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Effects of Biotic and Abiotic Setting on a Host-Pathogen Relationship: How Environmental and Community Characteristics Influence Infection Prevalence and Intensity of Amphibian Chytrid on California's Central Coast

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by

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Abstract

Effects of Biotic and Abiotic Setting on a Host-Pathogen Relationship:
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In the face of swift anthropogenic change, it is essential to examine the broad ecological context for species of concern using a variety of approaches in order to understand their interactions in a natural context. Host-pathogen relationships offer a close interaction to examine how each are acted upon by biotic and abiotic conditions. *Batrachochytrium dendrobatidis*, an emerging infectious disease of amphibians, has been implicated with wholesale loss and marked declines in amphibian species across the globe, thus understanding its dynamics across amphibian hosts and in complex, natural environments is a key area for conservation focus.

For this dissertation, I tested the importance of various biotic and abiotic factors in the relationship between *B. dendrobatidis* and three co-occurring amphibian hosts, as well as across metrics of host physiological health. In Chapter 1, I ranked environmental conditions that favor *B. dendrobatidis* success in two native and one introduced amphibian tadpole species. I found top models favoring *B. dendrobatidis* infection included: a) a positive relationship with amphibian community diversity, b) elevated *B. dendrobatidis* infection in co-occurring infected amphibian species, and c)

a varying, but strong relationship with assorted vegetative cover types. In Chapter 2, I asked which factor, *Bd* infection, amphibian community diversity, and predator diversity, best explained tadpole physiological metrics including, body condition, total white blood cell count, and neutrophil to lymphocyte ratio, for three species of tadpole.

The most important factors predicting tadpole body condition varied markedly by species. Tadpole body condition was positively correlated with *Bd* infection and predator diversity for the introduced *Lithobates catesbeiana*, negatively correlated with amphibian community diversity for *Rana draytonii*, and positively correlated with predator diversity index for *Pseudacris regilla*.

While the only factor that impacted neutrophil to lymphocyte ratios was *Bd* infection in bullfrogs, with white blood cell counts, I again saw a difference in the way the different species responded to the various stressors in their environment. *Bd* infection was the top driver in elevating white blood cell counts in *P. regilla*, while only amphibian diversity had this effect for *R. draytonii*. Predator diversity had a negative effect on white blood cell counts in *L. catesbeiana*. This diversity of responses is useful from a management perspective, as it may allow conservation practitioners to shift habitat suitability to species of interest. Given the differences in how the amphibians respond, a handful of concrete management recommendations for native amphibians emerge:

1. Decrease *Bd* by decreasing co-occurring species; provide ponds specialized for species in a site, thus decreasing in-pond diversity, but maintaining amphibian

diversity across the site

2. Increase sunning sites for *R. draytonii* and *P. regilla* and maintain habitat complexity

3. Create or maintain smaller, exposed, ephemeral ponds for *P. regilla*

4. Create or maintain larger, shallower, ephemeral ponds for *R. draytonii*

4. Improve water quality for *R. draytonii*

5. Decrease amphibian species diversity by controlling introduced species, particularly bullfrog tadpoles, adults, and introduced fish

My dissertation demonstrates the importance of taking a broad approach to examine ecological relationships by designing studies across various species, combining perspectives such as pathogen success and metrics of host physiological stress, and taking into account a suite of likely interacting biotic and abiotic factors. Broad studies such as this can help to avoid spurious conservation decisions given limited time and resources to protect rapidly declining species.

Dedication

For my grandfather, Arnold Wihtol, who always said “trust, but verify.”
Thank you for believing in me.

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And to all who have helped me in innumerable ways, thank you. I hope that this work serves to make the world at least a little better place.

Introduction

Few ecological relationships occur in a simple or uniform context. Instead they are nestled in a web of complex interactions acted upon by numerous biotic and abiotic influences (Connell, 1961; Polis, and Strong, 1996; Brown et al., 2001; Blaustein, and Kiesecker, 2002; Fair, and Ricklefs, 2002; Collins, and Storfer, 2003; Feyrer et al., 2007; Fausch et al., 2010; Hayes et al., 2010; Schweiger et al., 2010; Blaustein et al., 2011). Understanding how these components relate is essential to building a comprehensive view of how species are influenced by their environment, particularly in the face of rapid anthropogenic changes (Daszak et al., 2001; Jackson et al., 2001; Midgley et al., 2002; Travis, 2003). In turn, this broad picture can allow us to begin to understand threats to species of concern, rank the threats to their populations, and more effectively guide conservation efforts.

Host-pathogen systems are intimate associations between species that can allow careful examination of how multiple stressors may act upon their interaction and individual success (Sousa, and Grosholz, 1991; Collins, and Storfer, 2003; Sparling et al., 2003). Stressors may influence the strength and direction of species interactions at the individual and population level (Ruiz et al., 1999; Crain et al., 2008; Crain et al., 2008), while the strength of influence by stressors can vary by species; some species are more robust when challenged with particular environmental stresses than others (Sparling et al., 2003; D. Vinebrooke et al., 2004; Crain et al., 2008). To unravel the complexity of these systems, it is essential to take a holistic approach that incorporates a multitude of environmental variables and compares their

impacts on various host and pathogen metrics (Blaustein, and Kiesecker, 2002; Taylor et al., 2005; Blaustein et al., 2011).

For my dissertation, I focused on gaining insight on the interaction between an emerging infectious disease, *Batrachochytrium dendrobatidis (Bd)*, a community of three co-occurring larval amphibian hosts, and a suite of biotic and abiotic characteristics. I took a two-pronged approach to better understand how multiple stressors impact both host and pathogen, first examining pathogen success in its host when faced with multiple stressors, and second examining metrics of host physiology when faced with multiple stressors including *Bd* infection.

Previous studies on impacts of environmental cofactors on *Bd* infection prevalence and intensity of infection in hosts demonstrated a complex set of interactions influenced by environmental temperature, humidity, and rainfall (Woodhams et al., 2003; Berger et al., 2004; Piotrowski et al., 2004; Drew et al., 2006; Pounds et al., 2006; Rowley et al., 2006; Bosch et al., 2007; Kriger et al., 2007; Fellers et al., 2011), pond substrate (Fellers et al., 2011), pond depth (Fellers et al., 2011), length of pond shoreline (Fellers et al., 2011), pond permanence (Kriger and Hero, 2007), elevation (Kriger et al., 2007), and proportion of the pond covered in rooted vegetation (Fellers et al., 2011). In Chapter 1 of my dissertation, I used information theory metrics (AICc) to rank which biotic and abiotic variables or combination of variables favor *Bd* prevalence and intensity of infection in a suite of anuran larval hosts, including two native, *Rana draytonii* and *Pseudacris regilla,* and one introduced species, *Lithobates catesbeiana.* Given that the top ranked AICc

models explaining *Bd* infection included only biotic effects (infection in co-occurring tadpole host species, amphibian community diversity, and vegetative cover), I suggest that future studies should focus more strongly on biotic context when investigating *Bd'*s success or failure to generate serious declines in amphibian populations. More broadly, these results suggest that sites with multiple susceptible species favor *Bd* prevalence and intensity of infection, and that studies seeking guidance for conservation efforts in systems with introduced species and *Bd* infection should include co-occurring species' *Bd* infection metrics and measures of species diversity. In this system, conservation efforts may best be focused on control of the invasive *L. catesbeiana*, which would help relieve intraspecific competition and predation for the native tadpole species, and possibly decrease the *Bd* infection load from late summer through early winter season by effectively eliminating tadpoles in the pond sites during those seasons. Further, providing ponds individually specialized for native species preferences across sites would allow for decreased within-pond diversity while retaining diversity across sites.

Previous studies about tadpole growth and leukocyte levels in the face of infection with *Bd,* interspecific competition, and predation have demonstrated reduced tadpole growth with *Bd* infection (Parris and Beaudoin, 2004; Garner et al., 2009; Venesky et al., 2009), intraspecific competition (Relyea, 2003; Raffel et al., 2010), and both increased and reduced growth with predation (Alford, 1999; Paetow et al., 2012), while leukocyte levels changed with *Bd* infection (Davis et al., 2010; Fites et al., 2013; Gervasi et al., 2013), high densities of conspecifics (Davis, and

Maerz, 2009), and predators (Burraco et al., 2013). In Chapter 2 of my dissertation, I investigated whether infection with *Bd,* amphibian community diversity, and predator diversity, best explained three metrics of tadpole physiology, including body condition, white blood cell count, and neutrophil to lymphocyte ratios in the same suite of larval anuran hosts as in Chapter 1. The top-ranked models explaining body condition included positively correlated *Bd* infection and predator diversity for *L. catesbeiana*, weakly negatively correlated amphibian community diversity for *R. draytonii*, and weakly positively correlated predator diversity index for *P. regilla*. Further, I found that both *L. catesbeiana* and *P. regilla* had positively correlated leukocyte responses to *Bd* infection while *R. draytonii* did not have an obvious change in leukocyte levels with *Bd* infection. Further, amphibian diversity was negatively correlated with body condition while it was positively correlated with white blood cell counts in *R. draytonii,* lending support to providing multiple pond habitats specialized for particular species at sites to encourage decreased within pond amphibian diversity while maintaining it across sites. As biotic conditions effectively explained tadpole body condition while *Bd* infection was not a highly ranked predictor of body condition of the two native species, I suggest that control of the invasive species, *L. catesbeiana,* as well as producing specialized pond habitat may be an effective conservation action rather than focusing on *Bd.*

My approach of investigating the interaction of this host-pathogen system from the perspectives of pathogen success and metrics of host physiological stress across three species while taking into account a suite of likely interacting biotic and

abiotic factors allowed for a holistic view of this system. By taking a united approach, this dissertation adds to our growing understanding of the complexity of host-pathogen dynamics across species as well as the biotic and abiotic conditions that influence them. Together these chapters underscore the importance of taking into consideration multiple stressors that act both on host and on pathogens, on predator and on prey, on inter- and intraspecific competitors, and on other ecological relationships to investigate how these interactions in a natural context vary across species. As threatened amphibian populations face continuing change and numerous threats, this work reinforces that the ever-increasing list of threats they face needs to be investigated not just singly, but as a complex and dynamic system that varies across species, spatially, temporally, and with the multiple stressors that each population faces (Halpern et al., 2007; Blaustein et al., 2011). Incorporating this united approach will decrease the likelihood of reliance on spurious results when making conservation decisions with meager resources and as the clock ticks for imperiled species.

1. Chapter 1: Community Context and Environmental Influences on *Batrachochytrium dendrobatidis* Infection in Three Larval Anuran Species

1.1 Abstract

Species declines are often attributed to a complex array of factors, but few studies attempt to take a comprehensive view of potentially contributing factors, rank those factors, and understand how they may interact. Worldwide declines in amphibian species due to infection with the emerging infectious disease, *Batrachochytrium dendrobatidis*, present a complex and dynamic system to investigate the pathogen's impact across species and under varying biotic and abiotic conditions. Lotic amphibians, particularly their larval forms, occur in discrete sites, allowing for testing *Bd* infection prevalence and intensity across a variety of environmental conditions. In this study, I asked whether *Bd* causes mortality in tadpoles of a declining native amphibian, *Rana draytonii,* and I estimated *Bd* infection prevalence and intensity for three co-occurring amphibian species, *R. draytonii, Pseudacris regilla,* and *Lithobates catesbeiana* over two years across 39 pond sites. Further, I investigated whether *Bd* infection prevalence and intensity in these tadpole species correlated with biotic and abiotic conditions, specifically how they relate to: a) *Bd* infection in co-occurring species, b) amphibian community diversity, c) predator diversity, d) proxies for pond temperature (pond area, depth, periodicity, elevation, distance to the ocean, and solar masking by terrain), e)

vegetative cover, f) manageable factors (proximity to intensive horticulture, pond area and depth, pond periodicity), and g) property. To analyze my large environmental dataset, I used a two-stage selection process; first I winnowed variables that were not strongly correlated using one-way ANOVA, and I then used AICc to select the top-supported models explaining *Bd* infection prevalence and intensity in each species.

The late developmental stage *R. draytonii* tadpoles I tested did not experience mortality during the field-caging experiment, although they did not clear infection during the study. The fact that no individuals of this listed species succumbed to infection under field conditions was an important finding.

The factors that influence prevalence and infection intensity in the three study species varied significantly. For invasive *L. catesbeiana*, amphibian diversity was the top-ranked model explaining *Bd* prevalence. The second ranked model was a positive relationship between submerged vegetation *Bd* prevalence and a negative relationship between prevalence and both shoreline and willow vegetation. Infection intensity in this species was not linked to any of the environmental characteristics I studied.

For federally and state listed R. draytonii, the top models explaining patterns of *Bd* prevalence included a *Bd* in co-occurring species in both 2007 and 2008 and a positive relationship between prevalence and floating vegetation, shoreline vegetation and willows in 2007. There was a negative relationship between prevalence and submerged and emergent vegetation in 2007. In 2008, there was a positive correlation with *Bd* infection and both the hydroperiod of a pond and its size. The proximity of the pond to intensive horticulture had an interesting relationship, with sites with high

or no horticulture exhibiting increased levels of *Bd* and those with low exposure exhibiting reduced *Bd* levels. Intensity of infection was negatively correlated with shoreline and emergent vegetation and positively correlated with amphibian diversity.

For *P. regilla*, prevalence was negatively correlated with willows and positively correlated with proxies for pond coolness. *P. regilla Bd* prevalence showed a positive relationship with intensive horticulture at no and low exposure in 2008 and a negative relationship with high exposure to horticulture. In 2007, there was a positive relationship between *P. regilla* and *Bd* in co-occurring species. As with infection prevalence, infection intensity is negatively correlated with willows in 2008, though a positive relationship with amphibian diversity is the top ranked model.

