis* in red-legged frog (*Rana aurora*) tadpoles

Abstract:

The chytrid pathogen *Batrachochytrium dendrobatidis* (Bd) causes the disease chytridiomycosis in amphibians and has been linked to declines and extinctions of species. Chytridiomycosis outbreaks vary in space and time, and approaches to predicting the effects of Bd in an ecosystem have mainly focused on abiotic factors or biotic qualities intrinsic to the amphibian host. However, *Daphnia* are important zooplankton grazers of microbes in the water column, and are sympatric with declining red-legged frogs in British Columbia. Using experimental microcosms, I show that *Daphnia* are efficient grazers of Bd zoospores. In mesocosms, survivorship and body condition of tadpoles is decreased in the presence of Bd only when *Daphnia* are present. *R. aurora* tadpoles have dramatically increased growth in the presence of *Daphnia*, and, surprisingly, fed at high rates on *Daphnia*. I show that zooplankton components of the aquatic community have an important role in the outcome of Bd infection in tadpole communities, and that the working assumption of herbivory of anuran tadpoles in mesocosm model systems is inappropriate.

Introduction:

Amphibians are in decline (Stuart et al. 2004) and the emerging infectious disease chytridiomycosis has been implicated as the cause of catastrophic declines and

extinctions of many species (Berger et al. 1998, reviewed by Skerratt et al. 2007). Chytridiomycosis is caused by the fungus *Batrachochytrium dendrobatidis* (Bd), which behaves as a novel pathogen in many areas, and leads to population declines in susceptible species (Lips et al. 2005, Rachowicz et al. 2005, Skerratt et al. 2007, Lips et al. 2008). Treating Bd as an invasive pathogen may be critical for protecting many of the world's amphibian species where Bd has not yet arrived, such as Mainland Asia and Madagascar (Kriger and Hero 2009). However, the persistence of Bd in populations following declines, and the high prevalence of Bd in amphibian populations that are apparently stable (Retallick et al. 2004, Puschendorf 2006) suggest that Bd will exhibit more heterogeneous and subtle effects where it has become endemic (Garner et al. 2009, Fisher et al. 2009).

In British Columbia, Bd is widespread, but observations of mortality due to chytridiomycosis are uncommon (Govindarajulu, pers. comm.). The red-legged frog, *Rana aurora,* is in decline in BC (Conservation Data Centre, BC Ministry of Environment). *R. aurora* is commonly infected with Bd (Govindarajulu, pers. comm.), although the susceptibility of the species to chytridiomycosis remains untested. Congeners such as the yellow-legged frog, *Rana muscosa,* a more southern species, are highly susceptible to chytridiomycosis as adults, but less so as tadpoles, which lack the keratinized skin necessary for progression of the disease (Rachowicz and Vrendeburg 2004). Studying *R. aurora,* which presently coexists with Bd, provides an opportunity to study factors that modify Bd infection to influence the long-term persistence of infected populations, and can provide information critical for protecting this declining species.

Daphnia are keystone zooplankton species in shallow freshwater ecosystems (Sarnelle 2005) and graze efficiently on particles in the water column including algae, bacteria and fungal spores (Kagami et al. 2004, Sarnelle 2005). Recent work shows that *Daphnia* graze on chytrid zoospores and may influence disease dynamics of chytrid parasites of diatoms (Kagami et al. 2004, 2007) or increase their own rates of disease by ingesting spores of fungal parasites (Hall et al. 2007). Bd disperses via flagellated motile aquatic zoospores and transmission of Bd between amphibian hosts can be independent of contact between hosts (Parris and Beaudoin 2004, Rachowicz and Briggs 2007).

Mesocosm experiments on anuran larvae are an established method of testing ecological theory (e.g. Werner and Anholt 1996) and, more recently, assessing the impact of factors contributing to amphibian declines (e.g. Boone and Semlitsch 2003, Parris and Beaudoin 2004, Parris and Cornelius 2005, Rohr and Crumrine 2005, 2008, Relyea, 2009). *Daphnia* are generally included in mesocosm experiments and are recognized as important to their function. Their inclusion may be as part of a general added "zooplankton" component (e.g. Boone and Semlitsch 2003, Rohr et al. 2008) or specific (e.g. Werner and Anholt 1996). When zooplankton are explicitly monitored in amphibian mesocosm studies, it is typically in response to factors (i.e. pesticides) that are expected to affect both amphibian and zooplankton components of communities (Boone and Semlitsch 2003, Boone et al. 2007, Relyea and Diecks 2008, Relyea 2009). Anuran tadpoles are usually considered to be herbivorous, detrivorous or microphagous filter feeders (reviewed by Alford 1999, Semlitsch 2000), and the effects of changing

zooplankton abundance on tadpole growth in mesocosms are ascribed to trophic interactions affecting periphyton production – the preferred food of anuran tadpoles (e.g. Relyea and Diecks 2008). Still, there are increasing reports of carnivory and cannibalism from many species of anuran larvae (Petranka and Kennedy 1999, Altig et al. 2007, Schiesari et al. 2009), yet mesocosm studies continue to view tadpoles primarily as herbivores.

There are two parts to this study. First, I quantify the ability of *Daphnia* to remove Bd zoospores from the water column of microcosms. Second, I conduct a factorial mesocosm experiment to assess the effects of *Daphnia* on the transmission of Bd, and of *Daphnia* and Bd on survivorship, growth and body condition in *R. aurora* tadpoles.

Materials and Methods:

Bd Isolation, Growth and Detection

I used a local isolate of Bd because differences in the virulence of isolates have been detected and I wished to consider Bd effects among Bd and red-legged frogs that are likely to coexist in nature (Fisher et al. 2009). I cultured Bd from an infected bullfrog (*Lithobates catesbeianus*) tadpole collected near Nanaimo, BC following the methods of Longcore et al. (1999). I identified the isolate as Bd microscopically and using qPCR (named PTH 001; Boyle et al. 2004).

I produced Bd zoospores by growing the isolate on 1% tryptone agar for 4-5 days at 23 °C, then flooding plates with 10 mL NAYA® spring water and collecting the zoospore-containing supernatant (Longcore et al. 1999). I counted zoospores on a hemocytometer slide after staining 1:1 with acidic Lugol's solution. Throughout the study, I amplified Bd following the DNA extraction methods and real-time quantitative PCR (qPCR) procedure of Boyle et al. (2004) using a Stratagene MX4000 system and reagents supplied by Applied Biosystems (Foster City, CA). Genomic standards of known Bd zoospore genome equivalents (GEs) were prepared according to Boyle et al. (2004), and were subjected to qPCR alongside samples to quantify zoospore DNA (genome equivalents) where appropriate.

Daphnia Feeding Experiment:

To test the ability of *Daphnia* to filter Bd zoospores from the water column I filled ten 20 mL glass vials with 10 mL of NAYA® spring water and added 0 to 14 lab-reared *Daphnia pulex* taken at random from culture to each vial to test the effect of Daphnia on zoospores in microcosms. I allowed *Daphnia* to acclimatize in vials for 30 minutes, then added 1000 µL of water containing ~ 2 x 10^7 Bd zoospores to each vial. After five hours, I pipetted 1500 µL of water from the surface of microcosms into 2 mL cryotubes. Cryotubes were centrifuged at high speed (13000 g x 10 min), the supernatant discarded, and the pellet DNA extracted. Zoospore genome equivalents (GEs) in samples were quantified using qPCR.

Mesocosm Experiments:

To assess the role of *Daphnia* in Bd-infected *R. aurora* communities, I set up mesocosms in round polyethylene tanks 1.8 m in diameter and 0.60m deep ('cattle tanks') placed outdoors in a fenced field on the University of Victoria grounds. I used a crossed factorial design to evaluate the impacts of Bd and *Daphnia,* alone and in combination, on *R. aurora* tadpoles in the mesocosms, for a total of four treatments. Each treatment was replicated six times using a randomized complete block design for a total of 24 experimental tanks. Treatments were blocked with respect to a line of trees at the edge of the field that I felt might affect light conditions in tanks. The block effect was not significant and is therefore not considered further.

I disinfected mesocosm tanks with 10% bleach (NaOCl), rinsed them thoroughly with water and allowed them to air dry prior to use. I filled tanks to a depth of 0.33m with tap water on April 12 and added to each tank 1 kg of autoclaved deciduous leaf litter (primarily *Acer macrophyllum*) on April 15. I added 0.5 mL of concentrated *Chlorella vulgaris* algae and 50 g of Hagen® rabbit pellets to each tank on May 3. At this time and continuing every 10 days throughout the experiment, I added $NaNO_3$ and K_3PO_4 at an atomic ratio of 40:1 N:P to a concentration of 33 $\mu g \ P \cdot L^{-1}$ to discourage the growth of toxic cyanobacteria (Anholt 1994). I added *Daphnia* to mesocosms beginning May 7, with repeat additions over the course of a week. In total, ~15 large *Daphnia magna* and ~30 *Daphnia pulex* at various sizes were added to each tank in *Daphnia*-present treatments. Throughout the experiment I kept tanks securely covered with 40% shade

cloth affixed to weighted lids (Werner and Anholt 1996) to prevent predation on tadpoles and insect oviposition in the tanks.

I collected seven red-legged frog egg masses from a pond near Bamfield, BC and five from near Port Renfrew, BC in March 2009. I kept eggs outdoors in shaded plastic wading pools (hereafter referred to as rearing tanks) until all tadpoles had hatched. At hatching, tadpoles from all egg masses were mixed. As tadpoles reached Gosner stage 25 (Gosner 1960), the actively foraging stage, I added crushed rabbit chow to tanks for food. When all hatchlings were at Gosner stage 25-26 on May 5, I haphazardly assigned 55 tadpoles to each mesocosm tank, taking care that the tanks received tadpoles of similar size. I sacrificed ten tadpoles from the rearing tanks to confirm via qPCR that infection was absent in rearing tanks. I also assigned 60 tadpoles to 4 12 L plastic tanks for exposure to Bd (15 tadpoles / tank).

I introduced Bd to mesocosm tanks by infecting a subset of tadpoles, and adding the infected tadpoles to the mesocosms (Rachowicz and Briggs 2007). I added ~5×10^7 Bd zoospores to each 12 L tank in which tadpoles had been placed for Bd-exposure every other day for 8 days. Ten tadpoles sampled haphazardly from the exposed tanks were sacrificed at the end of the infection period and tested for Bd infection using qPCR. A 70% infection rate (n = 10) indicated that my method produced infection in a majority of tadpoles. I added 5 tadpoles from the Bd exposed group or 5 from the uninfected rearing tanks (retested for Bd via qPCR, n=20) into the appropriate Bd-present or Bd-absent mesocosm tanks on May 29. I marked tadpoles added at this time with an injection of

fluorescent elastomer (Northwest Marine Technology Inc., Washington, USA) to allow them to be identified at the end of the experiment. Tanks had a total of 60 tadpoles per mesocosm, representing the low end of *R. aurora* tadpole densities observed in the wild (Govindarajulu 2004).

I censused *Daphnia* in *Daphnia*-present treatments on June 30 by sinking a circular 250 µm mesh sieve 7.5 cm in diameter (Fisher Scientific) to the bottom of tanks, waiting 10 seconds and drawing the sieve to the surface. I rinsed *Daphnia* from the sieve with water, and returned samples to the lab for counting under a dissecting microscope. Samples were taken at the east side of tanks, 10 cm from the tank edge and in the shade. All *Daphnia*-absent tanks remained free of *Daphnia* to the end of the experiment.

I terminated the experiment beginning on July 3, dismantling tanks over the course of three days, when some individuals had metamorphosed in the majority of tanks. The effect of day dismantled was not significant in analyses and was excluded from final models. I collected tadpoles from tanks with a dip net that I disinfected with a 10% household bleach solution between tanks. I euthanized tadpoles with an overdose of buffered MS-222 (Tricaine methanesulfonate), transferred them to individual polyethylene bags, and froze them at –20 °C until further processing.

Red-Legged Frog Measurements:

I removed individual frogs from freezing under sterile conditions, and while frozen measured snout-vent-length (SVL), mass and Gosner stage (following Gosner 1960).

Tadpoles that had at least one emerged forelimb were considered to have successfully metamorphosed. I used sterile technique at all times to avoid cross contamination between tadpoles.

To detect Bd infection, I dissected the mouthparts of tadpoles (Gosner stage <42) or took toe clips from the hind limb of metamorphs in infected treatments for all animals in infected treatments. I randomly subsampled up to 10 tadpoles (depending on survivorship; Gosner stage 36-41) from each uninfected tank to confirm that they remained uninfected during the experiment.

Data Analysis:

Daphnia Feeding Trials

For *Daphnia*-zoospore feeding trial data, I converted qPCR C_t values (threshold cycle; the PCR cycle at which target DNA abundance reaches a certain threshold; a measure of target DNA abundance using qPCR -- see Boyle et al. 2004 for further explanation) to zoospore genome equivalents using a standard curve derived from standards of 10 to 10 000 genome equivalents ($R^2 = 0.9991$) (Boyle et al. 2004). Standards were orders of magnitude higher than others have used (Boyle et al. 2004) but were specified because they covered the range of GE values of the DNA samples. Genome equivalents were plotted as a function of *Daphnia* number in microcosms and appeared log-linear. I therefore applied a linear model to log-transformed GE values as a function of *Daphnia* in microcosms to assess the relationship between *Daphnia* and zoospores.

Red-legged frog Survival and Growth:

I stopped all treatments before metamorphosis was complete to allow assessment of Bd infection prevalence at the tadpole stage. To control for potentially confounding effects of developmental stage in tanks, analyses of SVL and body condition were done on the residuals of generalized additive models (GAMs) of SVL and body condition against Gosner stage (Gosner 1960) for all tadpoles in the experiment. Tadpoles added later to tanks to initiate the infection treatment were typically smaller and at earlier developmental stages, and were excluded from analyses on SVL and body condition.

I initially planned to use *Daphnia* presence or absence as a categorical predictor variable in the analysis, but found that including the actual number of *Daphnia* present in tanks as a covariate increased the explanatory ability of the model. I modeled the survival of tadpoles using a generalized linear model (GLM) with a logit link and quasibinomial error terms to account for overdispersion of the data (dispersion parameter = 7.73), and number of *Daphnia* and Bd presence as predictors. I assessed the significance of model parameters using analysis of deviance (ANODEV; Crawley 2005). I tested the response of SVL to treatments using ANCOVA, and body condition using the response of mass to treatments while including SVL as a covariate in the ANCOVA (Garcia-Berthou 2001).

To test for an overall effect on survival, SVL and condition, I used a multivariate analysis of covariance (MANCOVA) to assess the effects of treatments on the response variables simultaneously. I tested for an effect of *Daphnia* on Bd transmission among tadpoles

using a GLM with binomial error terms. All analyses were done using R v. 2.9.2 (R Foundation for Statistical Computing, 2009)

Results:

Daphnia Feeding Trials

Daphnia had a large negative effect on the persistence of Bd zoospores in the water column. The relationship between number of *Daphnia* present and zoospores removed from the water column was well described by a log-linear model ($P < 0.0001$, $R^2 = 0.966$; Fig. 1), with individual *Daphnia* having proportionately less effect on zoospores at higher *Daphnia* densities. *Daphnia* densities of one *Daphnia* / mL removed 99.8% of zoospores from the water column relative to *Daphnia* absent microcosms over the duration of the 5 hour experiment. At densities of more than one *Daphnia* / mL (10 / microcosm), zoospore concentrations fell below the established detection limit of 667 GE/mL (100x dilution factor × lowest amplified standard of 10 GE / 1.5 mL water sampled).

Red-legged frog Survival and Growth and Bd Transmission:

Overall, 69% of tadpoles survived until the end of the experiment. Survival decreased with exposure to Bd when *Daphnia* were present, but was unchanged when *Daphnia* were absent (ANODEV, Bd x *Daphnia* interaction, $F_{1, 20}=8.265$, P= 0.0092, Fig. 2A).

SVL increased with increasing *Daphnia* ($F_{1,20} = 34.07$, $P < 0.0001$, Fig. 2B). This effect was accentuated in the presence of Bd (Bd x *Daphnia* interaction, $F_{1,20} = 5.01$, $P = 0.0367$). Body condition increased dramatically in the presence of *Daphnia* ($F_{1,19} = 86.38$ $P < 0.0001$) but was unaffected by Bd.

MANCOVA of SVL, mass and survivorship as response variables and number of *Daphnia* per tank and Bd presence as predictor variables confirmed *Daphnia* density had a significant effect (Wilks' $\lambda = 0.2695$, $F_{3,18} = 16.26$, $P < 0.0001$) and there was a significant *Daphnia* × Bd interaction (Wilks' $\lambda = 0.5975$, $F_{3,18} = 4.04$, $P = 0.0230$).

Overall 7.6 ± 2.1 % (mean ± SE) of surviving tadpoles in infected treatments were positive for Bd. However, there was no discernible difference in infection rates between *Daphnia* present and absent treatments (GLM, $P > 0.50$). It was notable that the tank with the lowest survival had the highest, although still low, infection rate (out of 9 survivors, 2 were infected = 22% prevalence). All of the tadpoles tested for Bd from Bd-absent treatment tanks were negative.

Discussion:

The presence of *Daphnia* had a dramatic beneficial effect on *R. aurora* larvae and influenced the outcome of Bd exposure. Tadpoles reared with *Daphnia* were more than twice as massive as those in *Daphnia* absent treatments. The effects of Bd were less dramatic, but introduction of Bd into *Daphnia*-present tanks resulted in a 30 % average reduction in survivorship. The magnitude of the combined effects of *Daphnia* and Bd is

clearly seen when considering the total amphibian biomass in tanks (Fig. 3). Mass at metamorphosis is an oft-used measure of fitness of amphibians and correlates with lowered age of reproduction (reviewed by Semlitsch 2000) and decreased risk of mortality post-metamorphosis (Altwegg 2002a, 2002b). The presence of *Daphnia* at tadpole stages is therefore expected to be important to the overall fitness of *R. aurora.*

Anuran tadpoles are usually considered to be herbivorous, detritivorous and microphagous suspension feeders (Dickman 1968, Seale and Beckvar 1980, Alford 1999) with some exceptions (e.g. Pfennig 1989) and are nearly always considered herbivorous in mesocosm studies (e.g. Relyea and Diecks 2008). *R. aurora* is no exception (Dickman 1968). However, recent work has shown that tadpoles of many species forage opportunistically on aquatic invertebrates, and that the functional role of tadpoles in aquatic ecosystems requires re-evaluation (Petranka and Kennedy 1999, Altig et al. 2007, Schiesari et al. 2009). In this experiment, a regression of tadpole mass against number of *Daphnia* in all tanks yields an $R^2 = 0.78$ ($P < 0.0001$). This strong relationship suggested to me that tadpoles were foraging on *Daphnia*. To confirm this, I dissected four tadpoles haphazardly sampled from different *Daphnia* present tanks; these tadpoles had consumed as many as 14 (mean ± SE = 8 ± 3) distinguishable *Daphnia* per cm of intestine. To put this in perspective, I measured the total intestine length (28.4 ± 1.73 cm, n = 3) of haphazardly selected tadpoles and used a conservative estimate of a general tadpole clearance rate of 8 hours (Petranka and Kennedy 1999) to calculate that tadpoles can be expected to consume on average 28 ± 9 *Daphnia* per hour while foraging or 448 ± 144 *Daphnia* in a 16 hour day of foraging in these mesocosms. While this is clearly a rough

estimate, it does suggest the magnitude of *R. aurora* foraging on *Daphnia* can be much larger than previously imagined.

Daphnia were clearly important to performance of tadpoles in this experiment, but the effect of Bd was less pronounced. Bd infection prevalence at the end of this experiment was low relative to similar published experiments, at ~8%. Parris and Cornelius (2004) and Parris and Beaudoin (2004) recorded from 60 to 100% infection prevalence in metamorphs at the end of mesocosm experiments, but infection was achieved by including a caged infected adult frog or toad in mesocosm tanks. Rachowicz and Briggs (2007) also showed higher levels of Bd transmission in *R. muscosa* using tadpoles as Bd vectors, similar to this study, but they used more than an order of magnitude higher tadpole density than used here (up to 4.6 tadpoles/L vs. < 0.1 tadpole/L). They also found that in field enclosures the density of initially infected tadpoles was not a strong predictor of final Bd infection rates, suggesting that factors other than infection levels of tadpoles are driving infection patterns in more natural systems. My results are therefore not inconsistent with other studies, and suggest that Bd is not easily transmitted between tadpole hosts in the absence of other, likely more keratinized vectors, such as post-metamorphic amphibians.

That Bd had distinguishable per-capita effects on tadpoles at such low infection rates was unexpected. However, recent work suggests that the presence of Bd in tadpole communities can impose fitness costs on individuals that may be uncoupled with detectable presence of infection (Garner et al. 2009). This may potentially be due to

energetically costly immune defences that require life-history trade offs on the part of the tadpoles (Garner et al. 2009). Here, I used the most sensitive known methods to detect Bd infection (Hyatt et al. 2007), and although each sample was tested just once it is unlikely that I substantially underestimated infection rates (Kriger and Hero 2006, Garner et al. 2009). This study adds to the body of literature that suggests mortality and infection in tadpoles may be uncoupled under certain situations, and suggests that infection prevalence alone may not be a good indicator of the impact of Bd in a tadpole community.