Given our findings that multiple environmental factors were significantly related to *Bd* infection in our three study species and that the significant factors vary by species, management recommendations to benefit native amphibians begin to emerge. Our results reinforce the need to design studies in natural habitats that incorporate multiple factors across species and years to avoid focusing conservation efforts on factors that may not provide the desired conservation outcome.

1.2 Introduction

With recent rates of extinction accelerating in wild populations, it is essential to understand the factors responsible for population declines in order to slow or inhibit further loss (Singh, 2002; Stuart et al., 2004). Yet in surprisingly few cases is there a comprehensive understanding of these factors, their relative importance, or their interactions (Blaustein and Kiesecker, 2002). Many studies have looked at

factors involved in species declines separately (Hayes et al., 2002; Johnson and Sutherland, 2003), and very few have looked at two or more impacts to gain an understanding of how they work together and which play the greatest role in declines (but see (Kiesecker, 2002; Relyea, 2004)). This tendency to focus narrowly on one causal factor is especially evident in the field of disease ecology, where the role of pathogens in governing the ecological distributions, population dynamics, and community structures of host species is increasingly clear (Dobson and Hudson, 1992; McCallum and Dobson, 1995). Disease and parasite effects are predicted to be especially strong for species with small populations and/or restricted ranges (Anderson et al., 1986) and when acting synergistically with other stressors (Blaustein and Kiesecker, 2002; Blaustein et al., 2003; Johnson and Chase, 2004; Kiesecker et al., 2004). Further, unlike the assumption of density dependence in most classic disease models (e.g., (Anderson and May, 1978)), when pathogens are not operating in a density dependent fashion, they can be powerful drivers of host extinctions (de Castro and Bolker, 2005).

Amphibian populations are declining at a greater rate than any other vertebrate group on the planet (Stuart et al., 2004), with a rate of extinction estimated to be about 200 times higher than the natural background frequency (McCallum, 2007). A third of amphibian species are threatened with extinction and over 40% are experiencing population declines (Stuart et al., 2004). No single factor is likely to be responsible for the wholesale losses of multiple amphibian species; more likely is that some or all of these factors are working in concert, each contributing differently to

declines in different populations (Blaustein and Kiesecker, 2002; Collins and Storfer, 2003; Wilcox, 2006). Likely contributors to amphibian declines include habitat loss and fragmentation (Blaustein et al., 1994; Corn, 2000; Green, 1997), compromised water quality (Berrill et al., 1994; Harris et al., 1998; Hayes et al., 2002; Brodman et al., 2003; Houlahan and Findlay, 2003), introduced predators (Fisher and Shaffer, 1996; Lawler et al., 1999; Knapp et al., 2007), and emerging infectious diseases (Daszak et al., 1999; Blaustein and Kiesecker, 2002).

The interactions between disease, habitat restriction, and other environmental impacts are of particular concern for amphibian species, many of which now rely upon extremely small, heavily impacted patches of habitat and are threatened by multiple emerging diseases (Daszak et al., 1999). Indeed, several studies have highlighted the interactions between disease, pathogens and other stressors impacting amphibian survival (Linder et al., 2003; Relyea, 2003). Overall, the impact of pathogens on amphibian populations worldwide appears to have increased dramatically over the past ten to fifteen years, with wholesale loss of species and precipitous local declines attributed to pathogens (Daszak et al., 1999; Johnson and Sutherland, 2003).

Host-pathogen dynamics involve a variety of host defenses and pathogen counter offenses, with both predator and prey being influenced by a complex of environmental conditions (Tinsley, 1995; Dybdahl and Lively, 1998; Rollins-Smith et al., 2002; Thrall et al., 2002; Davidson et al., 2007). Seasonality, temperature, and humidity are a few of the environmental factors that can influence spatial and

temporal dynamics of host-pathogen relationships (Weng et al., 2002; Berger et al., 2004; Piotrowski et al., 2004; Carey et al., 2006; Pounds et al., 2006; Bosch et al., 2007; Kriger and Hero, 2007). Contaminants (Kiesecker, 2002; Linzey et al., 2003; Taylor et al., 2004; Forson and Storfer, 2006; Davidson et al., 2007), eutrophication (Johnson and Chase, 2004), climate change (Pounds et al., 2006; Bosch et al., 2007), predators (Taylor et al., 2004; Johnson et al., 2006), and intra- and inter-specific competitors (Parris and Beaudoin, 2004) are also implicated in changing host-pathogen dynamics.

One example, the interaction between pathogenicity of the amphibian pathogen, *Batrachochytrium dendrobatidis* (*Bd*), and temperature patterns, shows both the power and difficulty of investigating drivers of pathogen effects. In some studies conducted over large geographic areas, temperature variables seem to strongly predict differences in *Bd* effects (Pounds et al., 2006; Drew et al., 2006; Bosch et al., 2007), but no clear patterns emerged in another broad scale survey (Alexander and Eischeid, 2001). Smaller scale studies have led to a much clearer understanding of the interaction of temperature and *Bd* in specific species; *Bd* thrives between 17 and 23C and mortality is associated with lower temperatures, while temperatures above this range less often lead to mortality in infected animals (Woodhams et al., 2003; Berger et al., 2004; Piotrowski et al., 2004; Rowley and Alford, 2007; Kriger and Hero, 2007). Thus, understanding the role a pathogen plays in host populations relies in part on clarifying the complexity of host-pathogen dynamics.

Bd is a water-born fungus that attacks keratin, a protein found in the skin of adult frogs and the mouthparts of amphibian larvae, causing the potentially lethal disease known as amphibian chytrid (Berger et al., 1998). It is hypothesized that *Bd*'s lethality to post-metamorphic amphibians comes through disruption of epidermal function, causing osmotic imbalance (Voyles et al., 2007). Amphibian chytrid has been implicated in the decline of many amphibian populations globally, but recent research also suggests that the disease is widespread in apparently healthy North American populations and that the pathway to *Bd*'s lethality is complex (Ouellet et al., 2005). For example, for *Bd* infection to be fatal, it may require other stressors such as predators, climate change or pesticide exposure (Davidson et al., 2007) to in some hosts, while in other species, such as *Lithobates catesbeiana*, the American bullfrog, mortality is not a given. Host individuals may serve as carriers of *Bd* and infected tadpoles may experience mortality (Daszak et al., 2004; Garner et al., 2006; Paetow et al., 2013).

Several studies have looked into factors that may play a role in the intensity and prevalence of *Bd* infection, as well as its outcome for the infected species. Temperature and humidity, and related factors such as seasonality and altitude, have been the focus of a number of studies, with conditions favorable for *Bd* growth also leading to greater infection prevalence and intensity of infection in several species (Woodhams et al., 2003; Berger et al., 2004; Drew et al., 2006; Pounds et al., 2006; Rowley and Alford, 2007; Bosch et al., 2007). Competition between *Bd*-infected tadpoles led to decreased growth (Parris and Beaudoin, 2004), while exposure to

contaminants in conjunction with *Bd* infection lead to sometimes-counterintuitive outcomes (Parris and Beaudoin, 2004; Davidson et al., 2007). Interestingly, one study suggests that *Bd* may not survive well in freshwater sites that are ephemeral, thus amphibians inhabiting those sites may be exposed to less pathogen (Kriger and Hero, 2007). Additional factors that have been linked to disease outcomes in other host-pathogen systems have been little investigated with *Bd*, namely predation pressure and interspecific community structure (but see (Parris and Beaudoin, 2004; Reeder et al., 2012)).

Overall, there have been few studies that simultaneously investigate multiple biotic and abiotic factors that may play a role in *Bd* infection in an amphibian community (Fellers et al., 2011). While treatment of wildlife pathogens at the population level can be problematic and expensive, alleviating the synergistic threats may allow the host population to persist even when faced with a potentially devastating pathogen. An example of this phenomenon is a study that strongly suggests that the population effects of avian malaria can be mitigated by controlling invasive rodent populations (Kilpatrick, 2006).

Many studies have investigated the impact of various environmental pressures on amphibians. Predation by aquatic insects can influence amphibian population size and community structure (Pearman, 1995), while pond depth can impact survival and growth of larval amphibians, and surface area or pond size may impact community structure (Pearman, 1995). Various fishes are known predators of tadpoles (Hoyle and Keast, 1987; Lawler et al., 1999; Eklöv and Werner, 2000; Komak and

Crossland, 2000; Laurila et al., 2006). Whether predation stress and other environmental pressures may impact *Bd* infection prevalence and intensity in amphibians, particularly a community of amphibians, has been much less explored (Kilpatrick, 2006).

I focused on three co-occurring frog species that inhabit freshwater ponds, two native and one introduced. Of particular interest are California red-legged frogs (*Rana draytonii*) that have been extirpated from over 70% of their historical range. Much of their remaining habitat is in agricultural regions, often using agricultural drainages and ponds (Fellers, 2005). Its populations have been declining since the 1880's, and despite protection efforts at the Federal and State level, they have continued to decline (Fellers, 2005).

There has been one study investigating the fate of *Bd*-infected *R. draytonii* that found no mortality in captive animals over 18 months (Padgett-Flohr, 2008), but it is still not clear whether *R. draytonii* in a wild setting with continuous exposure to the pathogen experience detrimental effects from infection with *Bd*. A closely related species, *Rana muscosa,* appears to be strongly affected (Rachowicz et al., 2006), while American bullfrogs, *Lithobates catesbeiana*, are thought to be carriers of the fungus (Daszak et al., 2004).

The two other focal species for this study co-occur with *R. draytonii* but have distinct life histories and habitat associations. Pacific chorus frogs (*Pseudacris regilla*) are a smaller native pond-breeding frog, abundant throughout their range (Rorabaugh and Lannoo, 2005). They spend much of their post-metamorphic lives in

upland habitat, rather than in the ponds (Rorabaugh and Lannoo, 2005). *P. regilla* have been found to act as a carrier for *Bd* and populations may have reduced levels of infection relative to other species in the western United States (Fellers et al., 2011; Reeder et al., 2012). American bullfrogs (*Lithobates catesbeiana*) are an introduced ranid that poses a threat to native amphibians through predation, competition, and shared pathogens (Daszak et al., 2004; Casper and Hendricks, 2005). Both *R. draytonii* and *P. regilla* tadpoles typically metamorphose by the end of summer in the same year the eggs were laid, although *R. draytonii* tadpoles have been known to overwinter in permanent ponds (personal observation) (Fellers et al., 2001). *L. catesbeiana* tadpoles, on the other hand, typically require two summer seasons to metamorphose, making them dependent on permanent ponds.

One of the greatest differences between the three frogs relates to breeding habitat. *L. catesbeiana* and *P. regilla* will breed in over 90% of the ponds where adults are found, while *R. draytonii* breed in fewer than 40% of the ponds where they occur (D'Amore 2007; D'Amore et al., 2009). *R. draytonii* avoid breeding in ponds that are close to roads or are surrounded by cultivated land, but surprisingly the amount of agricultural runoff a freshwater site received is not a significant predictor of site occupancy by non-breeding adults (D'Amore et al., 2009).

The differences in life histories and habitat associations of these three species allowed us to explore a variety of hypotheses that may be related to *Bd* infection prevalence and intensity in these tadpoles. To investigate whether *R. draytonii* tadpoles that have high intensity of *Bd* infection in the field will be more

likely to die than uninfected or those that have low levels of *Bd* infection, I placed *R. draytonii* tadpoles in field cages until they metamorphosed, taking *Bd* samples at the beginning and end of their tenure. I tested *R. draytonii* tadpoles for *Bd* in three ponds three times throughout a single summer season to characterize the change in their *Bd* infection prevalence and intensity.

Additionally, I estimated *Bd* infection prevalence and intensity in three species of free-ranging co-occurring tadpoles, *R. draytonii*, *P. regilla*, and the introduced *L. catesbeiana,* across a suite of ponds. I hypothesize that infection prevalence and intensity will be higher in *R. draytonii* in the presence of infected *L. catesbeiana* tadpoles, but will be relatively unaffected by infection levels in *P. regilla*. To consider whether *R. draytonii Bd* infection prevalence and intensity would be positively correlated with predators – and hence host stress levels -- I collected data on predator diversity at the pond sites where I sampled the three species of amphibians for *Bd*. I also collected data on factors associated with pond temperature at each of these pond sites, such as distance from the ocean, terrain masking, vegetation, elevation, pond size and depth. I hypothesize that *Bd* infection prevalence and intensity will be negatively correlated with pond temperature. Finally, I examined whether *Bd* infection intensity and prevalence was negatively correlated with annual or occasional pond dry-down or other manageable pond characteristics such as pond size and proximity to intensive horticulture (row crops and golf courses).

1.3 Methods

Study Sites

I sampled tadpoles in ponds in 2007 and 2008, in 3 regions along the Central Coast of California (Figure 1): the Wilder Sand Quarry (WQ); the Elkhorn Region (ER), including the Elkhorn Ranch, Elkhorn Slough National Estuarine Research Reserve and surrounding areas; and Santa Lucia Conservancy (SLC). In 2007 I sampled 21 ponds across the ER sites. In 2008, I resampled 12 of the ER ponds (the remainder were dry or inaccessible) along with an additional 25 ponds from WQ and SLC that were new to my study, expanding my sample that year to a total of 37 ponds. Although *Bd* infection prevalence and intensity were not highly correlated between years in the 12 ponds that I sampled across the two years (prevalence R^2=0.1117, intensity R^2=0.1217), I opted to treat the data conservatively and analyze the data separately by year.

Does Bd cause mortality in R. draytonii tadpoles and what are the infection dynamics over time?

To characterize how *Bd* infection influences health and survival in tadpoles through metamorphosis, I placed 51 *R. draytonii* tadpoles individually in field cages in 6 ponds from late July 2008 until they reached adulthood, monitoring overall health and *Bd* infection intensity in infected individuals through metamorphosis.

Each field cage (1m high x 0.5m wide x 0.5m deep) was constructed with a PVC frame and lined with stiff black plastic ¼" poultry netting and a standard insect

fiberglass window screen liner (18x16 mesh). This design ensured tadpoles remained in their individual cages, excluding competing tadpoles and predators, while still exposing the tadpole to its natal pond water, including its nutrients and microbes. Cages opened at the top, allowing addition of food and monitoring of tadpole health. I placed cages in the ponds using anchoring shafts sunk into in the pond substrate; I secured the cages vertically so that none were closer than 20 cm apart and placed them with 0.75 m of the cage submerged. When possible, I chose locations that would receive a combination of light and shade through the day allowing tadpoles to thermoregulate. To allow continued water flow, plant growth was discouraged along the screen of each cage by rubbing the surface of the cage with a collecting net weekly.

I provided tadpoles a consistent diet of fish food and rabbit chow pellets every two to three days and, once the tadpoles' mouthparts began to metamorphose, I added terrestrial invertebrates found along the edges of the ponds to the cages as well. It is unlikely that the metamorphosing tadpoles consumed these invertebrates as they are thought to not eat between the time their mouthparts begin to metamorphose and their tails are fully reabsorbed, but I provided them to ensure they did not lack for food resources. As the tadpoles moved toward metamorphosis, I added sticks at the water's surface that I braced across the cages for metamorphosing tadpoles to pull up on and perch out of the water.

Tadpoles were not added to the field cages until the end of July because federal and state permits for this portion of the study were issued late in the field

season, at which point tadpoles had naturally developed to Gosner developmental stages (Gosner, 1960) 35 to 44. Tadpoles remained in the cages until they were Gosner developmental stage 44 to 46, when their front legs had emerged and most of their tail had been reabsorbed (Table 1).

I examined each tadpole prior to placement in the field cages and again just prior to release. During each examination I recorded length, weight, Gosner developmental stage, and any visually evident abnormalities. Additionally, to detect *Bd* infection, I swabbed the mouthparts of each tadpole by gently moving a sterile rayon-tipped swab (Medical Wire and Equipment 113) across their mouthparts six times (Obendorf and Dalton, 2006); the swab was then sealed in a sterile microcentrifuge tube and transported to the lab for storage and infection intensity analysis. Metamorphs were swabbed gently but firmly by rubbing a sterile swab across their bellies, feet, and thighs six times each.

To look at the relationship between infection intensity and health, I calculated the mean *Bd* infection intensity and prevalence in the tadpoles for each pond at the beginning and the end of the field-caging period. To estimate change in *Bd* infection intensity, prevalence, and associated mortality for each pond, I then subtracted the ending infection mean from the beginning infection mean.

How does *Bd* prevalence and *Bd* intensity of infection change in free-ranging *R. draytonii* tadpoles in ponds over the course of the summer season?

In order to determine whether or not *Bd* infection prevalence and mean infection intensity increase during the summer season as the larvae mature and are

exposed longer to *Bd* zoospores, as hypothesized, I sampled free-ranging *R. draytonii* tadpoles at three ponds in the ER on three different dates through the 2008 summer.

For twenty *R. draytonii* tadpoles per pond, I measured length and weight and recorded Gosner developmental stage. Using the method noted above, I sampled each tadpole for *Bd* by swabbing its mouthparts. I spent as many as five hours collecting twenty tadpoles by dipnet. I were able to capture a full sample size in each pond for each time series except on the final sample date in one pond—this pond was unexpectedly dry, and I were only able to capture four tadpoles on that date. To get a representative subset of the population, I sampled from throughout the ponds, but occasionally tadpoles were aggregated. When this occurred, I continued to sample the entire pond in an attempt to gather tadpoles from throughout, but sometimes had to sample more extensively from these aggregations to obtain twenty individuals. To minimize the potential spread of pathogens, tadpoles were handled with clean gloves and placed into individual sterile containers. Additionally, all field gear was sterilized between pond visits using a standard protocol (Speare et al., 2004; U. S. Fish and Wildlife Service 2005).

Is *Bd* infection in three species of free-ranging tadpoles correlated with presence of and *Bd* infection in other amphibian species?

Amphibian Diversity and *Bd* Infection

To test whether *Bd* infection prevalence or intensity is correlated with amphibian community diversity and co-occuring species' *Bd* infection levels, I looked at *Bd* infection intensity and prevalence in up to twenty individual *R.*

draytonii, L. catesbeiana, and *P. regilla* tadpoles per pond for the 2007 and 2008 summer seasons (Table 2 and Table 3).

I followed the capture and sampling methods described above in "Time Series Sampling of Free-Ranging CRLF Tadpoles." Although my analysis focused on the three frog species, I were also interested in how the overall diversity of the amphibian community may impact *Bd* infection prevalence and intensity. In each pond, I noted the presence of all amphibian species, including: any stage of *L. catesbeiana*, *R. draytonii*, *P. regilla*, *Taricha torosa*, *Taricha granulosa*, *Anaxyrus boreas halophilus*, and *Ambystoma californiense* (Table 4). To simplify the data on the five species of amphibians I found in various ponds, I made a composite variable for use in ANOVA analysis reflecting the number of amphibian species, which I call the "Amphibian Diversity Index" set equal to the total number of amphibian species minus 1 (for the dependent variable species). While I encountered six species total across all the ponds, the amphibian diversity index ranged from 0 (meaning only the dependent variable species was present) to three; I never found more than four different amphibian species in any single pond.

To investigate if *Bd* infection prevalence affected co-occurring species' infection prevalence, I categorized *Bd* infection prevalence for each species of frog (*R. draytonii*, *L. catesbeiana*, and *P. regilla*) per pond into one of three groups: NP (not present) if species was not present, LP (low presence) if species was present and *Bd* prevalence was low (0 to 0.05), or HP (high presence) if species was present and

Bd prevalence was moderate to high (>0.05). I used these categories for ANOVA analyses.

Predator Influence on *Bd* Infection

For each pond, I noted the presence or absence of several common aquatic invertebrate predators (e.g. *Bellastoma spp., Dytiscida spp., Notonectidae spp., Hirudinea spp., Lethocerus spp., Odonata spp., Ranatra spp*., and *Procambarus spp.*) by sampling with a fine-meshed net at three intra-pond locations and identifying the invertebrates in each sample and recorded incidental encounters while dipnetting for tadpoles. I also noted the presence of predatory fish species (e.g. sticklebacks (*Gasterosteus aculeatus*), gambusia (*Gambusia affinis*), bass (*Micropterus spp.*), catfish (*Amiurus spp.*), and bluegill (*Lepomis spp.*)).

In order to ask more general questions about the influence of predators on *Bd* infection patterns in tadpoles, I made a composite variable, "Predator Index," equal to the sum of the number of predatory invertebrates and fish species found in each pond with differential weighting of 0.5 for each species present in the pond that prey on only small tadpoles and 1 for each species that eats all stages of tadpoles (Table 5). These pond-specific index scores ranged from 0 to 3.

Environmental Influence on *Bd* Infection

To determine whether *Bd* infection prevalence or intensity correlated with pond characteristics, including pond periodicity, temperature proxies, vegetative cover, and several manageable factors, I collected data on a variety of additional biotic and abiotic parameters for each pond I surveyed by year (Table 4). I used

various combinations of factors in different models to analyze the predictive power of these environmental factors individually and in combination.

I visually estimated percentage of pond surface covered by categories of vegetation including: submerged vegetation (percent coverage across entire pond), floating vegetation (percent coverage across entire pond), emergent vegetation (percent coverage across entire pond), shoreline vegetation (percent coverage of shoreline), and, above the shoreline, willow (percent coverage of pond perimeter). I considered making a composite variable for vegetative cover to increase the power of my analyses, but preliminary examination of the data indicated that different vegetation measures were uncorrelated. Thus I decided to maintain separate categories for vegetative cover.

I estimated terrain masking, defined as the percent of daylight hours that the sun is blocked by landforms and preventing direct illumination, using the MicroDEMS mapping program and the method described by Leszek Pawlowicz on the Free Geography Tools site (http://freegeographytools.com/2007/determining-sun-blockage-by-topography). I first input latitude and longitude of each pond site, and selected the annual option in the "direct illumination" box in the Horizon Options. From the output, I calculated average annual terrain masking in hours by averaging the daily percentage of hours the pond was masked by terrain and multiplied it by the average number of hours of sunlight per day at that site.

To answer my question about whether there are manageable factors that influence *Bd* prevalence and intensity, I considered pond periodicity, size, and

proximity to intensive horticulture to be manageable pond characteristics. Pond length and width were measured using a 100m long tape. I ground-truthed and fine-tuned all of these spatial estimates using the closest Google Earth (version 5.2) historical images to the date of surveys, taken from 2006 to 2009. I also used Google Earth to obtain estimates of the elevation for each pond and, using the ruler tool, measurements for the shortest straight-line distance to the ocean. Pond length and width were strongly correlated (Figure 2), so I multiplied them together to obtain a single rough estimate of pond surface area that I used for subsequent analyses. I additionally categorized depth in each pond (less than 1m, 1-2m, greater than 2m).

To determine pond periodicity, I used prior pond surveys, observation between survey years, landowner interviews, and historical imagery on Google Earth to determine whether ponds dried down during or within the three years prior to the surveys.

I categorized influence on the pond by intensive horticulture, namely row crops and golf courses, into three levels. No influence (NP=no proximity) was defined as a pond with no close proximity to intensive horticulture; indirect influence (LP=low proximity) was defined as a pond in close proximity to intensive horticulture and receiving indirect runoff or spray (i.e., the pond was lower in elevation but did not receive the bulk of its runoff from intensive horticulture); and direct influence (HP=high proximity) was defined as a pond being in close proximity to intensive horticulture and receiving direct runoff that constituted a large water source for the pond. Again, I used data from the date of survey as well as historical

images in Google Earth that were closest to the date of survey and observation of the terrain to help place ponds into these three categories.

Treatment of Pond Data

I performed a two-stage model selection process on the various categories of data I collected for each of the three species (*L. catesbeiana*, *R. draytonii*, and *P. regilla*) by pond to address questions of how amphibian and predator diversity and environmental factors influence trends in *Bd* infection prevalence and intensity within the tadpole populations. First, I used one-way ANOVA with each explanatory factor singly to examine effects on *Bd* infection prevalence and intensity for each species in 2007 and 2008. I included any parameter that had an R^2 greater than 0.05 to construct 8 models that included different sets of factors, minus the parameters that were rejected in the first selection process (Table 6). Due to limited sample size (ponds sampled per year), I only tested simple models that embodied a single general type of effect (e.g. diversity of co-occurring amphibians) at a time, and did not consider models that included multiple classes of explanatory factors. I ran ANOVAS on each model and used AICc weights calculated using the outputs from the ANOVAS to evaluate the quality of fit for each model against the other models. For some dependent variables, sample size is small, limiting my ability to test or to make strong inferences about different effects, particularly in the infection intensity models. Once I had the top ranked models, there were several models with categorical variables for which I wanted to know the direction of correlation; to this end, I performed Tukey-Kramer HSD tests with these categorical independent variables.

I also ran models of the effects of undefined spatial variation (i.e., which general area each pond occurs in) to test for spatial differences in incidence that were not explained by quantified causal factors.

Bd Sample Analysis

I transported swab samples to the lab and kept them in a freezer at -20°F until analysis. To extract the DNA from the swabs in their eppendorf storage tubes, I added 40µL of PrepMan Ultra to cover the swab tip, heated the samples to between 95 and 100°C for two minutes, placed them in a centrifuge for 3 minutes at 13000 RPM, and placed the resulting fluid in a sterile Eppendorf tube. I then diluted this sample tenfold with sterile deionized water. I analyzed the resulting diluted samples according to the standard procedure outlined by Boyle et al. 2004 (Boyle et al., 2004), using a quantitative Polymerase Chain Reaction (qPCR) assay, running each sample in triplicate with the internal positive control described by Hyatt et al. 2007 (Hyatt et al., 2007).

I had no false positives in negative controls, indicating my tests provided good specificity (Hyatt et al., 2007) and giving us confidence to classify indeterminate results, those with only 1 or 2 positives of the triplicate sample run, as positives, effectively increasing the sensitivity of the test (Boyle et al., 2004; Hyatt et al., 2007). I averaged the positive results of the triplicate run for positive samples to obtain a single genetic equivalent result for the sample.

The qPCR returns a raw genetic equivalent score. I multiplied this score by a dilution factor, the amount the sample had been diluted during extraction and addition

of the quantitative PCR reagents. This returned the corrected zoospore genetic equivalent score (z_{swab} score), a quantitative metric for infection intensity for that individual. Typically the dilution factor was 80x, but when the internal positive control indicated inhibition in the sample, I diluted the sample by an additional factor of 10. Any sample with z_{swab} score greater than 0 was treated as a positive sample (the lowest positive z_{swab} score I obtained was 0.008, with only 9 of the 358 positive samples with a z_{swab} score less than 1).

Bd Infection Prevalence and Intensity Estimates

I used pond-specific prevalence of infection for several of my analyses. *Bd* prevalence for the dependent variable species was calculated as the percent of infected animals of said species in the pond based upon qPCR results. To normalize data for analysis, I took the natural log of the prevalence data +1.

Because there were instances where one or more of the three tadpole species of interest were not present, the *Bd* prevalence calculations for non-dependent variable species were treated categorically. Prevalence was categorized into three tiers: NP designates a species that was not present in the pond, LP and HP indicate a species was present with a *Bd* prevalence less than 5% and greater than 5%, respectively. This allowed us to compare instances where not all three species were present to those when they were all present, allowing us flexibility in exploring my questions and a larger dataset to work with.