The low Bd transmission rates in this study made it difficult to substantiate the effect of *Daphnia* on the transmission of infection, but may reflect realistic conditions. The clear Bd × *Daphnia* interaction on survival suggests that *Daphnia* are important in the outcome of Bd-exposure in these communities, although the mechanism behind this remains speculative. Future studies engineered to have higher Bd transmission rates may clarify my original hypothesis.

Previous studies examining the role of zooplankton in amphibian mesocosms usually do so in response to pesticides that simultaneously affect amphibians and zooplankton (e.g. Boone and Semlitsch 2001, 2002, 2003, Rohr and Crumrine 2005, Relyea and Diecks 2008, Relyea 2009). The effects of declining zooplankton on amphibians in these studies are explained by invoking food-web dynamics driving periphyton production, for both observed effects. My results suggest that effects of declining zooplankton on tadpoles may equally be explained through the loss of zooplankton as a high quality food source.

The inclusion of *Daphnia* caused a marked decrease in phytoplankton in tanks and likely increased periphyton growth by improving water clarity. The relative contributions of periphyton and animal food sources to *R. aurora* and anurans in general remain to be clarified, but tadpoles in this study consumed large numbers of *Daphnia*. The zooplankton component in this experiment was simplified to a single genus, but studies with more diverse communities have noted similar effects on tadpoles (Rohr and Crumrine 2005). This experiment was not designed to measure the degree to which trophic cascades were responsible for observed effects on tadpoles. Although mesocosm studies have been criticized for their lack of generality to natural systems (Skelly and Kiesecker 2002), isotopic studies on wild amphibian larvae have also suggested that animal food sources are important to tadpoles (Schiesari et al. 2009). Clearly the role of zooplankton as food sources in amphibian model systems and for anuran larvae in general requires further study.

Overall this study showed that *Daphnia* change the per-capita effects of Bd in tadpole communities, and that *Daphnia* are critical components of amphibian mesocosm systems. Mesocosm studies should not assume herbivory on the part of anuran tadpoles, and should include assessments of trophic links that may be responsible for experimental results, as the influence of experimental factors on the zooplankton community may be unexpectedly important. The links between the biotic community and Bd infection remain to be clarified, but a comprehensive understanding of the epidemiology of chytridiomycosis will clearly require attention to biotic factors extrinsic to the host or pathogen.

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Appendix: Tables and Figures

Table 2.1. Results of MANCOVA and individual ANCOVAs to determine the effects of Bd and *Daphnia* treatments on tadpole survival, SVL and body condition. Analyses are done on the residuals of GAMs of SVL and body condition against tadpole stage to control for stage. Significant *P*-values ($P < 0.05$) are bolded.

Source of variation	***Wilks' lambda***	***P***
MANCOVA (df = 3, 18)		
Daphnia	0.270	**< 0.0001**
Bd	0.824	0.3093
Daphnia × Bd	0.598	**0.0234**
ANODEV (quasibinomial GLM)	*F*	
Survival		
Daphnia	3.78	0.0661
Bd	0.31	0.5812
Daphnia × Bd	8.29	**0.0092**

Table 1, cont'd

Source of variation	***F***	***P***
ANCOVAs		
SVL (df = 1, 20)		
Daphnia	34.25	**< 0.0001**
Bd	0.37	0.5746
Daphnia × Bd	5.01	**0.0367**
Condition (mass ~ svl) (df = 1, 19)		
Daphnia	86.38	**<0.0001**
Bd	24.36	0.7781
Daphnia × Bd	1.30	0.2684

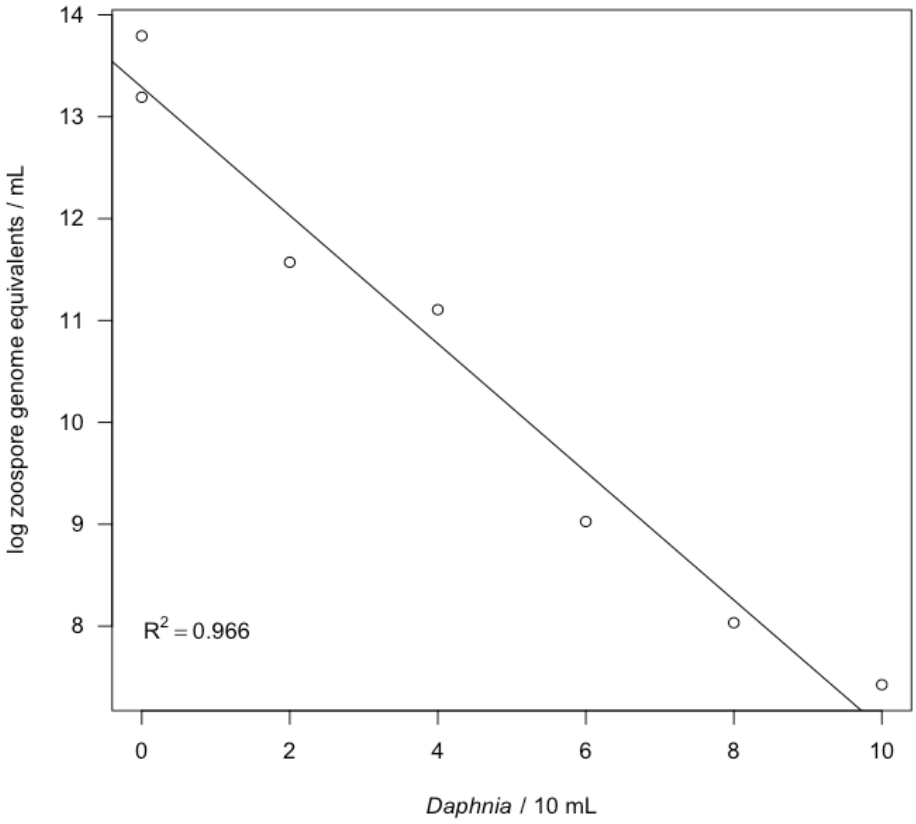

Figure 2.1. The decline of Bd zoospores in the presence of *Daphnia* in 10 mL microcosms after five hours. Zoospore genome equivalents are a measure of number of zoospores present, determined using qPCR.

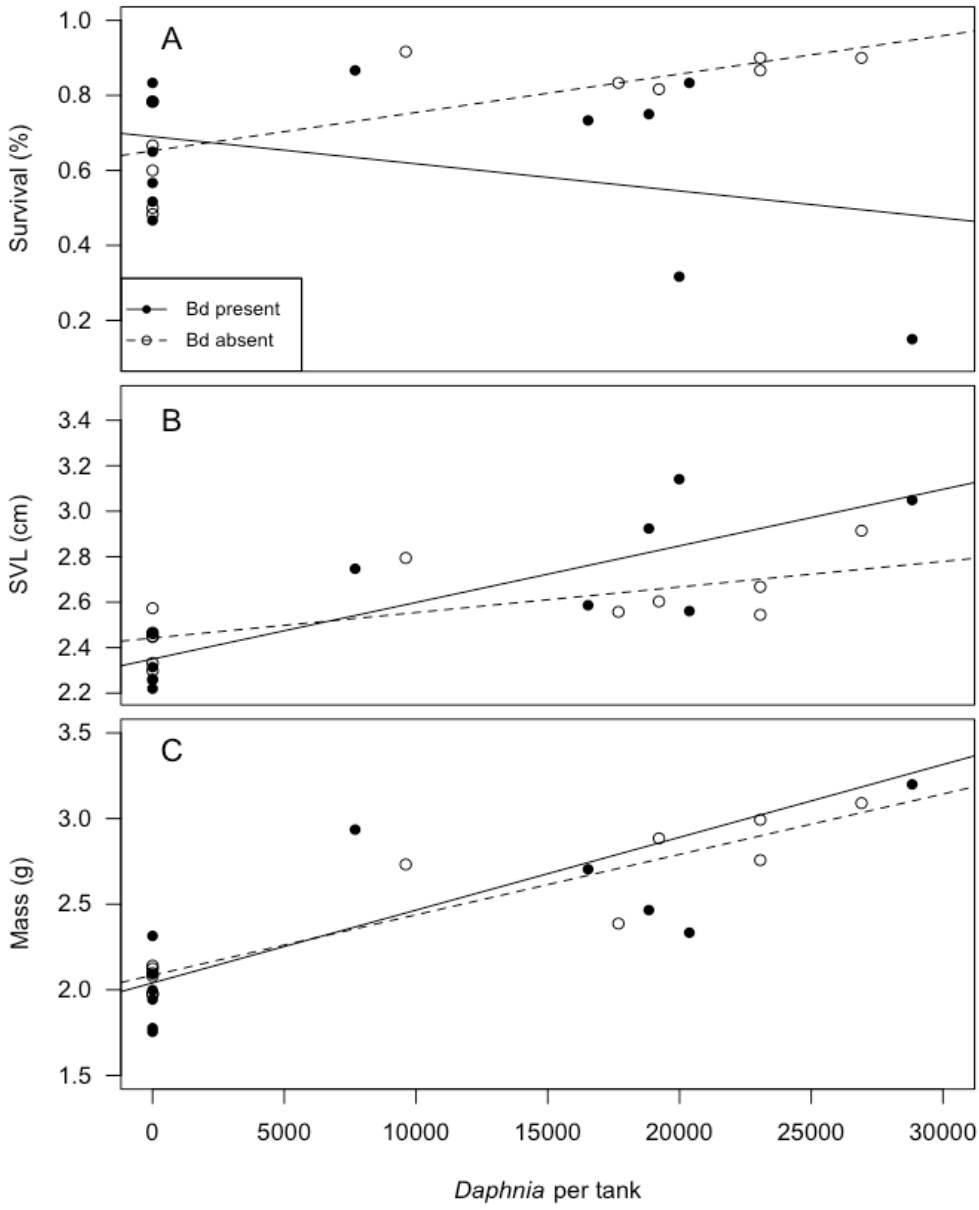

Figure 2. The response of *R. aurora* tadpoles to *Daphnia* and Bd exposure in experimental mesocosms. (A) Percent survival out of 60 tadpoles in each tank. (B) and (C) report SVL and mass as residuals of GAMs that are most easily interpreted as the response variable's departure from its predicted value at a given Gosner stage, to correct for stage. Mass (C) is analyzed including SVL as a covariate to assess tadpole body condition.