I made estimates of infection intensity for each pond by taking the average of the z_{swab} scores of positive *Bd* samples for each species in that pond. As with infection

prevalence, I converted these values into a categorical variable to allow us to explore a larger dataset: NP represents a species that was not present in the pond, LP and HP indicates a species was present with a z_{swab} score from 0 to 99 and above 99, respectively. There was only sufficient data to examine relationships for *R. draytonii* and *P. regilla* in 2008.

1.4 Results

Does *Bd* cause mortality in *R. draytonii* tadpoles and what are the dynamics over time?

Of the 51 field-caged *R. draytonii* tadpoles, 5 had positive *Bd* samples when they were placed in cages at the beginning and, by the end, those 5 along with an additional 33 tested positive for *Bd*. No tadpoles lost their infection; tadpoles either maintained their infection status or became infected over the course of the study. All tadpoles survived and reached metamorphosis. Mean *Bd* infection intensity and prevalence increased over the caging period with two exceptions—in one pond infection prevalence remained constant and in another pond mean infection intensity declined, though mean infection intensity stayed categorically moderate to high throughout (Table 7).

How does *Bd* prevalence and *Bd* intensity of infection change in free-ranging *R. draytonii* tadpoles in ponds over the course of the summer season?

Time series sampling of free-ranging *R. draytonii* tadpoles in which I sampled three ponds three times during a single summer season suggests a pattern of

increasing *Bd* infection prevalence and no clear pattern of increasing *Bd* infection intensity. In the first pond, I detected no infection during any of the three sampling dates. In the second, I found only one infected animal, which was on the last sample date. The last pond had increasing *Bd* infection prevalence through the summer, but no consistent pattern in *Bd* infection intensity (Table 8).

Is *Bd* infection in three species of free-ranging tadpoles correlated with presence of and *Bd* infection in other amphibian species?

Overall *Bd* infection prevalence across species and years was 18.5%. Consistent with other studies (Ouellet et al., 2005; Longcore et al., 2007; Pearl et al., 2007), *Bd* infection prevalence was highest in *L. catesbeiana* while it was lowest in *P. regilla*, except in ER in 2008 (Table 9). In 2008, the year I was able to sample three properties, *Bd* infection prevalence for all three species was lowest in ER and highest in SLC, except for *R. draytonii*, which was marginally higher at WQ (Table 9). Despite this, property was not significantly correlated with *Bd* infection prevalence or intensity, although it was marginally correlated for *L. catesbeiana* in 2008 (R^2=0.401, Table 10).

R. draytonii and *P. regilla Bd* infection prevalence did not appear to be strongly correlated with *L. catesbeiana Bd* infection prevalence, contrary to my hypothesis (Table 10).

Mean *Bd* infection intensity across species and properties was 81.71 z_{swab} score (s.d. = 448) (Table 9). Across all sites and species, individual *Bd* z_{swab} scores ranged from less than one to more than 7715 zoospores per swab. Mean *Bd* infection

intensity per site in *L. catesbeiana* ranged from z_{swab} score of 64 to 194, in *R. draytonii* z_{swab} score ranged from 116 to 784, and in *P. regilla* z_{swab} score ranged from 171 to 905. Interestingly, *L. catesbeiana* consistently ranked lowest in *Bd* infection intensity, except in 2008 when *P. regilla* had a lower *Bd* infection intensity at ER. Patterns of *Bd* infection intensity by species across property in 2008 were less clear than *Bd* infection prevalence, with *P. regilla* and *R. draytonii* having highest infection intensity at SLC where *L. catesbeiana* had its lowest infection intensity.

Extrinsic Characteristics Correlated with *Bd* Infection Prevalence and Intensity in Three Species of Tadpoles

From the factors with $R^2 > 0.05$ in the ANOVA models, I constructed multi-parameter models to test my other central hypotheses (Table 11). These multi-parameter models yielded very clear top models for each of the species/year combination, except for *P. regilla* 2008 and, to a lesser extent, *P. regilla* in 2007 (Table 12). I discuss these results in detail below.

Amphibian Diversity Index

Amphibian species diversity index was included in the top multivariate models for *L. catesbeiana* and *P. regilla* populations from 2008. Of the seven models included in the analysis of *L. catesbeiana* 2008 infection prevalence, the one including the amphibian diversity index is the best supported, with an AICc weight of 70% (Table 12). As amphibian diversity in a site increases, so does that site's *L. catesbeiana Bd* infection prevalence (Figure 3). Similarly, with *P. regilla* in 2008, of models I ran to examine the relationship between *Bd* infection intensity and the other

factors, the model including amphibian species diversity had the highest AICc weight at 99.8% (Table 22); the analogous model relating infection prevalence with the amphibian diversity index showed a positive correlation with an AICc weight of 10%.

Infection Prevalence of Other Species

Across the board, models relating *R. draytonii* and *P. regilla* infection prevalence and intensity suggest a positive correlation in infection between both species for both years.

The second most likely model explaining *R. draytonii Bd* infection prevalence in 2007 was *P. regilla Bd* infection prevalence at a low AICc weight of 2.9% (Table 12). In these models, *P. regilla Bd* infection prevalence was treated as a categorical variable, with NP indicating that *P. regilla* was not detected at the site, LP indicating *P. regilla* were at the site and had no or very low infection prevalence (less than 0.05), and HP indicating that *P. regilla* were at the site and had an infection prevalence of greater than 0.05 at the site. Post-hoc comparisons using the Tukey-Kramer HSD test indicated that the mean score for NP (M = 0.18, SD = 0.105) and LP (M = 0.047, SD = 0.043) were not significantly different (p=0.5). However, HP (M = 0.549, SD = 0.074) significantly differed from LP (p=0.0027) and marginally from NP (p=0.066) (Table 15). As *P. regilla Bd* infection prevalence increased in pond sites where the species co-occurred, so did *R. draytonii Bd* infection prevalence.

From the opposite perspective, of the 4 candidate models included in the analysis for *P. regilla* 2007 *Bd* infection prevalence, the *R. draytonii Bd* infection prevalence index model appears to be most descriptive (AICc weight of 66%, Table

12). *R. draytonii Bd* infection prevalence is a categorical variable, and post-hoc comparisons using the Tukey-Kramer HSD test indicated that the mean score for NP (M = 0.13, SD = 0.22) to LP (M = 0, SD = 0) and for NP (M= 0.13 , SD= 0.22) to HP (M= 0.46, SD= 0.40) were not significantly different (p= 0.56 and 0.09 respectively). However, HP significantly differed from LP (p= 0.03) (Table 19) indicating that when *Bd* infection prevalence in *R. draytonii* is above 0.05 in a pond, *P. regilla Bd* infection prevalence tends to also be higher.

For *R. draytonii* the following year (2008), the model based on *P. regilla Bd* infection prevalence ranked highest; it is 98.6% likely to be the best candidate model when compared to the other candidate models (Table 12). As noted, *P. regilla Bd* infection prevalence is a categorical variable, and post-hoc comparisons using the Tukey-Kramer HSD test indicated that the mean score for NP (M = 0.46, SD = 0.119) to LP (M = 0.042, SD = 0.048) and HP (M = 0.344, SD = 0.051) to LP are significantly different (p=0.094 and p=0.0008 respectively). However, NP and HP did not vary significantly (p=0.635) (Table 16). As *P. regilla Bd* infection prevalence increased at pond sites where they co-occurred, *R. draytonii Bd* infection prevalence also increased, a positive correlation. Unfortunately, for models of *P. regilla* 2007 infection intensity, my sample size was too small to perform AICc on the factors that emerged as significant in the first cut of variables via the single-factor models with an R^2 of greater than 0.05. Of the single-variable models that were most significant, they included *R. draytonii Bd* infection prevalence (Table 21).

For *P. regilla* that same year (2008), the second best model was based on *L. catesbeiana* and *R. draytonii Bd* infection prevalence indices; this model had an AICc weight of 13.9% (Table 12).

Manageable Factors

The second highest ranking model for *R. draytonii* 2008 infection prevalence was based on manageable factors, including pond area, periodicity, and intensive horticulture ranking. This model was given an AICc model weight of only 1% (Table 12). Of the factors included in this model, only horticultural intensity was significant (p=0.044) (Table 17). Post-hoc comparisons using the Tukey-Kramer HSD test indicated that the mean score for NP (M=0.24, SD=0.055) to HP (M=0.39 and SD=0.12) and for NP to LP (M=0.05, SD=0.096) were similar (p=0.38 and p=0.084), compared to LP and HP (p=0.049) (Table 18, Figure 4). Ponds with direct exposure to run-off were more strongly correlated with higher *Bd* infection prevalence levels than those with indirect run-off, and ponds that had no run-off were more strongly positively correlated with *Bd* infection prevalence than those that had indirect runoff.

The model for *P. regilla* 2008 infection prevalence that included parameters for manageable factors, namely intensive horticulture ranking, was one of the two highest-ranking models with an AICc weight of 33.6% (Table 12). The manageable factors model included only intensive horticulture ranking. Intensive horticulture rank is a categorical variable, and post-hoc comparisons using the Tukey-Kramer HSD test returned mean scores for NP (M=0.18, SD=0.21) to LP (M=0.02, SD=0.03) were marginally significant (p=0.07) compared with NP to HP (M=0.09 and SD=0.14) or

for LP to HP (p=0.56 and p=0.74) (Table 20), meaning that there was a marginally significant increase in *P. regilla Bd* infection prevalence between sites that had no exposure to agricultural or golf course runoff and those that had indirect exposure. Similarly, of the single-variable models that were most significant in describing *P. regilla* 2007 infection intensity, intensive horticulture ranking was among the top three (Table 21).

Manageable factors were not identified as significant predictive factors in models for *L. catesbeiana* in 2008 or *R. draytonii* in 2007.

Predator Index

Predator index factor was the basis for one of the three significantly ranked single-variable models run for *P. regilla* infection intensity in 2007 (Table 21). Predator index factor was not included in the highly ranked models for other tadpole species in either year or *P. regilla* in 2008.

Proxies for Temperature

The second best ranked model to explain *P. regilla* 2007 *Bd* infection prevalence included proxies for temperature—namely distance to ocean, pond area, and terrain masking—and had an AICc weight of 18.6% (Table 12). While none of the individual factors in the temperature model were significant, the overall model received some support (Table 20). The characteristics that lead to cooler ponds, larger pond area, closer to the ocean, and blockage of sunlight by the terrain were associated with higher *Bd* infection prevalence in *P. regilla* tadpoles. Supporting this evidence, *Bd* infection prevalence in *P. regilla* in 2008 was negatively correlated with these

temperature proxies; the model including temperature proxies had an AICc weight of 9% (Table 12).

Property

All but one of the models that included property as a factor received very low AICc weights. The model with the highest AICc weight (9%) that included property indicated a negative correlation between property and infection prevalence in *P. regilla* tadpoles from 2008 (Table 12).

Vegetation

Vegetation was the only universal variable to be included in the top-ranking models of each species in each year with a large enough sample size to analyze.

For *L. catesbeiana* in 2008, the model including vegetation characteristics has an AICc weight of 24% (Table 12). While *Bd* infection prevalence in *L. catesbeiana* appeared to increase in sites with more submerged vegetation, it decreased in those sites with more shoreline vegetation or willow. Of the three types of vegetation, submerged vegetation seems to be most strongly correlated with *Bd* infection prevalence in *L. catesbeiana* (ANOVA $F(1, 5)=8.603$, $p=0.033$) (Table 13).

Of the six models included in my analysis of *R. draytonii* 2007 infection prevalence, the vegetation model ranks most likely, with an AICc weight of 99.7% (Table 12). The most significant relationship in this model appears to be with emergent vegetation (ANOVA $F(1, 3)=69.31$, $p=0.0036$), with shoreline vegetation (ANOVA $F(1,3)=23.29$, $p=0.017$), submerged vegetation (ANOVA $F(1,3)=20.27$, $p=0.020$), and floating vegetation (ANOVA $F(1,3)=11.41$, $p=0.043$), also being

significant (Table 14). Submerged and emergent vegetation were negatively correlated with *R. draytonii Bd* infection prevalence, while floating, shoreline, and willow vegetation were positively correlated with *R. draytonii Bd* infection prevalence. Similarly, of the multivariate AICc models I ran to examine the relationship between *Bd* infection intensity in *R. draytonii* in 2008 and my other factors, the top model included vegetation with an AICc weight of 99.9% (Table 22).

For *P. regilla* in 2007, the model including vegetation had an AICc weight of 13% (Table 12). In the vegetative cover model, no factors were significant, although submerged vegetation ranked as marginally significant (Table 19). Interestingly, submerged vegetation was negatively correlated with *P. regilla Bd* infection prevalence (Figure 5), a trend I might expect if submerged vegetation decreases the temperature in ponds, thus facilitating optimal temperatures for *Bd* growth.

Infection prevalence in *P. regilla* in 2008 was negatively associated with vegetation (Figure 6) and the model including only willow has an AICc weight of 23.8% (Table 12). Of the multivariate AICc models I ran to examine the relationship between *Bd* infection intensity in *P. regilla* in 2008 and the other factors, the two top models included vegetation with paltry AICc weights of 0.02% (Table 22).

***R. draytonii* 2007 and *L. catesbeiana* 2008 Sample Size**

Unfortunately, my sample sizes for both *L. catesbeiana* in 2008 and *R. draytonii* in 2007 were too small to run AICc models on the factors that emerged as significant in the first cut of variables via the single-factor models for *Bd* with an R^2 of greater than 0.05. Of the single-variable models that were most significant, both

groups showed some correlation with emergent vegetation and horticulture intensity. Other significant models for *L. catesbeiana* in 2008 were based on pond area, and terrain masking and *R. draytonii* in 2007 additionally suggested some correlation with *P. regilla Bd* infection prevalence (Table 21).