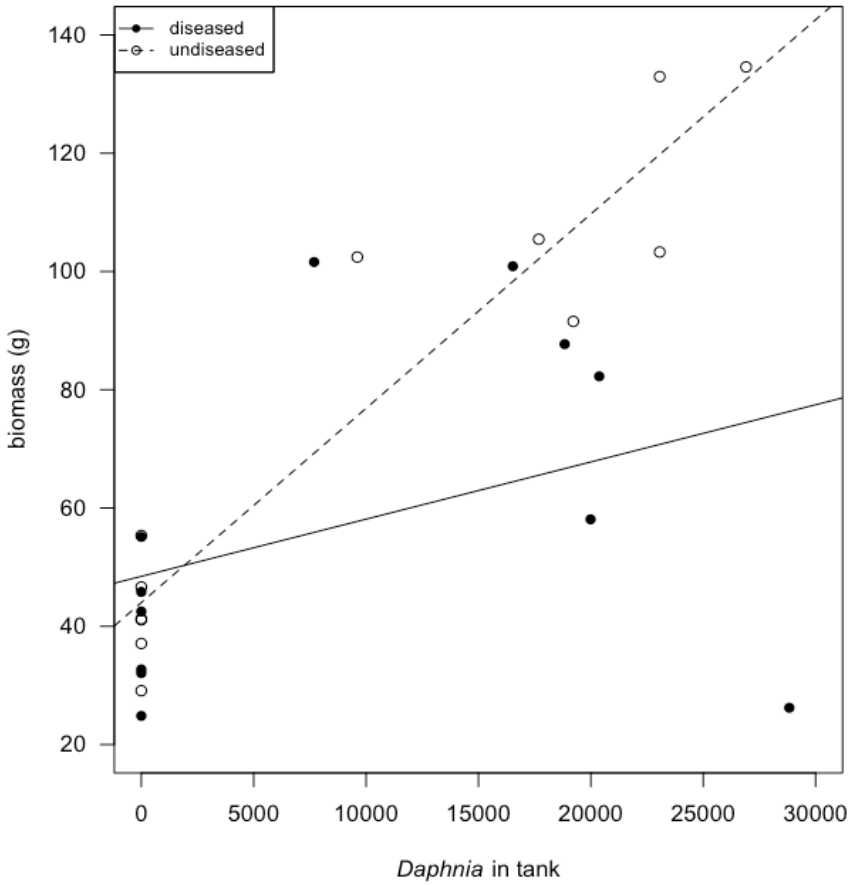

Figure 3. Total *R. aurora* biomass per tank at the end of the experiment, in response to *Daphnia* and Bd-exposure, shown for perspective on the magnitude of effects associated with treatments.

Chapter 3: Temperature mediates interspecific interactions and the effects of the pathogen *Batrachochytrium dendrobatidis* in *Rana aurora* tadpoles

Abstract:

Chytridiomycosis, an emerging infectious disease of amphibians caused by *Batrachochytrium dendrobatidis* (Bd), has caused amphibian population declines and extinctions at a global scale. Global climate change has been implicated as triggering outbreaks of chytridiomycosis, driving observed amphibian declines in parts of the world. Climate change is also predicted to affect a diversity of species by uncoupling the phenology of interacting species.

I manipulated temperature regimes experienced by red-legged frog (*Rana aurora*) tadpoles when exposed to Bd and sympatric Boreal chorus frog (*Pseudacris regilla*) tadpoles in outdoor experimental mesocosms. I found that Bd presence, temperature and chorus frog presence have non-additive impacts on the survivorship, snout-vent-length (SVL) and body condition of *R. aurora* tadpoles. Increased temperatures increased the body condition of *R. aurora* in general, but not when either Bd or *P. regilla* were present, and had little effect on these responses in *P. regilla*. *R. aurora* SVL responded differently to temperature change depending on the presence or absence of *P. regilla*. Both the addition of Bd and increased temperatures uncoupled the development rates of

R. aurora and *P. regilla* additively, with *P. regilla* developing more quickly in both cases. I tested for an influence of temperature and *P. regilla* presence on the transmission of Bd among tadpoles using qPCR, but found none. Infection prevalence of Bd at the end of this experiment was very low, showing little transmission of Bd between tadpoles in mesocosms. Overall, I found that increases in temperature within the Bd thermal optimum can increase the impact of Bd on its host, helping to explain findings that increasing temperatures can trigger outbreaks of chytridiomycosis.

Introduction:

Amphibians are the most threatened class of vertebrates, with over 40% of known species in decline (Stuart et al. 2004). Chytridiomycosis, an emerging infectious disease of amphibians caused by the chytrid fungus *Batrachochytrium dendrobatidis* (Bd) (Berger et al. 1998, Longcore et al. 1999) has been implicated as the cause of ongoing and catastrophic amphibian declines and extinctions (Skerratt et al. 2007, Wake and Vredenburg 2008).

Global climate change has been linked to declines and extinctions involving chytridiomycosis (Pounds et al. 2006, Bosch et al. 2008, Laurance 2008), but the role of climate change in the emergence and epidemiology of chytridiomycosis remains controversial, as studies showing this have been largely correlational. The best studied amphibian collapse linking climate change and the emergence of chytridiomycosis may be confounded with the introduction of Bd in the ecosystem (Pounds et al. 2007, Rohr et al. 2008, Lips et al. 2008). Similarly, laboratory experiments (Longcore et al. 1999,

Piotrowski et al 2004, Berger et al. 2005) and surveys (Berger et al. 1998, Berger et al. 2004, Kriger and Hero 2007, Kriger and Hero 2008) consistently indicate that Bd is expected to be more virulent at cooler (< 23 C) rather than warmer temperatures. Researchers have documented the persistence of amphibian populations in tropical lowlands infected with Bd, while adjacent montane populations at cooler temperatures have been extirpated by chytridiomycosis (Pounds et al. 1999, Puschendorf 2006). Yet, overall, chytridiomycosis outbreaks are often correlated with increasing temperature (Pounds et al. 2006, Bosch et al 2007).

Explanations for this apparent paradox include: increasing cloud cover due to climate change may favor chytrid growth (Pounds et al. 2006, Bosch et al. 2008), increased night-time temperatures may result in temperature convergence around a 'chytrid thermal-optimum' (Pounds et al. 2006), or increased temperature may generally stress amphibians, predisposing them to disease (Alford et al. 2007). Still, Rohr et al. (2008) note that evidence of increasing global temperatures driving chytridiomycosis outbreaks is weak, as many factors that increased concomitant with declines including 'banana and beer production' are better predictors of declines than temperature. Despite a growing literature on correlations between climate change and amphibian declines, there remain few experimental studies demonstrating plausible links between increasing temperature and the emergence of chytridiomycosis.

In addition to affecting disease, climate change may disrupt interactions between species by uncoupling the timing of development of interacting species (reviewed by Parmesan

2006). Bd may also affect species differently depending on their community ecology. For instance, De Castro and Bolker (2005) point out that it is theoretically improbable for a pathogen to drive a host to extinction, and yet extinction of species has been recorded repeatedly from outbreaks of chytridiomycosis (reviewed by Skerratt et al. 2007). Pathogen transmission is generally expected to be density dependent, but when hosts of differing susceptibilities to a disease are present in a community, more resistant species may act as disease reservoirs, enabling the extinction of more susceptible species (De Castro and Bolker 2005). On shorter timescales, such as the devopmental periods of tadpoles, differences between individuals in disease susceptibility are important in determining the spread of a disease in a population (De Castro and Bolker 2005). The species composing a community may therefore be critical in determining the outcome of climate change on amphibian communities, the effects of chytridiomycosis, and interactions between the two.

In British Columbia, the red-legged frog, *Rana aurora*, is in decline (BC Ministry of Environment, Conservation Data Centre), and is commonly infected with Bd (Govindarajulu, pers. comm.). Congeners such as *Rana muscosa*, the yellow-legged frog, are highly susceptible to chytridiomycosis (Rachowicz et al. 2006). The Boreal chorus frog, *Pseudacris regilla,* is sympatric with the red-legged frog and is resistant to chytridiomycosis (Blaustein et al. 2005, Garcia et al. 2006), and populations are stable in BC.

To experimentally assess the interacting roles of climate and amphibian community composition on the effects of Bd in *R. aurora* communities, I reared tadpoles in a factorial mesocosm experiment, where I manipulated temperature, chorus frog presence and exposure to Bd. I measured the survivorship and growth of tadpoles. I assessed the infection prevalence of Bd at the end of the experiment using quantitative PCR to examine how temperature and community composition affect the transmission of Bd.

Methods:

To assess the influence of temperature and the presence of chorus frog larvae in Bd-infected and uninfected *R. aurora* communities, I established mesocosms in 1.6 m diameter round polyethylene tanks ('cattle tanks') in a fenced field on the University of Victoria grounds. Using a factorial design, I subjected each tank to one of eight possible treatments (Table 1). I replicated each treatment six times, for a total of 48 tanks, arrayed in a randomized complete block design. Treatments were blocked with respect to a line of trees at one edge of the field that I felt might influence light availability in tanks. This block effect was non-significant and excluded from the final models.

Prior to the experiment, I disinfected tanks with a 10% household bleach (NaOCl) solution, rinsed them thoroughly and air-dried them. I filled tanks to a depth of 33 cm with tap water in April and allowed them to stand for three days prior to adding 1 Kg of autoclaved leaf litter (primarily *Acer macrophyllum*), 50g of Hagen® rabbit pellets, and a 0.5 mL aliquot of concentrated *Chlorella vulgaris* algae. Beginning on May 3, and continuing every ten days for the duration of the experiment, I added $NaNO_3$ and K_3PO_4

at an atomic ratio of 40:1 N:P to a concentration of 33 µg $P \cdot L^{-1}$ to discourage the growth of toxic cyanobacteria (Anholt 1994). I added lab-reared *Daphnia* to all tanks on May 7, adding ~15 large *Daphnia magna* and 30 *Daphnia pulex* to each tank. Although similar mesocosm studies generally use wild-collected plankton to establish aquatic communities, I avoided this approach as I was concerned with introducing Bd into tanks unintentionally (Johnson et al. 2003, Walker et al. 2007). Throughout the experiment, I kept tanks securely covered with 40% shade cloth affixed to weighted lids to prevent predation on tadpoles, insect oviposition in tanks, and overheating.

I collected nine *R. aurora* egg masses from a pond near Bamfield, B.C. (48º 58' 46" N, 125º 7' 16" W), and five near Port Renfrew, B.C. (46º 29' 43" N, 124º 16'1 8" W) in March 2009. I mixed egg clutches, and kept eggs outdoors in shaded plastic wading pools (hereafter, 'rearing tanks') until eggs had hatched and tadpoles had developed to the actively foraging Gosner stage 25 (Gosner 1960). I collected 15 *P. regilla* egg masses from Sooke, B.C. (48º 20' 13" N, 128º 38' 37" W) in early April, and raised them under the same conditions in separate tanks. Once tadpoles had reached Gosner stage 25, I added rabbit pellets to rearing tanks to provide food. On May 5 I added *R. aurora* tadpoles to the mesocosms; 55 tadpoles were added to treatments with only *R. aurora* and 25 tadpoles were added to treatment tanks that included chorus frog larvae.

As differences in the virulence of Bd isolates have been observed (Fisher et al 2009), I isolated Bd locally from an infected bullfrog (*Lithobates catesbeianus*) tadpole captured near Nanaimo, BC following the methods of Longcore et al. (1999). The identity of the

isolate as Bd was confirmed microscopically and using qPCR (named PTH 001; Boyle et al. 2004)

I produced Bd zoospores by growing Bd for 3-5 days on 1% tryptone agar, flooding plates with NAYA® water and collecting the zoospore-containing supernatant (Longcore et al. 1999). I quantified zoospores in the supernatant by counting on a hemocytometer slide after staining 1:1 with Lugol's solution.

To generate Bd treatment tanks, I infected five tadpoles by directly exposing them to Bd zoospores for 10 days. Exposed tadpoles were held in 12 L tanks at a density of 15 per tank. I added ~5 x 10^7 Bd zoospores to each 12 L tank every other day for ten days. I fed tadpoles ground rabbit pellets *ad libitum* during this time.

At the end of the exposure period, I confirmed successful Bd infection in a sample of exposed tadpoles using qPCR (70% infection; n = 10). I added either five *R. aurora* tadpoles from the Bd-infected group or five uninfected tadpoles from the rearing tanks (0% infection based on qPCR, n=20) (June 2) to Bd-present and Bd absent treatments, respectively. I marked all tadpoles added at this time with an injection of fluorescent elastomer to allow later identification. This resulted in 60 total tadpoles total per tank.

To generate a temperature effect, half of the mesocosm tanks were buried 40 cm in the ground (Table 1) to insulate them from the higher and more variable air temperature over the course of the experiment, creating the low temperature treatment. The high

temperature treatment tanks remained above ground. I recorded temperature differences between treatments with iButton (Maxim Integrated Products, Inc.) temperature loggers that recorded the temperature hourly at the bottom eastern edge of each tank, beginning shortly before the introduction of tadpoles to tanks.

I censused *Daphnia* in tanks on July 1 by dropping a 7.5 cm diameter 250 μm collecting sieve to the bottom of tanks, waiting 10 s, and drawing the sieve to the surface. Samples were collected from the east side of tanks, in the shade. I flushed *Daphnia* from the sieve with distilled water and returned samples to the lab for counting under a dissecting microscope.

Chorus frog tadpoles began to metamorphose the last week of June. I checked tanks every other day for chorus frog metamorphs and removed and euthanized metamorphs to prevent them escaping tanks, although they appeared unable to escape tank covers. I ended the experiment on July 3 when some *R. aurora* tadpoles in the majority of tanks had begun to metamorphose (one forelimb present). Over the course of three days, all tadpoles from tanks were removed with a dip net and euthanized with an overdose of buffered MS-222 (Tricaine methanesulfonate). I disinfected dipnets with 10% bleach solution between tanks, and stored euthanized tadpoles on ice in individual polyethylene bags until returning them to the laboratory and freezing them at –20 °C. Treatment tanks had a large amount of organic material that was mixed by dip-netting, making it impossible to see the tank bottom and some tadpoles were therefore missed during the initial dip netting. These tadpoles (0.43 ± 0.069, mean ± SE per tank) were collected on

subsequent days, and were included in the analysis of survivorship, but excluded from growth rate analysis as they spent longer in tanks.

For each tadpole collected at the end of the experiment, I recorded Gosner stage (Gosner 1960), mass, and snout-vent length (SVL) while frozen. For all tadpoles in Bd-present treatments and a random subsample of tadpoles (up to 10 per tank, depending on treatment, Gosner stages 36-41) from Bd-absent tanks, I dissected mouthparts (Gosner stage <42) or took toe-clips under sterile conditions (Gosner stage ≥42) for qPCR detection of Bd. The majority of DNA samples from one infected tank, as well as ten samples from another infected tank were lost due to error, so these tanks were excluded from analyses on Bd transmission. All analyses were done on means of response variables for each tank.

Bd detection

I tested for the presence of Bd using quantitative real-time PCR after Boyle et al. (2004). Briefly, I extracted DNA from dissected mouthparts of tadpoles or toe clips of metamorphs by combining frog parts with 40 µL PrepMan Ultra (Applied Biosystems) and 30 mg silica/zirconium beads in a 2 mL cryotube and disrupting samples in a MiniBeadbeater (Biospec Products). Samples were homogenized for 45 s in the Beadbeater, centrifuged (13 000g x 30 s), homogenized and centrifuged again and incubated at 100 C for 10 minutes. I cooled samples to room temperature, centrifuged samples (13 000g x 3 min), and pipetted off 20 µL of the DNA-containing supernatant.

I detected Bd DNA in samples using TaqMan qPCR in a Stratagene MX4000 system with the primers ITS1-3 (5'-CCT TGA TAT AAT ACA GTG TGC CAT ATG TCC-3'), 5.8S Chytr (5'-AGC CAA GAG ATC CGT TGT CAA A-3') and the minor groove binding probe Chytr MGB2 (5'- 6FAM CGA GTC GAA CAA AAT MGBNFQ –3') (Applied Biosystems; Boyle et al. 2004) in 25 µL reactions (12.5 µL TaqMan Master Mix (Applied Biosystems), 0.45 µL of primers (900 nM), 0.625 µL of MGB probe (250 nM) 5.975 µL water and 5 µL DNA (diluted 10^{-1}). I used a temperature profile of 2 minutes at 50°C, 10 min at 95°C followed by 50 cycles of 15 sec at 95°C and 1 min at 60°C (Boyle et al. 2004). I included positive controls of Bd (isolate PTH 001 or JEL 423) as well as negative controls during DNA extraction and qPCR.

Data Analysis

I analyzed survival of *R. aurora* at the end of the experiment first using a generalized linear model with quasibinomial errors to account for overdispersion. The data were overdispersed (dispersion parameter = 7.39), indicating that extra-binomial variation in survival may have been due to unmeasured factors or poor model performance (Crawley 2005). However, tadpole survival data collected from tank means is typically overdispersed (Eberhardt 1978, Govindarajulu 2004). Still, because of a lack of confidence in the GLM due to overdispersion, I evaluated treatment effects using a randomization test on ANOVA, comparing observed treatment F-values to empirical distributions derived from 10 000 randomizations of the data (Manly 2001). All analyses were done using R v.2.9.2. (http://cran.r-project.org).

I used a substitutive design for this experiment in that I removed *R. aurora* tadpoles from treatments that had chorus frogs added. This design is sometimes considered inferior to additive designs in which the number or *R. aurora* would be kept constant for measuring the effects of interspecific competition, in that the effect of chorus frogs is confounded with *R. aurora* density (Snaydon 1991, Joliffe 2000, but see Cousens and O'Neill 1993). In this experiment, using an additive design was not possible as it would have increased the density of Bd-susceptible hosts (McCallum et al. 2001) and made meaningful comparisons of differences in infection rates across treatments impossible. Also, survival in tanks was not complete, so density dependent effects had the potential to confound analyses irrespective of the design. To address these issues, I included the number of surviving *R. aurora* as a covariate in analyses of SVL and body condition.

Similar mesocosm studies to this one are generally stopped as each tadpole reaches metamorphosis (e.g. Boone et al. 2007). Because I stopped this experiment with tadpoles at various stages, I controlled for the effect of stage by doing analyses of SVL and mass on the residuals of a generalized additive model of SVL or mass against Gosner stage.

I analyzed SVL using analysis of covariance (ANCOVA). For SVL and body condition, I included number of *Daphnia* in tanks as a covariate in ANCOVA initially, as I expected *Daphnia* abundance would explain variation in this responses (Chapter 2). As the three-way interaction term between experimental factors was non-significant, I simplified the model using the *step()* function in R which sequentially removes parameters from a model if removal results in an improved (lower) AIC score.

I analyzed *R. aurora* body condition in tanks by analyzing the effect of treatments on tadpole mass while including SVL as a covariate in ANCOVA (Garcia-Berthou 2001) and simplified the initial model using the *step()* function in R. Because I analyzed data from the same individuals in multiple analyses (survival, SVL and condition), I Bonferroni corrected the significance level of tests and evaluated significance at $P < 0.0167$. I also tested the response of *P. regilla* survival, SVL and body condition to treatments.

Although there was no clear effect of treatments on developmental stages of *R. aurora* in tanks (ANCOVA, $P > 0.05$), treatments appeared to affect the development of chorus frogs. For each tank containing chorus frogs, I subtracted the mean Gosner stage of red-legged frogs from the stage of chorus frogs to see if treatments affected the development of species differently. I included chorus frog metamorphs removed prior to the end of experiments in this analysis as Gosner stage 46 (Gosner 1960). I tested the effects of temperature and Bd presence while controlling for *R. aurora* survival on these differences using ANCOVA, followed by model simplification based on AIC.

Results:

Burying tanks successfully produced measurable differences in temperatures of tanks (Figure 1). Bd treatments were also effective in that infected tadpoles were detected in Bd-present treatment tanks and no infected tadpoles were found in Bd-absent treatments, indicating that cross-contamination did not occur.

The overall survival of *R. aurora* tadpoles in this study was 69%. There was a significant three-way interaction between temperature, disease and chorus frog presence on *R. aurora* survival (ANOVA Randomization F = 10.68, P = 0.002, Table 2). This three-way interaction occurred because in the presence of Bd survival was largely unaffected by the temperature treatment, but in the absence of Bd survival increased at high temperature when chorus frogs were absent and decreased at high temperature when chorus frogs were present (Figure 2). However, the overall explanatory ability of the model was low (adjusted $R^2 = 0.10$), suggesting that unmeasured or random sources of variation contributed much more to survivorship than treatments, possibly explaining the overdispersion of the GLM.

SVL was greater in the high temperature treatment relative to the low temperature treatment when chorus frogs were absent, but when chorus frogs were present this pattern was reversed (Figure 3A) (ANCOVA, temperature x chorus frog interaction $F_{1,43} = 7.52$, P = 0.009; Table 3). An effect of Bd on SVL was eliminated from the model during simplification. Survival, as a covariate, was highly significant as expected, while the *Daphnia* covariate was eliminated from the model.

Body condition of *R. aurora* tadpoles increased substantially when chorus frog tadpoles were added to tanks (Table 4) (ANCOVA, chorus frog main effect, $F_{1,39} = 49.41$, P <0.001, Figure 3C). There was a significant interaction between temperature and chorus frog presence ($F_{1,39}$=10.41, P = 0.003). In the absence of chorus frogs, body condition was greater in the high temperature treatment, but when chorus frogs were present body

condition was higher in the low temperature treatment (Figure 3C). There was also a significant interaction between temperature and Bd presence ($F_{1,39}$=8.87, P = 0.005). Body condition increased in the high temperature treatment with Bd absent, but decreased in the high temperature treatment when Bd was present (Figure 3B). Covariates of number of *Daphnia*, *R. aurora* survival and SVL were highly significant and/or retained in the model, as expected. Chorus frog survival was unaffected by treatments (GLM, P > 0.05 for all effects).