1.5 Discussion

Does *Bd* cause mortality in *R. draytonii* tadpoles and what are the dynamics over time?

Our field-cage study investigating whether *Bd* infection influences survival in field-caged *R. draytonii* tadpoles found no mortality over the course of the experiment. Though counter to my prediction that heavily-infected tadpoles would experience mortality, this is not completely unexpected. While some studies have found mortality in tadpoles infected with *Bd* in the lab (McMahon et al., 2013; Kleinhenz et al., 2012), several researchers observed no mortality in tadpoles of various species that were infected with *Bd* (Rachowicz and Vredenburg, 2004; Blaustein et al., 2005; Stockwell et al., 2010).

Vrendenberg and colleagues found that populations of post-metamorphic *Rana muscosa* experienced population crashes when a large proportion of animals reached a threshold level of *Bd* infection intensity that correlated with a z_{swab} score of 10,000 (Vredenburg et al., 2010). While it appears these lethal *Bd* thresholds are species-specific, few of the tadpoles I tested approached these high levels of zoospore equivalents (Table 7). It should be noted that these observed z_{swab} score thresholds

come from studies of post-metamorphic frogs rather than tadpoles. Whether this number can translate to tadpoles is questionable, but it does point out that the levels of infection detected in the tadpoles were relatively low.

Bd prevalence increased in field-caged tadpoles between the first and second sampling, just prior to release (Table 7). This may indicate that *R. draytonii* tadpoles tend to develop more intense infections later in their development. Alternatively, it may reflect that *Bd*-infection is easier to detect in later stage or larger sized tadpoles or that a tadpole is more likely to become infected with *Bd* the longer it remains in the water or a field cage. It is possible that some of the tadpoles at the beginning of the study may have had light infections that were not detected by the sampling method, a very gentle swab of their mouthparts, although this is unlikely due to the extreme sensitivity of the testing method (Hyatt et al., 2007). Additionally, the stress of being in field cages (e.g. decreased ability to thermoregulate, caged in an area of the pond that happened to have a high *Bd* load) may have made the tadpoles more susceptible to high infections. It is also possible that the tadpoles became infected while in the field cages by *Bd* zoospores moving in the water column or other small organisms carrying *Bd* zoospores, without direct contact with other tadpoles. I noticed free-ranging young-of-the-year frogs in several ponds using the field cages as perches. Swimming tadpoles and young-of-the-year amphibians could both contact the field cage screening, allowing fairly close, indirect contact potentially facilitating infection of the field-caged tadpoles. Regardless of the details of the experiment, it is a significant conclusion for *R. draytonii* conservation that no infected tadpoles died

during the course of the field caging, indicating that mid- to late-developmental stage tadpoles may not experience significant mortality due to *Bd* infection in field conditions.

As a caveat, my results are limited by the study's experimental design. Due to logistical constraints, I placed mid- to late-stage tadpoles, Gosner developmental stage 35 to 44, in the field cages (Table 1). Placing egg clutches or very young tadpoles (roughly Gosner developmental stage 26) into the cages would have provided a broader view of the potential impact of *Bd* infection on tadpoles in the field.

How does *Bd* prevalence and *Bd* intensity of infection change in free-ranging *R. draytonii* tadpoles in ponds over the course of the summer season?

I expected to see a clear increase in *Bd* infection intensity and *Bd* infection prevalence in *R. draytonii* tadpoles over the sampling period. Had I sampled more ponds, I may have detected a clearer pattern. All samples from one of the three ponds tested negative, and I was left to extrapolate from a sample of two ponds (Table 5). In each of those two ponds there was a general increase in *Bd* infection prevalence, although the trend in one pond was based on a single positive sample on one date. There was no clear trend for *Bd* infection prevalence. The results for *Bd* infection intensity are encouraging for a future study, but I do not feel comfortable making any generalization on the course of *Bd* infection prevalence or *Bd* infection intensity in *R. draytonii* tadpoles over time based on my data.

It is interesting to compare the results from two ponds in which I conducted both field-caging experiments and time-series sampling. I would expect *Bd* infection prevalence to increase in *R. draytonii* tadpoles during the course of both experiments. The general trend is the same in these two ponds, East Raleigh and PMP: *Bd* infection prevalence increased over the time of the study, while *Bd* infection intensity decreased. Of note, though, *Bd* infection prevalence was six times greater in the field caged tadpoles compared to the data from time-series sampling of tadpoles in those same ponds. In the field cage study, East Raleigh *Bd* infection prevalence at time of release is 100% with an average *Bd* z_{swab} score of 262.46; PMP *Bd* infection prevalence at time of release is 100% and with an average *Bd* z_{swab} score of 1689.91.

There may be several reasons for greater *Bd* infection prevalence and intensity in the field caged tadpoles, as noted in the field-cage sampling section above. The difference could also be influenced by the small sample size of field caged tadpoles (9 field caged tadpoles in each of the two ponds, compared to 20 tadpoles in each of the repeat-sampling sessions), or due to stage when swabbed (the field caged animals were already metamorphosing and I swabbed their bellies, thighs, and feet while the tadpoles from all animals sampled in the multi-sampled ponds were still tadpoles and I only swabbed their mouthparts). One study is consistent with the last explanation, with *Bd* infection loads of recently metamorphosed frogs of various species topping out an order of magnitude higher than the tadpoles of the same species (Stockwell et al., 2010).

Is *Bd* infection in three species of free-ranging tadpoles correlated with presence of and *Bd* infection in other amphibian species?

Bd infection prevalence was consistently highest in *L. catesbeiana* and lowest in *P. regilla*, except in ER in 2008 (Table 7). High *Bd* infection prevalence in post-metamorphic *L. catesbeiana* compared to *P. regilla* is consistent with other studies (Ouellet et al., 2005; Longcore et al., 2007; Pearl et al., 2007) and may arise because post-metamorphic *L. catesbeiana* tend to spend most of their time in water where *Bd* zoospores are circulating while *P. regilla* spend much of their time in the upland habitat. In tadpoles, this trend may be related to *L. catesbeiana*'s larger mouthpart size, or that they spend much more time in the pond before metamorphosing compared to *P. regilla* tadpoles, and thus they are exposed to *Bd* zoospores floating in the water for a longer period of time.

Bd z_{swab} scores ranged up to 7715. While Vrendenburg's rule of 10,000 (Vredenburg et al., 2010), which states that for some species of frogs, *Bd* infection is lethal when the z_{swab} score reaches 10,000, has not been tested on tadpoles, I can assume that the high values for *R. draytonii* at the SLC are likely detrimental for the developing tadpoles since the *Bd* breaks down the keratin in their mouthparts and inhibits effective foraging (Venesky et al., 2010). In the vast majority of tadpoles I tested, though, infections were far below this threshold.

Surprisingly, I did not find that the infection intensity or prevalence of the invasive carrier of *Bd*, *L. catesbeiana*, was strongly correlated with prevalence or intensity of infection in either of the two native species. This is particularly

unexpected given that *R. draytonii* and *L. catesbeiana* adults inhabit many of the same ponds, and I et al. have observed many *R. draytonii* and *L. catesbeiana* juveniles and adults in physical contact in the field (Pearl et al., 2005; D'Amore et al., 2009). Given my findings of seasonally increasing number of infections in field-caged *R. draytonii* tadpoles, I would expect that exposure to water with zoospores, rather than direct contact, would be all that is necessary to infect the tadpoles. However, my sample only had 2 ponds that contained both *R. draytonii* and *L. catesbeiana* tadpoles, thus limiting my power to test this relationship.

It is interesting that in the 12 ponds I sampled two years in a row, *Bd* infection and prevalence were not strongly correlated. I expected that there would be strong correlation given that *R. draytonii* and *L. catesbeiana* adults tend to exhibit site fidelity (D'Amore et al., 2009) and that many of the ponds I resampled were perennial ponds. This may indicate that the dynamics of *Bd* infection in adults and tadpoles are fairly decoupled that *Bd* infection dynamics within pond appear to be complex and worth further investigation. It may also indicate that *P. regilla* behavior is driving *Bd* dynamics.

Amphibian diversity was a strong candidate in explaining *L. catesbeiana Bd* infection prevalence in 2008; the more diverse the co-occurring amphibian community, the greater the *Bd* infection prevalence in *L. catesbeiana* tadpoles. This is an interesting relationship, given that *Bd* levels in co-occurring *P. regilla* species did not appear to influence *L. catesbeiana Bd* infection prevalence. This may indicate that *R. draytonii* strongly influence *Bd* prevalence in *L. catesbeiana* or possibly that other

amphibian species that I did not test have a strong influence on *Bd* infection prevalence in *L. catesbeiana*. I also found that amphibian species diversity strongly explained *Bd* infection prevalence in *P. regilla* in 2008 and, to a lesser extent, amphibian diversity was also correlated with *P. regilla Bd* infection prevalence in 2007 (R^2=0.45). Given that *P. regilla* tended to occur in most of the ponds (Table 3), this is not a surprising result; *P. regilla* likely come into contact with many species due to their flexibility in breeding pond choice (D'Amore et al., 2010). Further, *P. regilla* are a highly vagile species, and are hypothesized to be responsible for the spread of *Bd* through the Sierra Nevada Mountain Range (Reeder et al., 2012).

In 2008, I found that *P. regilla Bd* infection prevalence was strongly positively correlated with *R. draytonii Bd* prevalence and fairly strongly related to *R. draytonii Bd* infection intensity in 2007 and 2008 (R^2=0.42 and 0.26 respectively). In 2007, I found that *P. regilla Bd* infection prevalence was best explained by *R. draytonii Bd* infection prevalence, with a strong positive correlation, as well as strongly correlated with *R. draytonii Bd* infection prevalence (R^2=0.71). This makes sense as I often found these tadpoles in close proximity in the ponds, inhabiting similar areas (Hemingway, personal observation). Alternatively, these relationships may result from one or the other species serving as a primary infection source for the other, or from both species mutually supporting higher infection rates. Regardless, this is a very interesting set of results, both counter to our original hypothesis that *L. catesbeiana Bd* infection levels would be strongly correlated with native species'

infection levels, but instead it appears there is a relationship between *P. regilla* and *R. draytonii Bd* infection levels.

Extrinsic Characteristics Correlated with *Bd* Infection Prevalence and Intensity In Three Species of Tadpoles

Contrary to my hypothesis that stress from multiple predator sources would lead to increased *Bd* infection levels, predator diversity did not appear to be closely correlated with any *Bd* infection prevalence or intensity in any species/year combination except slightly in *P. regilla* in 2007 (R^2=0.65). While one study found that predator stress could cause slowed growth in tadpoles that had *Bd* (Parris and Beaudoin, 2004), predator stress may not impact acquisition or growth of *Bd* in tadpoles. *Bd* grows on the mouthparts of tadpoles, causing damage and potentially decreasing their foraging efficiency (Venesky et al., 2009) and intestine length (Venesky et al., 2013). Thus, while being harassed by predators may further decrease an infected tadpole's ability to eat and grow, being harassed by predators may not significantly increase a tadpole's likelihood of becoming infected with *Bd*.

Environmental Characteristics' Influence on *Bd* Infection Prevalence and Infection Intensity In Three Species of Free-Ranging Tadpoles

Pond characteristics, particularly vegetative cover, provided some of the strongest explanatory models for *Bd*, including *R. draytonii* in 2007, *L. catesbeiana* in 2008, *P. regilla* in 2007 and *P. regilla* in 2008. Across these models, the results were not always intuitive or consistent. I assumed that there would be a relationship to

temperature in pond microhabitat based on the type of vegetation in the ponds (emergent, shoreline, willow, and floating vegetation leading to cooler temperatures and submerged vegetation leading to increased temperature) and that this would be correlated with *Bd* infection levels. Given the inconsistent results, it seems that an alternative explanation is more likely, such as variation in how tadpole species use available microhabitats (Alford, 1986; Loschenkohl, 1986) influencing likelihood of encounters with *Bd* zoospores.

Pond area was significantly correlated only with *L. catesbeiana Bd* infection intensity in 2008, but was not significant in any other species' *Bd* infection intensity or prevalence. Many studies have found a significant relationship between *Bd* infection and temperature (Berger et al., 2004; Drew et al., 2006; Kriger and Hero, 2007; Bosch et al., 2007; Richards-Zawacki, 2010; Forrest and Schlaepfer, 2011), and I was surprised temperature proxies, such as pond area and depth, terrain masking, and distance from the ocean were not significant in explaining more *Bd* infection in more species and years. In fact, temperature proxies were the second most likely model in explaining *P. regilla Bd* infection prevalence in 2007, but only at 32.3%, and terrain masking was only weakly correlated with *L. catesbeiana Bd* infection intensity in 2008. Further, while I may have expected infection to increase through the season or differ significantly by year, Julian date was also not strongly correlated with *Bd* infection prevalence in any species.

Kriger found a non-random distribution of *Bd* across amphibian breeding habitats, positively correlated with permanent water and with stream breeding frogs

more likely to be infected than pond breeding species (Kriger and Hero, 2007). Further, Longcore et al. found that amphibian species that hibernated in aquatic habitats had significantly higher *Bd* infection prevalence than those species that hibernated in terrestrial habitats (Longcore et al., 2007). Thus I expected to find that tadpoles that were in perennial ponds would have higher *Bd* infection prevalence and intensity than those in ephemeral ponds. I did not find any spatial correlations either between properties nor an association between pond ephemerality and *Bd* infection intensity or prevalence.