Both the presence of Bd and the high temperature treatments increased the developmental stage of chorus frogs at the end of the experiment (Table 5, Figure 4). When comparing the mean Gosner stage of chorus frogs in a tank to that of red-legged frogs, it is clear that Bd ($F_{1,20}$ = 5.51, P = 0.029), and temperature ($F_{1,20}$ = 9.17, P = 0.006) both had positive effects on chorus frog but not red-legged frog development (Table 4; controlling for *R. aurora* survivorship). As early-metamorphosing chorus frogs were included in the analysis at only stage 46, this estimate is likely conservative as well.

Temperature Effects

Although the intent of burying tanks was to achieve an overall decrease in temperature, the effects of burying tanks on temperature were ultimately more complex (Figure 1). To investigate the aspects of temperature differences that were most important in driving observed changes, I calculated four descriptors of temperature for each tank, each adapted from hypotheses that have been proposed to explain the epidemiology of chytridiomycosis: mean temperature (Berger et al. 1998, Pounds et al. 2006), temperature

variability (chytrid thermal-optimum hypothesis; Pounds et al. 2006), hours at "high" temperatures (> 20°C; Ron et al. 2005) and hours at "low" temperatures (< 14°C) (Muths et al. 2008). I substituted each of these predictors in the place of the dichotomous temperature predictor in the final models selected for each response variable above. I compared AICc scores of these new models using AICc weights (ω-AICc; Burnham and Anderson 1998). For survival, I calculated AICc based on ANOVA, as the model residuals were normally distributed. Temperature loggers failed to record in three tanks, and these tanks were excluded from this analysis.

In general, AICc was able to distinguish the aspects of tank temperature that best predicted the observed responses to treatments. Survival was best predicted by the variation in temperature within tanks (ω-AICc = 69%), while SVL and body condition were better explained by hours above 20 C (ω-AICc = 46% and 66% respectively). Although the performance of these predictors could be ranked clearly amongst themselves, their performance was generally poorer than the performance of the dichotomous predictor in the model (i.e. higher AICc scores; Table 6). This is unsurprising given that the model was originally optimized based on the dichotomous predictor.

Bd Transmission

Rates of Bd infection were very low at the end of the experiment (mean prevalence ± SE = 2.7 ± 0.6 % for chorus frogs and *R. aurora* pooled), and the prevalence in *R. aurora* did

not differ with the temperature and chorus frog treatments (binomial GLM, P >0.5). No tadpoles tested positive in some initially exposed tanks (5/24 infected tanks). No tadpoles from Bd-absent control tanks tested positive for Bd, and no chorus frogs tested for Bd were positive.

Discussion

The impacts of Bd on *R. aurora* were contingent on the temperature treatment, with tadpole body condition negatively affected by the presence of Bd at higher, more variable temperatures. This divergence in the effects of Bd in tadpoles at different temperatures suggest that the balance between the *R. aurora* host and Bd pathogen is sensitive to the small changes in temperature applied here, with warmer temperatures favouring the pathogen.

R. aurora generally had greater survival in the high temperature treatments, although this effect was reversed in the presence of either Bd or chorus frogs. Although parameter estimates for these effects are significant, they represent relatively small effect sizes – the temperature treatment increased the mean survival of tadpoles by only 1%, and even interactions between disease and temperature resulted in only 5% differences in survival between treatments (Figure 5). This underscores the inherent variability in survival of tadpoles (Alford 1999), and that the treatments applied here did not have very large effects on survival relative to other measured responses. These effects were small enough that treatments were essentially indistinguishable using ANOVA.

Other researchers have found that including heterospecifics in amphibian communities may mediate the effects of Bd in the system, presumably through increasing competitive stress or shifting the strength of competitive interactions (Parris and Cornelius 2004). This experiment used a competitor species (*P. regilla*) that puts minimal competitive stress on *R. aurora* (Govindarajulu 2004), aiming to test the effects of mixed communities on Bd transmission instead of more classic interspecific competitive effects. However, for both SVL and body condition, *R .aurora* performed better at low than high temperatures in the presence of chorus frogs, suggesting that temperature in fact mediates aspects of competition between the two species.

There is increasing evidence that climate change is having unanticipated consequences by uncoupling the phenology of interacting species (reviewed by Parmesan 2006). Chorus frogs typically develop more quickly than *R. aurora*, but the experimental treatments applied differences in development rates. The addition of Bd to tanks also increased the development of chorus frogs relative to red-legged frogs. Bd may therefore have unanticipated effects in structuring amphibian communities and shaping interactions between species even when mortality is low.

The very low transmission rates of Bd throughout this study are consistent with the findings of the similar experiment discussed in Chapter 2, but are inconsistent with the dominant paradigm of Bd as highly infectious at all amphibian life history stages (e.g. Rachowicz and Briggs 2007). Possible explanations for this (e.g. low density of tadpoles used here, tadpoles as poor Bd vectors) are discussed in the previous chapter, but it is

worth noting that it is unlikely that temperature in the tanks limited the spread of Bd. Laboratory studies show optimal growth of Bd up to 25°C (Piotrowski et al. 2004), and temperatures in this study were always below this, at least near the tank bottoms. Basking by tadpoles on the surface may have exposed them to higher temperatures, but the lack of a clear effect of the temperature treatment on Bd transmission suggests temperature played a minimal role in the spread of Bd at this scale. There was little evidence that the inclusion of chorus frogs affected Bd transmission, although it was noteworthy that no chorus frogs became infected over the course of the experiment. It may be that chorus frogs are not only relatively resistant to chytridiomycosis, but to Bd infection as well, although this is difficult to infer from the generally low transmission rates observed here.

Despite the lack of discernible effects of temperature on the transmission of Bd, *R. aurora* tadpoles were clearly affected by the fungus. However, the addition of Bd to mesocosms had per-capita impacts on the development rates and body condition of tadpoles when infection prevalence was very low (i.e. few infected individuals contributed to mean scores). This suggests that Bd may behave as a stressor to *R. aurora* (Parris and Cornelius 2004), in addition to pathologies associated with infection (e.g. reduced foraging ability due to degradation of infected mouthparts; Berger et al. 1998), affecting the fitness of tadpoles that are not ultimately infected. This result is not without precedent; potentially, this stress could arise from failed colonization attempts by Bd that may require an energetically costly immune defence by tadpoles (Garner et al. 2009).

The results of this study differ from similar studies (Parris and Beaudoin 2004, Parris and Cornelius 2004) in that the introduction of Bd in mesocosms had apparently positive effects on some species (i.e. chorus frogs). This is not completely surprising, as experimental infections have failed to find effects of Bd infection on both tadpole and post-metamorphic *P. regilla* (Blaustein et al. 2005, Garcia et al. 2006). Because of this, it may be that Bd disproportionately affects species and shifts the strengths of interspecific competitive effects. In wild systems, there is evidence of chytridiomycosis facilitating the expansion of some species at the expense of others (i.e. the expansion of *Bufo bufo* populations at the expense of *Alytes obstetricans;* Bosch and Rincon 2008). This study suggests similar processes affect tadpole communities, but these processes may be less obvious than those affecting more susceptible life history stages, particularly over short timescales.

Throughout this experiment, water temperatures were mostly within the thermal optimum predicted for Bd growth by others (Piotrowski et al. 2004). Still, marginal increases in the mean temperature and the variation in temperature, within this Bd-optimum, changed the impact of Bd on the focal host, *R. aurora*. These findings help to explain the apparent paradox associated with the emergence of chytridiomycosis at higher temperatures. This study affirms that it is unlikely that laboratory experiments examining large changes in temperature on the host and pathogen alone will be able to completely explain the ecological and epidemiological impacts of climate change. I suggest that larger scale experimental manipulations, such as this one, are necessary to more

realistically predict the factors that contribute to the emergence of Bd as a mass killer of amphibians.

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Appendix : Tables and Figures

Table 1. Treatment combinations. Bd presence is crossed with low and high temperatures. For each of these combinations, there are treatments with 60 red-legged frogs (RF), or a mix of 30 RF and 30 chorus frogs (CF): a total of eight possible treatments.

	Bd absent		**Bd Present**	
	RF	**CF**	**RF**	**CF**
Low Temp	60	0	60	0
	30	30	30	30
High Temp	60	0	60	0
	30	30	30	30

Table 2. ANOVA of effects of treatments on proportion of *R. aurora* surviving to the end of experiment. P-values were generated using a randomization test.

Source of Variation	**df**	**F**	**P** (randomized)
Temp	1	0.04	0.84
CF	1	0.09	0.765
Bd	1	0.46	0.507
Temp x CF	1	1.96	0.173
Temp x Bd	1	0.08	0.791
TF x Bd	1	0.77	0.387
Temp x CF x Bd	1	8.85	**0.0043**
Residuals	40		

Table 3. ANCOVA results for the effects of temperature, chorus frogs and survival on *R. aurora* SVL (controlled for stage using GAM residuals). The effect of Bd was non-significant and removed from the model.

Source of Variation	**df**	**F**	**P**
Temp	1	0.65	0.420
CF	1	0.76	0.390
Survival	1	25.31	**<0.001**
Temp x CF	1	7.52	**0.009**
Residuals	43		

Table 4. ANCOVA results for the effects of temperature, chorus frogs and Bd on *R. aurora* body condition (mass ~ SVL; controlled for stage using GAM residuals). *R. aurora* survival has been included in the model to measure density dependence.

Source of Variation	df	F	P
Temp	1	0.01	0.912
Bd	1	0.35	0.558
CF	1	49.41	**<0.001**
Survival	1	32.72	**<0.001**
SVL	1	50.19	**<0.001**
Daphnia	1	0.88	0.353
Temp x Bd	1	8.87	**0.005**
Temp x CF	1	10.41	**0.003**
Residuals	39		

Table 5. ANCOVA results for the effects of treatments on difference between *P. regilla* and *R. aurora* developmental stages in tanks at the end of the experiment.

Source of Variation	df	F	P
Bd	1	5.51	**0.029**
Temp	1	9.17	**0.007**
Survival (RF)	1	3.67	0.070
Residuals	20		

Table 6. AICc and AICc-weights of models with different indices of temperature as explanatory variables. Initial AICc is the AICc of the model with the dichotomous temperature predictor. Mean is the mean of the temperature over the course of the experiment, while SD is the temperature standard deviation. >20°C and <14°C represent hours above and below 20°C and 14°C respectively.