Interestingly, only two of our manageable factors came up as significantly related to *Bd* infection prevalence and intensity. Horticultural intensity was the most likely model explaining *Bd* infection prevalence in *P. regilla* in 2008, and was significantly correlated with *P. regilla Bd* infection intensity in 2007 (R^2=0.71) and *L. catesbeiana Bd* infection intensity in 2008 (R^2=0.64). These results are intriguing and run counter our expectations; *R. draytonii Bd* levels were neither positively nor negatively correlated with proximity to horticulture. Further, in 2008 *P. regilla Bd* infection prevalence was negatively correlated with increased intensive horticulture runoff, although the explanatory power of the model that included horticultural intensity was not very strong (Figure 6). Thus, from our study, it appears the influence of horticultural intensity on *Bd* infection levels is marginal at best. This is supported by some recent studies between frogs and contaminants (Kleinhenz et al., 2012; Gahl et al., 2011; Venesky et al., 2012). While tadpoles experienced mortality

due to *Bd* infection, *Bd* infection was either not impacted by contaminants or mortality was actually decreased in the presence of contaminants.

While it is likely that I missed some variables that play important roles in predicting *Bd* infection prevalence and infection intensity, it is significant that our study points to several interesting relationships, particularly a seemingly reciprocal relationship between *P. regilla* and *R. draytonii Bd* infection. Furthermore, I find it hopeful for *R. draytonii* conservation that there was no mortality in the field caging experiment.

Ecological systems are complex by nature and are influenced by many factors, both biotic and abiotic, to varying degrees and with many interactions. Teasing out the factors that influence any one relationship, in this case a host-pathogen relationship, can be difficult at best. Our study of *Bd* and several amphibian species has increased our basic knowledge of how *Bd* infection impacts *R. draytonii* tadpoles as well as indicating several factors that are clearly and consistently related to *Bd* infection in tadpoles of several species. While our investigation of environmental factors that influence *Bd* prevalence and intensity of infection yielded several strongly supported patterns, these were not always consistent between species or years. This is not surprising given the complexity of the various factors interacting in the system and, once again, emphasizes the importance of multi-year studies to avoid emphasis of potentially spurious results.

1.6 Conclusions

To better understand extinction risk for amphibians and effectively target conservation actions, it is essential to implement an integrated approach to studying how *Bd* differentially impacts species depending on intrinsic traits and extrinsic threats (Murray et al., 2011). This approach is appropriate across conservation questions. I analyzed a variety of extrinsic threats across three species to better understand how each interacts with these in the context of *Bd* infection. Our study reinforces the inherent problems with a reductionist approach, while relying on information provided by narrow studies to inform our design and understand our results. Our sometimes-muddy results reinforce the need to design studies in natural habitats that incorporate multiple factors across species and years to avoid focusing conservation efforts on factors that may not provide the desired conservation outcome. Long-term studies provide the best information, but time is running short to conserve these species. By taking into account a multitude of interacting factors across habitats, sites, species, and time, we can better tease out the most important factors to focus conservation goals and most effectively benefit wildlife.

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1.8 Figures

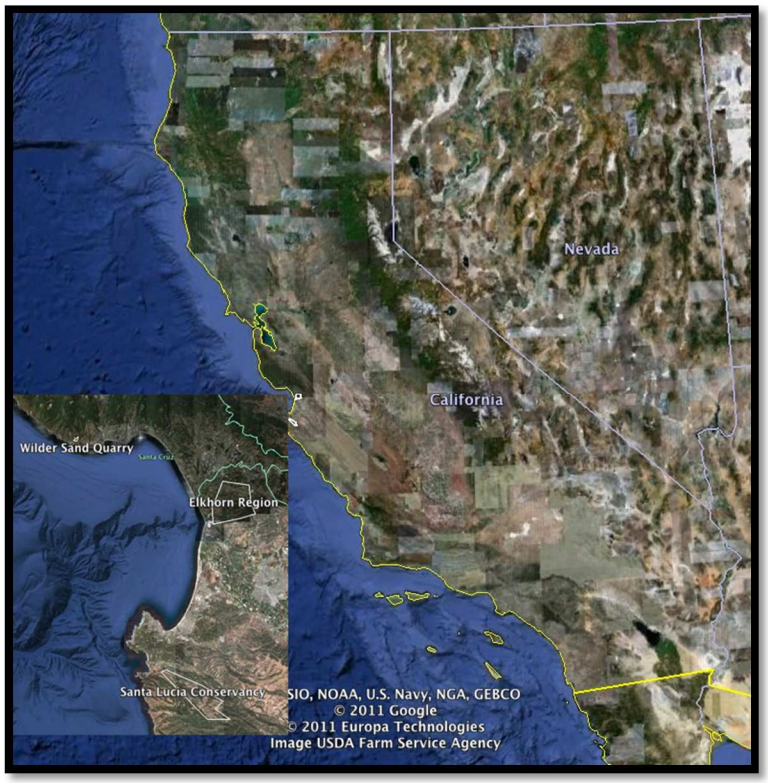


Figure 1: Map of California and study sites.

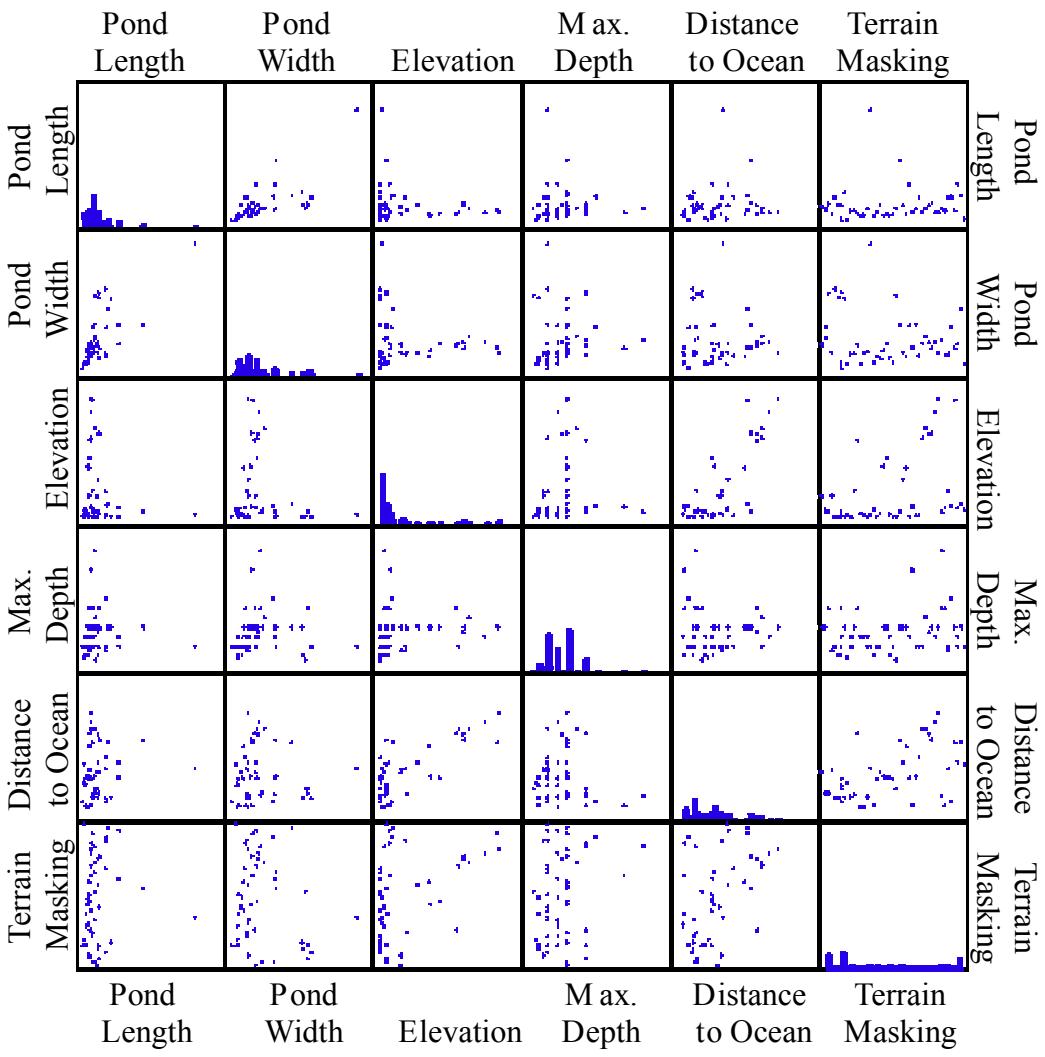


Figure 2: Scatterplot matrix of pond characteristics. Pond length and width appear strongly correlated.

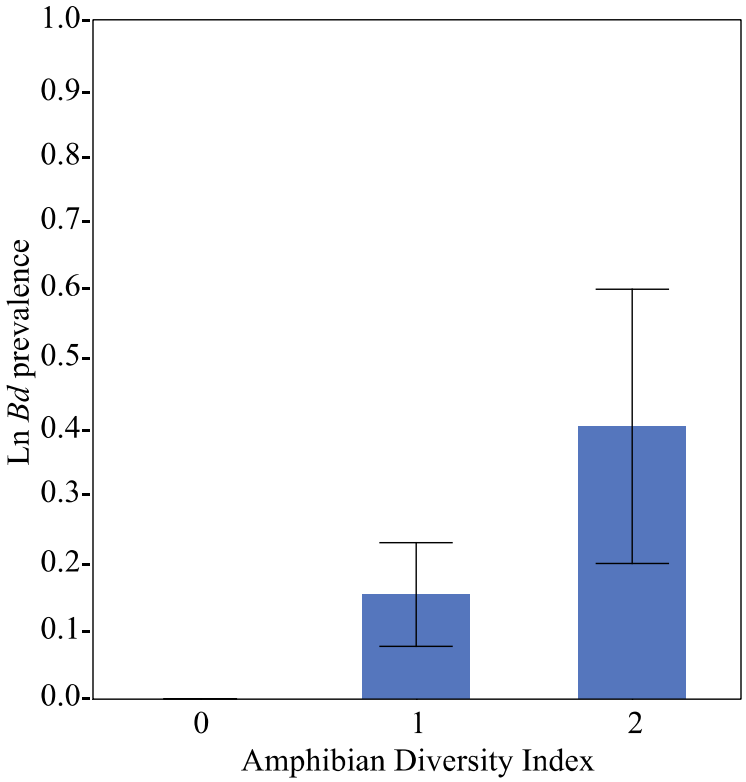
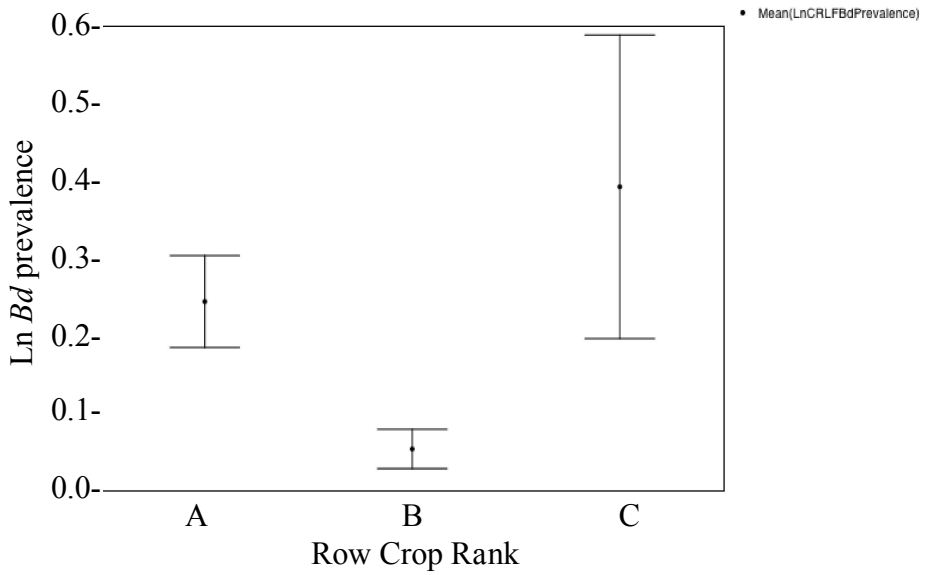


Figure 3: Amphibian diversity index by Ln *L. catesbeiana Bd* Prevalence for 2008



Each error bar is constructed using 1 standard error from the mean.
Figure 4: Plot for horticultural intensity rank and mean ln *R. draytonii Bd* infection prevalence in 2008. (A=no proximity to horticulture, B=low proximity to horticulture, C=high proximity to horticulture).

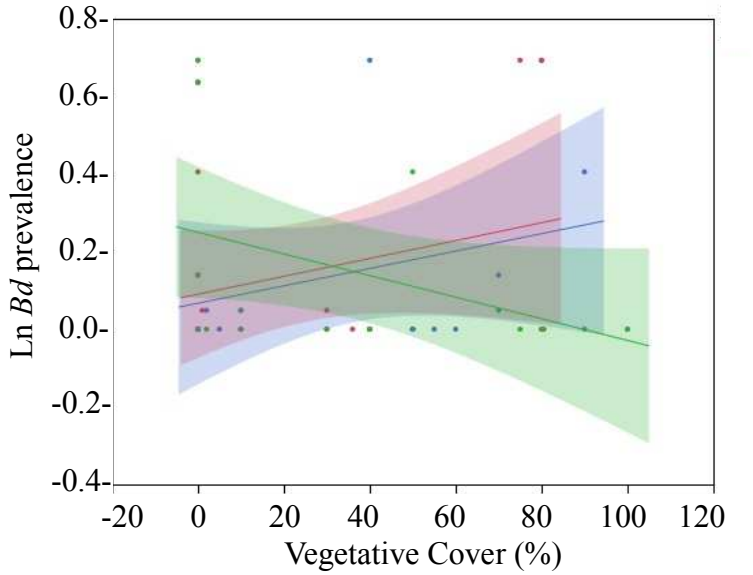


Figure 5: Percent vegetative cover by ln *P. regilla* Bd Infection Prevalence in 2007.

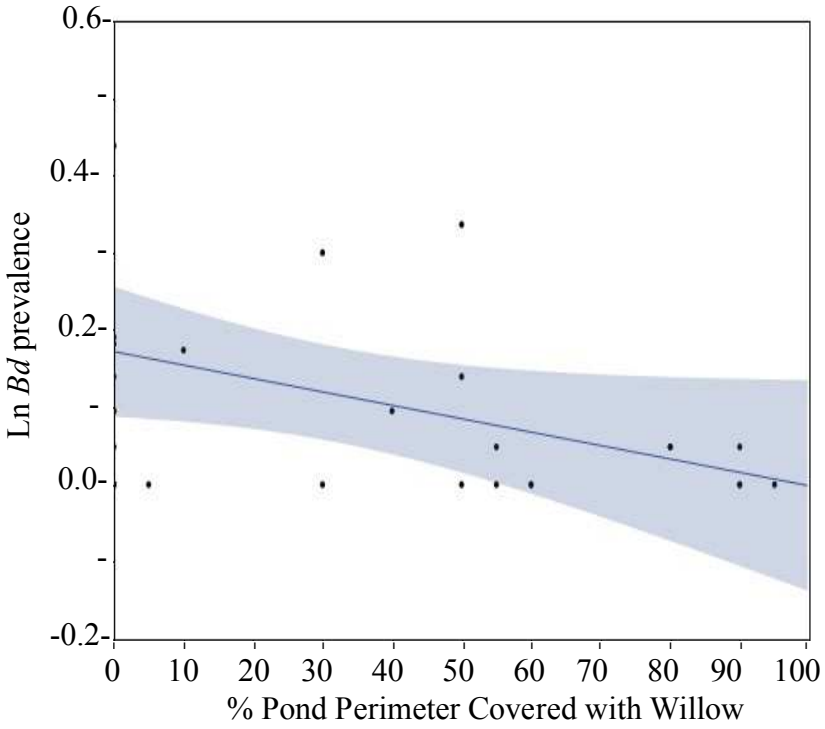
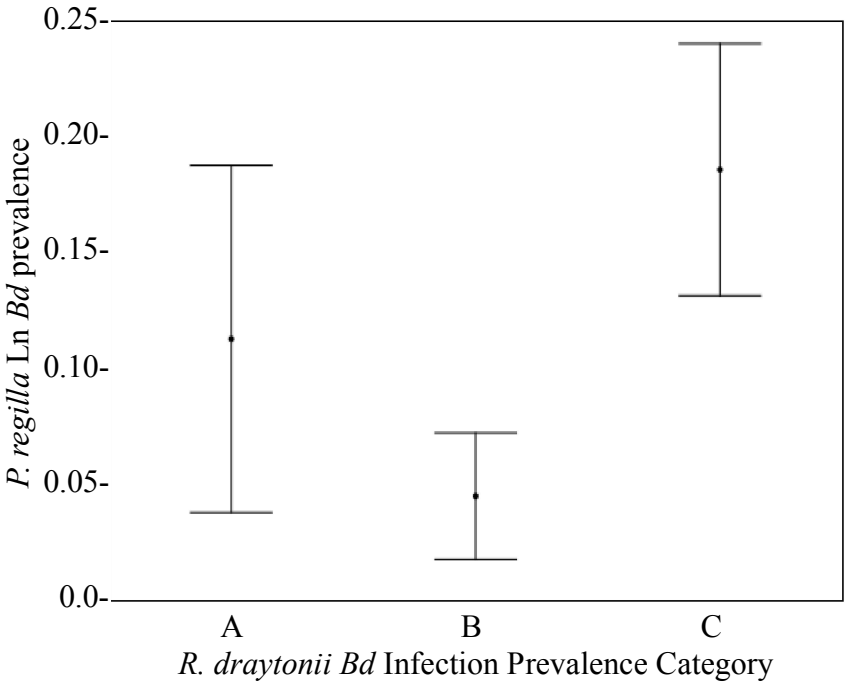
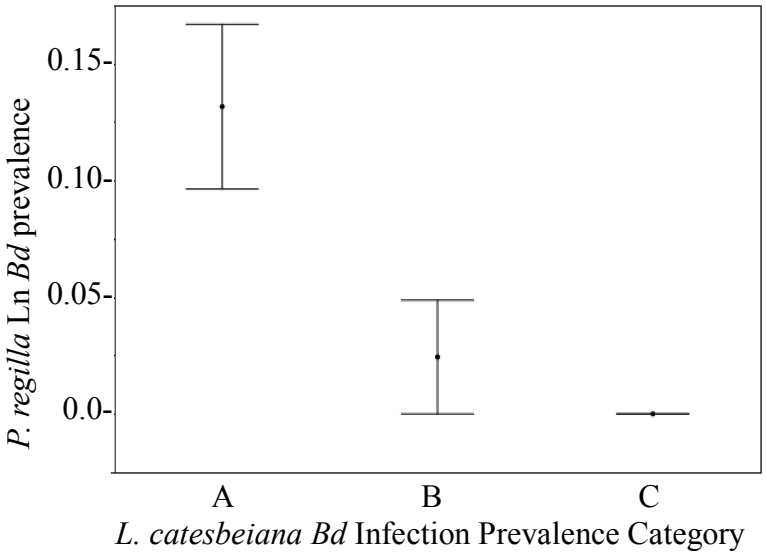


Figure 6: Percent Pond Perimeter Covered with Willow for Mean Ln *P. regilla Bd* Infection Prevalence in 2008.



Each error bar is constructed using 1 standard error from the mean.
Figure 7: Categorical *R. draytonii Bd* Infection Prevalence and Mean Ln *P. regilla Bd* Infection Prevalence in 2008 (*Bd* Infection Prevalence Categories: A=0% *Bd* prevalence, B=0>5% *Bd* prevalence, C=>5% *Bd* prevalence).



Each error bar is constructed using 1 standard error from the mean.
Figure 8: Categorical *L. catesbeiana Bd* Infection Prevalence and Mean Ln *P. regilla Bd* Infection Prevalence in 2008 (*Bd* Infection Prevalence Categories: A=0% *Bd* prevalence, B=0>5% *Bd* prevalence, C=>5% *Bd* prevalence*).*

1.9 Tables

Gosner Developmental Stage	# of Tadpoles At Entry	# of Tadpoles at Release
35	1	
36	1	
37	2	
38	3	
39	3	
40	8	
41	21	
42	5	
43	3	
44	4	32
45		18
46		1

Table 1: Gosner developmental stage of *R. dtaytonii* tadpoles at time of addition to the field cages and at release.

Species	Year	# of Ponds Sampled	Total # of Tadpoles Sampled Across Ponds
L. catesbeiana	2007	2	40
L. catesbeiana	2008	8	160
R. draytonii	2007	8	137
R. draytonii	2008	25	539
P. regilla	2007	17	314
P. regilla	2008	32	625

Table 2: Number of tadpoles of each species sampled by pond and year.

	Number of Ponds with Sp. Composition	
Species Composition	**2007**	**2008**
P. regilla	11	7
R. draytonii	1	2
L. catesbeiana	2	4
P. regilla and *L. catesbeiana*	0	2
P. regilla and *R. draytonii*	7	21
R. draytonii and *L. catesbeiana*	0	0
P. regilla, *R. draytonii*, and *L. catesbeiana*	0	2

Table 3: Number of ponds with given tadpole species composition sampled by year.

	R. draytonii		***P. regilla***		***L. catesbeiana***	
Parameters	**2007 (N=9)**	**2008 (N=25)**	**2007 (N=18)**	**2008 (N=33)**	**2008 (N=9)**	**2007 (N=2) ****
R. draytonii Bd	N/A	N/A	X	X	X	
L. catesbeiana Bd	*	X	*	X	N/A	
P. regilla Bd	*	X	N/A	N/A	X	
Julian Date	X	X	X	X	X	
Region/Site	*	X	*	X	X	
Pond Length (m)	X	X	X	X	X	
Pond Width (m)	X	X	X	X	X	
Elevation (m)	X	X	X	X	X	
Maximum Depth Estimate (m)	X	X	X	X	X	
Periodicity	X	X	X	X	X	
Shortest Straight-Line Distance to Ocean (m)	X	X	X	X	X	
Average Annual Terrain Masking (Hours, Average Annual Terrain Masking*# daylight hours)	X	X	X	X	X	
Intensive Horticulture Influence	X	X	X	X	X	
% Submerged Vegetation Cover	X	X	X	X	X	
% Floating Vegetation Cover	X	X	X	X	X	
% Emergent Vegetation Cover	X	X	X	X	X	
% Shoreline Vegetation Cover	X	X	X	X	X	
% Willow Shoreline Cover	X	X	X	X	X	
L. catesbeiana	X	X	X	X	N/A	
R. draytonii	N/A	N/A	X	X	X	
P. regilla	X	X	N/A	N/A	X	
A. macrodactylum	*	*	*	X	*	
T. torosa* and *T. granulosa	*	X	*	X	*	
A. boreas halophilus	*	X	*	X	*	
Invertebrate Predators	X	X	X	X	X	
Fish	X	X	X	X	X	
N=Number of Ponds for the Prevalence Models						

***Not Enough Data Points To Justify Inclusion In Analysis.**
****Not enough ponds to conduct analysis**

Table 4: Parameters included in analyses by study species and year.

	Tadpole Prey Size		
Predator	**Small**	**All Sizes**	**Source**
Belastoma sp., adult	X		(Brodie et al., 1983)
Coleoptera dytiscidae, adult	X		(Smith, 1983)
Coleoptera dytiscidae, larvae		X	(Licht, 1974; Brodie et al., 1983)
Hemiptera notonectidae	X		(Licht, 1974; Cronin and Travis, 1986)
Hirudinea	X		(Licht, 1974; Berven et al., 2001)
Lethocerus sp., adult		X	(Licht, 1974; Brodie et al., 1983)
Lethocerus sp., nymphal	X		(Brodie et al., 1983)
Odonata sp., dragonfly naiads	X		(Brodie et al., 1983; Smith, 1983)
Ranatra sp.	X		(Licht, 1974)
Procambarus sp.		X	(Axelsson et al., 1997; Gherardi et al., 2001; Renai and Gherardi, 2004)
Lithobates catesbeiana		X	(Lawler et al., 1999)
Ambystoma californiense	X		(Burger, 1950; Anderson, 1968; Webb and Roueche, 1971; Wilbur, 1972)
Taricha sp.	X		(Chandler, 1918; Neish, 1971)
Large Fish*: Lepomis macrochirus, Amiurus sp., Micropterus sp.*		X	(Hoyle and Keast, 1987; Eklöv and Werner, 2000; Towey 2007)
Gambusia affinis holbrooki	X		(Lawler et al., 1999; Komak and Crossland, 2000)
Gasterosteus aculeatus		X	(Laurila et al., 2006)

Table 5: Prey size preference of various aquatic predators used for estimating "Predator Index."

Model	Potential Components
Species Composition	Amphibian Diversity Index
Bd	*L. catesbeiana, R. draytonii, P. regilla* Categorical *Bd* Scores
Pond Dry Down	Pond Periodicity
Predators	Predator Index
Temperature Proxies	Pond Surface Area, Depth, Elevation, Distance to Ocean, Terrain Masking
Vegetation	Shoreline, Submerged, Floating, Emergent, and Willow Vegetation
Manageable Factors	Periodicity, Pond Area, Pond Depth, Intensive Horticulture (Row Crop/Golf Course Rank)
Spatial Autocorrelation	Property

Table 6: All Potential *Bd* Prevalence Multi-Variate Model Components

Pond	Initial Infection Intensity Mean (SE)	Final Infection Intensity Mean (SE)	Change in Infection Intensity Mean (SE)	Initial Infection Prevalence (SE)	Final Infection Prevalence (SE)	Change in Infection Prevalence (SE)
4-Birdhouse	1.04 (1.04)	2.79 (2.79)	1.75 (1.75)	0.1 (0.1)	0.1 (0.1)	0 (0)
East Raleigh	638.70 (423.09)	262.46 (161.55)	-376.24 (310.25)	0.22 (0.15)	1 (0)	0.78 (0.14)
N. Midway	0.24 (0.25)	613.26 (257.88)	613.01 (257.76)	0.1 (0.1)	1 (0)	0.9 (0.11)
Poor Mikey	0 (0)	1689.91 (660.44)	1689.91 (660.44)	0 (0)	1 (0)	1 (0)
West Raleigh	0 (0)	0.49 (0.49)	0.49 (0.49)	0 (0)	0.25 (0.25)	0.25 (0.25)
Yellow House	6.57 (6.57)	82.18 (37.19)	75.61 (32.23)	0.1 (0.1)	0.7 (0.15)	0.6 (0.16)

Table 7: Change in *Bd* infection prevalence and mean infection intensity in field caged *R. draytonii* tadpoles during the 2008 field season.

Pond Name	East Raleigh			PMP			Rookery 3		
Date Sampled (2008)	**7/2**	**7/29**	**8/18**	**6/13**	**7/15**	**8/2**	**6/23**	**7/14**	**8/11**
Infection Intensity	0	315.53	28.90	0	0	1.67	0	0	0
Infection Prevalence	0	0.25	0.45	0	0	0.05	0	0	0

Table 8: Time series sampling of free-ranging CRLF tadpoles in 3 ponds during the 2008 field season.