Parameter	**AICc**	**ΔAICc**	**w-AICc**
Survival (Initial AICc= -138.0)			
SD	-138.8	0	0.69
>20 C°C	-136.96	1.84	0.28
<14 C°C	-132.23	6.57	0.03
Mean	-129.96	8.84	0.00
SVL (Initial AICc= -181.42)			
>20°C	-178.08	0	0.46
Mean	-177.64	0.44	0.37
SD	-175.71	2.37	0.14
<14°C	-173.14	4.94	0.04
Body Condition (Initial AICc= -128.77)			
>20°C	-119.6	0	0.66
SD	-117.94	1.66	0.29

Mean	-113.28	6.32	0.03
<14°C	-112.69	6.91	0.02

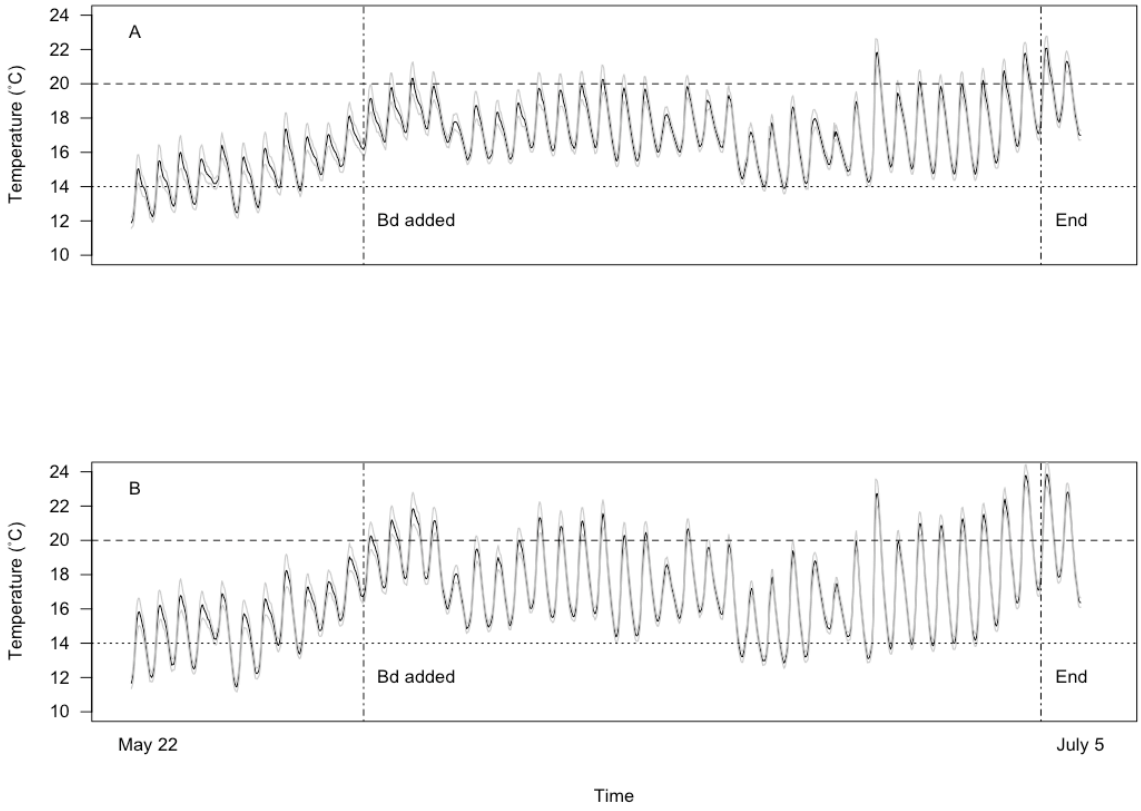

Figure 1. Temperature of water in tanks during experiment for (A) buried tanks and (B) unburied tanks. Black lines represent the means of all tanks for a treatment, while grey lines represent the standard error of the mean. 20°C and 14°C are marked with dashed and dotted lines respectively.

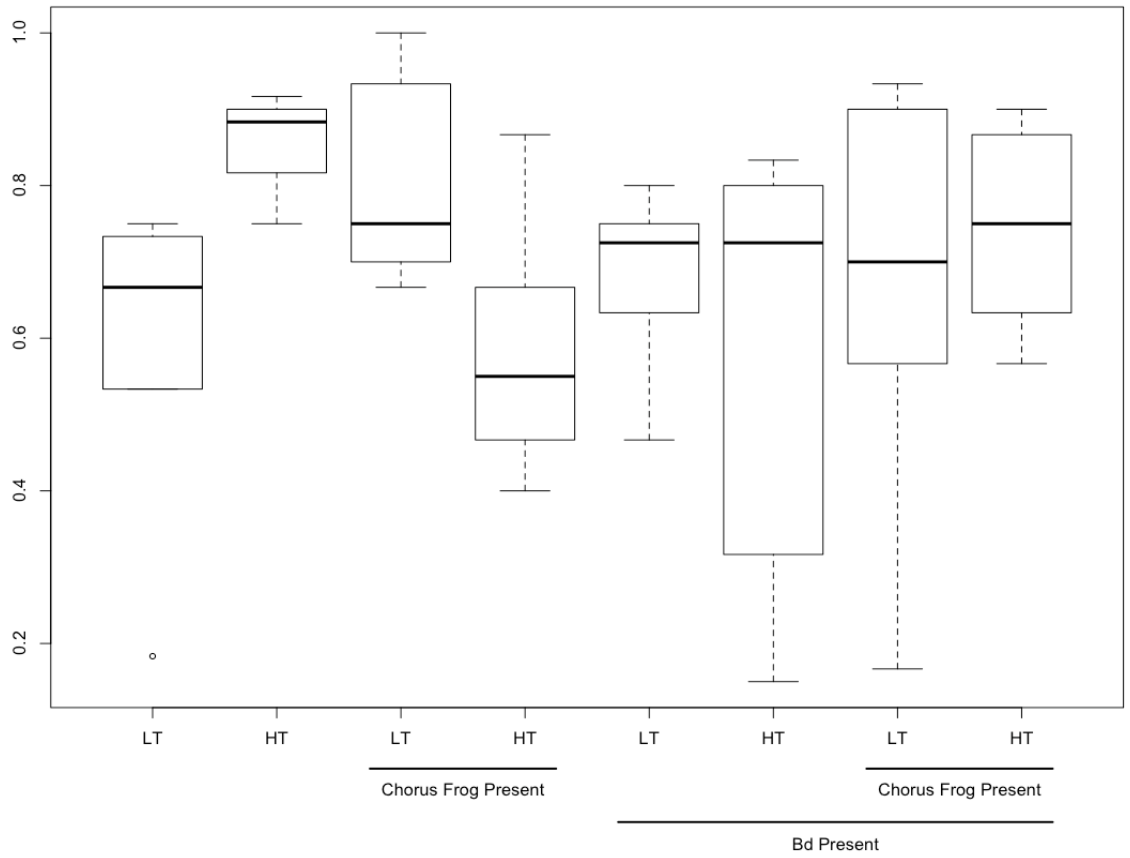

Figure 2. Proportion of *R. aurora* surviving to end of experiment (out of 60, or 30 in chorus frog present treatments). LT and HT denote low and high temperatures treatments respectively.

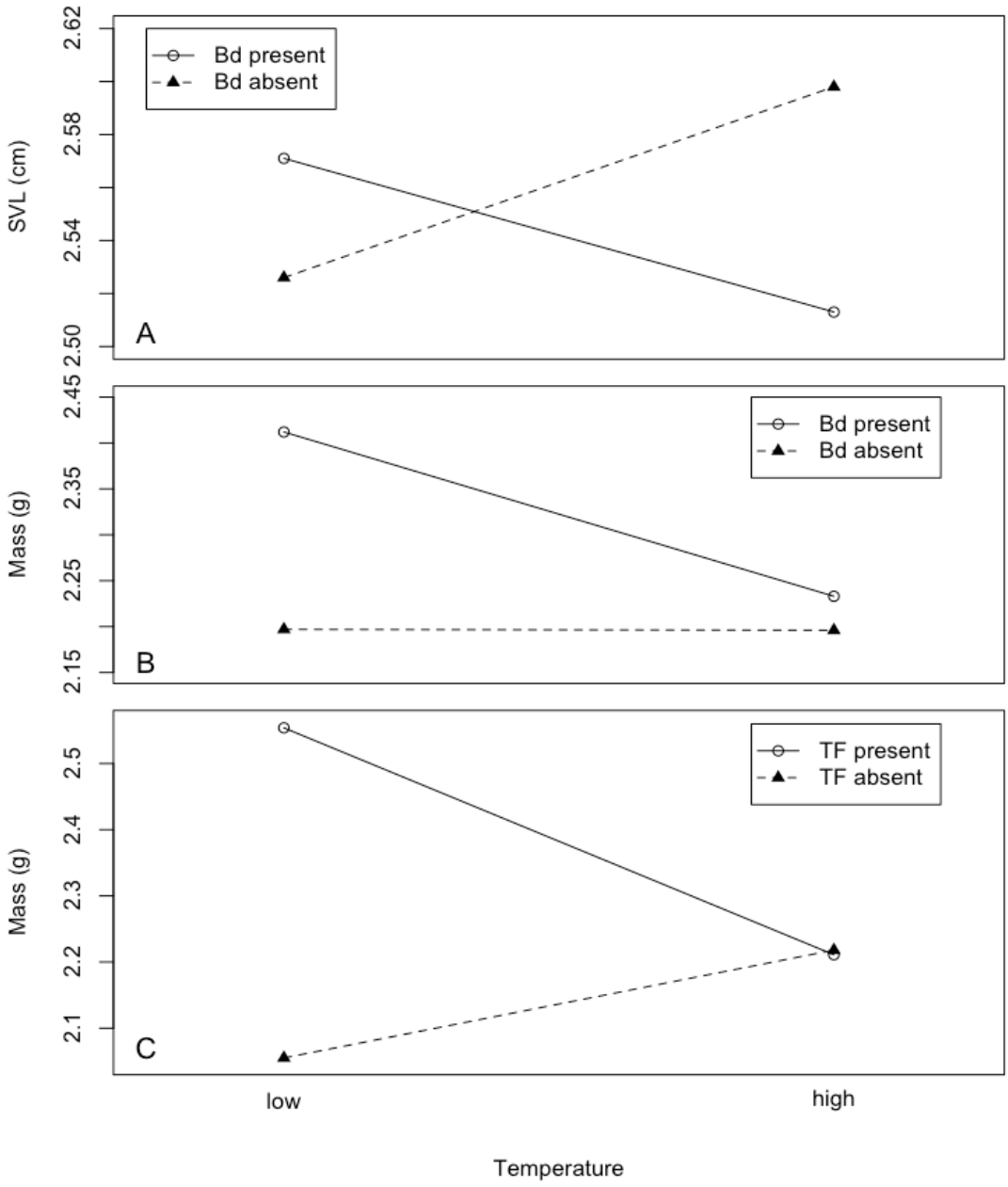

Figure 3. Two way interactions between experimental factors on tadpole SVL and mass. (A) Interaction between temperature and the presence of Bd on SVL. (B) Interaction between temperature and Bd and (C) temperature and Chorus frog presence on mass. Data shown are uncontrolled for tadpole stage, and in the case of mass, SVL (as analyses of body condition are done on mass ~ SVL). When including these factors, interactions are qualitatively similar but become stronger.

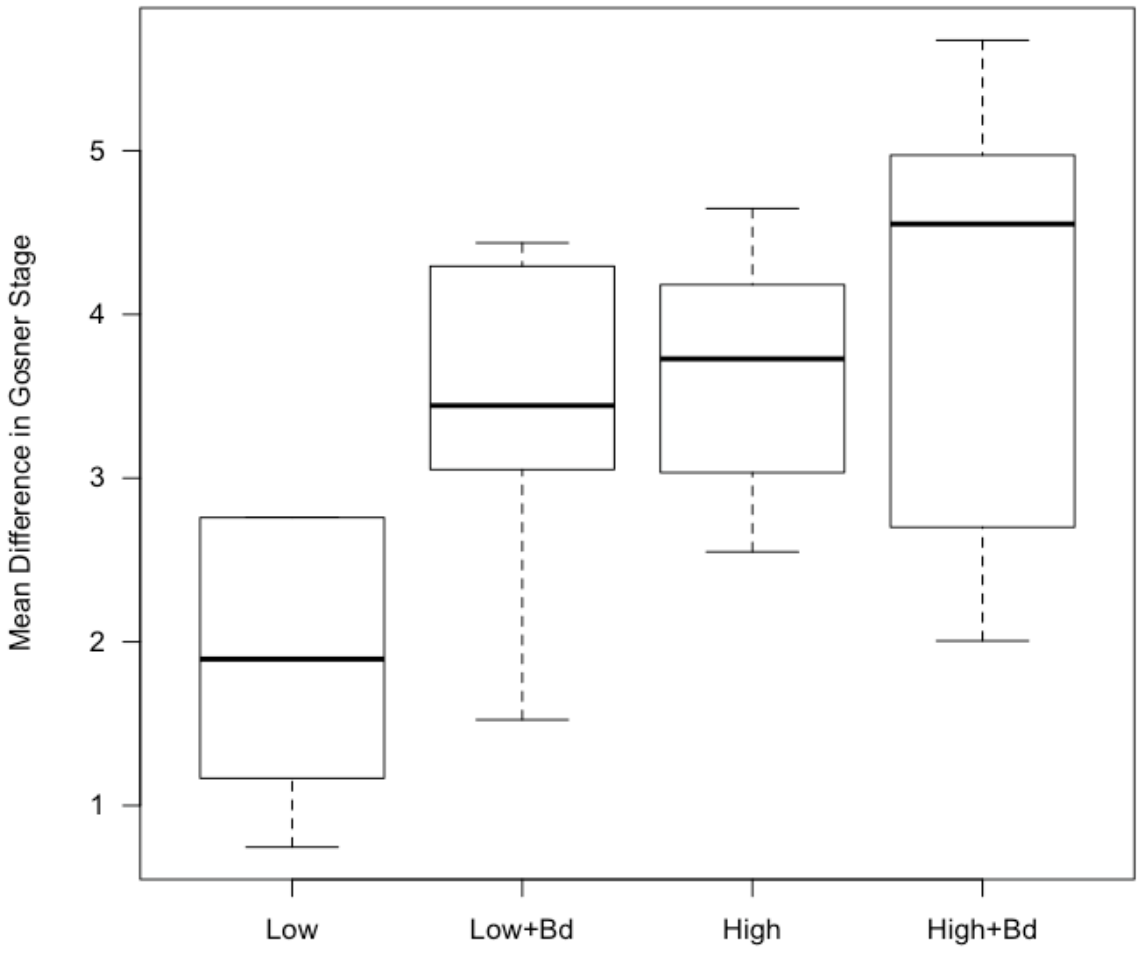

Figure 4. Box-plot of mean differences between *P. regilla* and *R. aurora* (Gosner stage of *P. regilla* - *R. aurora*) Gosner stages in treatments (N = 24). The overall mean *R. aurora* is stage 39.

Batrachochytrium dendrobatidis and *Rana aurora*: Effects of environmental factors in mesocosms

The expression and effects of disease in an ecological community are complex (Casadevall and Pirofski 2003, Beldomenico and Begon 2010), and chytridiomycosis is no exception. Although early reports of chytridiomycosis focused on mass-die offs of amphibians (Berger et al. 1998, Bosch et al. 2001, Lips et al. 2006, Rachowicz et al. 2006) evidence is accumulating that Bd remains, endemic in communities, after initial declines (Retallick et al. 2004, Puschendorf 2006,), and over time can contribute to the decline of initially resistant species (Garner et al. 2009).

Viewing Bd as a novel infectious disease that spreads rapidly may allow the best management strategies in parts of the world where there are high numbers of potentially susceptible species and Bd has not been detected (e.g. Kriger and Hero 2009). However, in many parts of the world Bd is present, and the protection of these ecosystems requires no less priority. The studies presented here studies confirm a role of the environment in the outcome of Bd infection.

In laboratory and mesocosm studies at the tadpole stage, Bd infection has been shown to have generally subtle effects relative to the high mortality rates observed in post-metamorphic stages. These range from little discernible effect (Rachowicz and Vredenburg 2004) to decreased mass at metamorphosis (Parris and Beaudoin 2004, Parris and Cornelius 2004), increased time to metamorphosis (Parris and Baud 2004), and

increased mortality (Blaustein et al. 2005, Garner et al. 2009), with effects varying by species.

In the studies presented here, tadpoles responded similarly to the presence of Bd throughout the experiments. In Chapter 2, Bd exposure resulted in increased body length in *Rana aurora* tadpoles, but increased mortality when *Daphnia* were present. These results were similar in the larger experiment described in Chapter 3, although the presence of Bd interacted with the different experimental factors: temperature and the presence of chorus frogs. These experiments ended before all *R. aurora* had metamorphosed. Metamorphosis is energetically costly (Steiner and Van Buskirk 2008) and small body size may lead to mortality during or shortly after metamorphosis (Altwegg 2002a, Altwegg 2002b). These results may therefore be conservative with respect to mortality that ultimately results from both Bd exposure and the absence of *Daphnia* to *R. aurora*.

It is difficult to infer the ecological and evolutionary effects of Bd exposure from these experiments, but Bd presence in *R. aurora* tadpole communities incurred per-capita fitness costs, even in the absence of direct infection of all individuals. This result is initially counterintuitive, but it is supported by other studies (Garner et al. 2009) in which Bd exposure results in life history tradeoffs that decrease the fitness of tadpoles and impact individuals in a population that are not ultimately infected. Over multiple generations, these fitness costs may accrue and lead to the overall decline of populations

that initially appeared resistant to Bd infection (i.e. declines of the common toad, *Bufo bufo*, in central Spain; Bosch et al. 2007, Garner et al. 2009).

The low Bd transmission rates in this experiment were unexpected. Potential reasons for them are discussed in more detail in preceding chapters. Bd is considered highly infectious to all amphibian life history stages (Rachowicz and Vredenburg 2004, Rachowicz and Briggs 2007, Kriger and Hero 2009), but it is clear that it does not transmit easily under all conditions. While preventing the introduction of Bd to naïve ecosystems remains a conservation concern, contrasting these studies with other mesocosm studies on Bd (Parris and Beaudoin 2004, Parris and Cornelius 2004) demonstrates that the method of Bd introduction into a study system is important. Metamorphosed amphibians appear to be much better at disseminating Bd than tadpoles. This is consistent with current understanding of Bd growth physiology, in that keratinized tissue is limited in tadpoles (Piotrowski et al. 2004). However, studies examining the spread of the disease through communities should make explicit the assumptions supporting different methods of Bd infection in experiments, and how these bear on the hypotheses being tested. Similarly if Bd infection prevalence is related to the presence of multiple life-history stages in a community, the demographics of amphibian populations may critical in determining the resilience of species to Bd threats.

The importance of *Daphnia* to *R. aurora* tadpoles was clearly demonstrated in Chapter 2. *Daphnia* are considered keystone species in some aquatic ecosystems, and I propose that they represent keystone species in amphibian tadpole communities as well. Inclusion of

Daphnia markedly increased the size and body condition of tadpoles, beyond even what was expected based on mesocosm studies of others (e.g. Relyea and Diecks 2008). High rates of tadpole feeding on *Daphnia* were evident from the experiment, and counter to the prevailing view of anuran tadpoles as predominately herbivorous (Alford 1999). The perception of anuran tadpoles as herbivores is incorrect, at least for *R. aurora*, and other studies suggest this is true for many species (Altig et al. 2007). The interpretation of mesocosm studies in which multiple trophic levels are affected by treatments therefore requires caution. Dramatic tadpole responses to experimental factors that are sometimes observed in mesocosms (Skelly and Kiesecker 2001) may even be due in part to the high numbers of zooplankton that mesocosms support (personal observation). This is supported by studies on wild tadpoles, that also suggest zooplankton are important components of tadpole (Schiesari et al. 2009).

In Chapter 3, temperature influenced important aspects of the growth and development of *R. aurora* tadpoles in the presence of Bd, including their relationship to sympatric chorus frogs. In general, tadpoles benefitted from increasing temperatures, but these benefits were reversed in the presence of Bd, suggesting that increased temperatures within the thermal optimum for Bd (Piotrowski et al. 2004) benefit the fungal pathogen more than the host. This potentially explains how subtle temperature increases from global climate change can paradoxically increase the virulence of Bd infection (Pounds et al. 2006, Bosch et al. 2007, Laurance et al. 2008). Similarly, both increased temperature and Bd benefitted chorus frogs as chorus frogs developed more quickly in the presence of these factors, apparently at the expense of *R. aurora* tadpole growth. The uncoupling of species

level responses to experimental factors suggests that there is little generality in the responses of species, and that the amphibian community will respond as a whole to perturbation from temperature change and Bd presence. This underscores the complexity of a system that I designed specifically to be simple: the response of either species to Bd infection was contingent on all the factors included in the experiment.

Altogether these studies demonstrate links between environmental factors and the outcome of *R. aurora* exposure to Bd in mesocosms. Complex interactions between multiple factors mediated the host response to exposure, and more thorough understanding of the importance of these factors will be necessary to adequately protect declining amphibian studies. Further studies would benefit from following these animals post-metamorphosis. There is a paucity of literature tying tadpole characteristics to performance at later life history stages (Smith 1987, Semlitsch et al. 1988, Alford 1999, Altwegg 2002, Garner 2009). Still, these studies help interpret increasingly contradictory correlational studies on Bd and on amphibian declines in general.

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