Species	Year	Property	Total Positive / Total Swabbed	Mean *Bd* Infection Prevalence	Mean Intensity of Infection in Tadpoles (z_{swab} score)	*Bd* z_{swab} Range
L. catesbeiana	2007	ER	11/41	0.27	63.92	0.21-579.94
	2008	ER	4/60	0.07	194.68	10.96-738.78
		SLC	47/100	0.47	106.52	0.11-1219.67
R. draytonii	2007	ER	30/137	0.22	554.9	0.91-3865.66
	2008	ER	40/295	0.14	305.26	1.65-3159.26
		SLC	62/166	0.37	783.78	0.008-7715.31
		WQ	32/79	0.41	116.03	0.55-740.12
P. regilla	2007	ER	43/313	0.14	904.73	0.93-6055
	2008	ER	30/335	0.09	174.58	3.26-1208.32
		SLC	46/198	0.23	344.43	4.07-2232.86
		WQ	15/92	0.16	170.69	3.37-1118.22

Table 9: *Bd* Infection Prevalence and Intensity (as measured by z_{swab} score) in tadpoles by year and property (ER=Elkhorn Region, SLC=Santa Lucia Conservancy, WQ=Wilder Quarry).

Parameter	BF 2008 ***	CRLF 2008	CRLF 2007	PTF 2008	PTF 2007
CRLF *Bd* Categorical	0.441	N/A	N/A	0.115	0.353
PTF *Bd* Categorical	**	0.515	0.851	N/A	N/A
BF *Bd* Categorical	N/A	**	*	0.051	*
Amphibian Diversity	0.311	0.100	0.293	0.073	**
Predator Index	**	0.103	0.068	**	**
Property	0.401	0.229	*	0.096	*
Julian Date	0.220	**	**	**	**
Depth	**	**	0.066	**	**
Pond Surface Area	0.213	0.095	0.251	**	0.115
Periodicity	0.075	0.108	**	**	**
Distance To Ocean	0.323	**	**	**	0.113
Elevation	0.429	0.094	**	0.066	**
Intensive Horticulture Rank	0.704	0.224	**	0.159	0.111
Emergent Vegetation	**	0.117	0.169	**	**
Floating Vegetation	**	**	0.152	**	0.083
Shoreline Vegetation	0.156	**	0.314	**	**
Submerged Vegetation	0.617	**	0.798	**	0.176
Terrain Masking	**	0.050	0.064	**	0.060
Willow	0.205	0.073	0.082	0.115	0.065

*Did not include in test due to insufficient data.

**R2<0.05

***Did not include Bullfrogs in 2007 in tests because I did not test sufficient ponds with *L. catesbeiana* tadpoles.

Table 10: R^2 Values for single parameter models that were used in the selection of factors to go into *Bd* infection prevalence multivariate models. BF=*L. catesbeiana,* CRLF=*R. draytonii,* PTF=*P. regilla.*

Model Hypothesis	BF 2008	CRLF 2007	CRLF 2008	PTF 2007	PTF 2008
Species Composition	Amphibian Diversity	Amphibian Diversity	Amphibian Diversity		Amphibian Diversity
Bd	CRLF Bd Categorical	PTF Bd Categorical	PTF Bd Categorical	CRLF Bd Categorical	BF Bd Categorical*CRLF Bd Categorical
Pond Dry Down	Periodicity		Periodicity		
Predators		Predator Index	Predator Index		
Temperature Proxies	Distance To Ocean* Elevation* Pond Area	Depth* Pond Area* Terrain Masking	Elevation* Pond Area	Distance To Ocean* Pond Area* Terrain Masking	Elevation
Vegetation	Shoreline* Submerged* Willow	Emergent* Floating* Shoreline* Submerged* Willow	Emergent* Willow	Floating* Submerged* Willow	Willow
Manageable Factors	Periodicity* Pond Area* Intensive Horticulture Ranking	Depth* Pond Area	Pond Area* Periodicity* Intensive Horticulture Ranking	Pond Area* Intensive Horticulture Ranking	Intensive Horticulture Ranking
Spatial Autocorrelation	Property		Property		Property

Table 11: Components included in each multivariate model tested for *Bd* Prevalence. BF=*L. catesbeiana,* CRLF=*R. draytonii,* PTF=*P. regilla.*

Year/Species/Covariates	n	p	ΔAICc	AICc weight	R^2
***L. catesbeiana* 2008**					
Amphibian Species Diversity	9	4	0	0.7	0.70
Vegetation: Shoreline* Submerged* Willow	9	5	2.157	0.238	0.67
***R. draytonii* 2007**					
Vegetation: Emergent* Floating* Shoreline* Submerged* Willow	9	7	0	0.997	0.96
Categorical *P. regilla* Bd Prevalence	9	4	11.701	0.0029	0.85
***R. draytonii* 2008**					
Categorical *P. regilla Bd* Prevalence	25	4	0	0.986	0.52
Manageable Factors: Pond Area* Periodicity* Intensive Horticulture Influence	25	6	9.032	0.011	0.39
***P. regilla* 2007**					
Categorical *R. draytonii Bd* Prevalence	18	4	0	0.663	0.35
Temperature Proxies: Distance To Ocean* Pond Area* Terrain Masking	18	5	2.542	0.323	0.32
***P. regilla* 2008**					
Manageable Factors: Intensive Horticulture Influence	32	4	0	0.336	0.16
Vegetation: Willow	32	3	0.683	0.239	0.12

Table 12: The top 2 AICc candidate models for *Bd* Prevalence for each species in each year (n=number of *Bd* prevalence scores for that species/year combination included in model, p=number of parameters in model).

Model Parameter	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Submerged Vegetation	1	0.32603	0.32603	8.603	0.03252 *
Shoreline Vegetation	1	0.00021	0.00021	0.0056	0.9432
Willow	1	0.01257	0.01257	0.3317	0.58961
Residuals	5	0.18949	0.0379		

Table 13: ANOVA table of *L. catesbeiana Bd* infection prevalence model including vegetation for 2008.

Model Parameter	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Submerged Vegetation	1	0.067594	0.067594	20.2741	0.020458 *
Floating Vegetation	1	0.038052	0.038052	11.4132	0.043144 *
Emergent Vegetation	1	0.231092	0.231092	69.3139	0.003632 **
Shoreline Vegetation	1	0.077643	0.077643	23.2882	0.016959 *
Willow	1	0.021026	0.021026	6.3067	0.086837
Residuals	3	0.010002	0.003334		

Table 14: ANOVA table of *R. draytonii Bd* infection prevalence model including vegetation for 2007.

Level	- Level	Difference	Std Err Dif	Lower CL	Upper CL	p-Value
HP	LP	0.5026558	0.0858455	0.239269	0.7660427	0.0027
HP	NP	0.3669846	0.1287683	-0.028096	0.7620649	0.0656
NP	LP	0.1356712	0.113563	-0.212757	0.4840993	0.4981

Table 15: Tukey-Kramer HSD table of *P. regilla Bd* infection prevalence categories for *R. draytonii Bd* infection prevalence in 2007. (NP=0% *Bd* prevalence, LP=0>5% *Bd* prevalence, HP=>5% *Bd* prevalence)

Level	- Level	Difference	Std Err Dif	Lower CL	Upper CL	p-Value
NP	LP	0.4199428	0.1283893	0.097421	0.7424648	0.0094
HP	LP	0.3013385	0.0701693	0.125069	0.4776084	0.0008
NP	HP	0.1186042	0.1292203	-0.206005	0.4432138	0.635

Table 16: Tukey-Kramer HSD table of *P. regilla Bd* infection prevalence categories for *R. draytonii Bd* infection prevalence in 2008. (NP=0% *Bd* prevalence, LP=0>5% *Bd* prevalence, HP=>5% *Bd* prevalence)

Model Parameter	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Pond Area	1	0.12242	0.122418	3.1421	0.09153
Periodicity	1	0.09636	0.096359	2.4732	0.13149
Intensive Horticulture Rank	2	0.28481	0.142407	3.6552	0.04436*
Residuals	20	0.77921	0.038961		

Table 17: Means for One Way ANOVA for Manageable Factors for *R. draytonii Bd* infection prevalence in 2008.

Level	- Level	Difference	Std Err Dif	Lower CL	Upper CL	p-Value
HP	LP	0.4148874	0.1631519	0.002116	0.8276587	0.0487
NP	LP	0.2380078	0.1048777	-0.027331	0.5033464	0.0838
HP	NP	0.1768796	0.1300857	-0.152235	0.5059939	0.3802

Table 18: Tukey-Kramer HSD table of Intensive Horticulture Rank categories for *R. draytonii Bd* infection prevalence in 2008. (NP=no proximity, LP=low proximity, HP=high proximity).

Level	- Level	Difference	Std Err Dif	Lower CL	Upper CL	p-Value
HP	LP	0.4620981	0.1633587	0.037779	0.8864176	0.0321*
HP	NP	0.3341597	0.1472496	-0.048317	0.7166361	0.0916
NP	LP	0.1279384	0.1225191	-0.190301	0.4461780	0.5616

Table 19: Tukey-Kramer HSD table of *R. draytonii Bd* infection prevalence categories for *P. regila Bd* infection prevalence in 2008. (NP=0% *Bd* prevalence, LP=0>5% *Bd* prevalence, HP=>5% *Bd* prevalence)

Model Parameter	Df	Sum Sq	Mean Sq	F value	Pr(>F)
PondArea	1	0.13320	0.133201	2.3752	0.1456
DistanceToOcean	1	0.17296	0.172961	3.0842	0.1009
TerrainMasking	1	0.06878	0.068776	1.2264	0.2868
Residuals	14	0.78513	0.056080		

Table 20: Means for One Way ANOVA for Temperature Proxies for *P. regilla* 2007 *Bd* prevalence

Model Parameter	Df	Sum Sq	Mean Sq	F value	Pr(>F)
SubmergedVeg	1	0.20369	0.203694	3.5295	0.08127 .
FloatingVeg	1	0.04915	0.049146	0.8516	0.37174
Willow	1	0.09925	0.099248	1.7197	0.21083
Residuals	14	0.80798	0.057713		

Table 21: Means for One Way ANOVA for Vegetative Cover for *P. regilla Bd* infection prevalence in 2007

Level	- Level	Difference	Std Err Dif	Lower CL	Upper CL	p-Value
NP	LP	0.1593162	0.0691644	-0.011497	0.3301291	0.0712
NP	HP	0.0885168	0.0856448	-0.122997	0.3000308	0.5622
HP	LP	0.0707994	0.0944966	-0.162576	0.3041743	0.7365

Table 22: Tukey-Kramer HSD table of Intensive Horticulture Rank categories for *P. regilla* 2008 *Bd* prevalence. (NP=no proximity, LP=low proximity, HP=high proximity).

Parameter	BF 2008 ***	CRLF 2008	CRLF 2007	PTF 2008	PTF 2007
Amphibian Diversity	**	0.08	0.23	0.12	0.45
CRLF *Bd* Categorical	**	*	*	0.18	0.72
PTF *Bd* Categorical	**	0.26	0.42	*	*
BF *Bd* Categorical	*	**	*	**	*
Periodicity	0.27	0.08	0.20	**	**
Predator Index	0.16	**	**	**	0.65
Pond Area	0.53	0.13	0.07	0.06	**
Elevation	0.39	**	0.08	0.15	0.07
Depth	**	**	0.07	**	**
Terrain Masking	0.50	**	0.28	**	**
Distance To Ocean	**	**	**	**	**
Intensive Horticulture Rank	0.64	0.18	0.44	0.13	0.71
Floating Vegetation	0.07	**	**	**	**
Shoreline Vegetation	**	0.13	0.09	**	0.09
Emergent Vegetation	0.69	0.40	0.36	**	**
Submerged Vegetation	**	**	0.06	**	0.20
Willow	0.14	**	**	0.06	**
Julian Date	**	**	0.14	**	**
Property	0.27	0.10	*	0.06	*

Table 23: R^2 Values for single parameter models that were used in the selection of factors to go into *Bd* infection intensity multivariate models (BF=*L. catesbeiana,* CRLF=*R. draytonii,* PTF=*P. regilla).*

***Not Enough Data Points To Justify Inclusion In Analysis.**

****Not enough ponds to conduct analysis**

Year/Species/Covariates	n	ΔAICc	AICc weight
***R. draytonii* 2008**			
Vegetation: Emergent Vegetation* Shoreline Vegetation	19	0	0.999
Species Diversity: Amphibian Community Composition	19	35.726	0.001
***P. regilla* 2008**			
Species Diversity: Amphibian Community Composition	18	0	0.998
Vegetation: Willow	18	12.442	0.002

Table 24: The top 2 AICc candidate models for *Bd* Infection Intensity for each species in 2008.

2. Chapter 2: Response of Body Condition and Leukocytes to *Batrachochytrium dendrobatidis* Infection, Predation, and Competition in Three Species of Anuran Larvae

2.1 Abstract

Knowledge of organisms' physiological responses to changes in the environment can help us understand how alterations challenge species to respond and can better help us predict how changes impact various species. Lotic tadpoles are particularly plastic in many of their responses to environmental changes, occur in discrete ponds that function as replicates, and thus present a particularly suitable opportunity to explore ecological relationships under complex, natural conditions. In this study, I investigated the relationship of body condition, white blood cell counts, and neutrophil to lymphocyte ratios to three stressors, infection with an emerging infectious disease called *Batrachochytrium dendrobatidis* (*Bd*), amphibian community diversity, and predator diversity in three co-occurring species of tadpole, including two natives, *Rana draytonii* and *Pseudacris regilla,* and one introduced, *Lithobates catesbeiana*. My study spanned 39 pond sites with multiple samples per site. Thus I took a mixed modeling approach treating pond as a fixed factor and tadpole body condition, white blood cell counts and neutrophil to lymphocyte ratios as random. I then used corrected Akaike's Information Criterion to select the most parsimonious models.

I found that *Bd* infection was positively correlated with body condition in *L. catesbeiana* tadpoles, whereas it was not among the top ranked models for *R.*

draytonii and *P. regilla*. The top-ranked models explaining tadpole body condition included *Bd* infection and predator diversity for *L. catesbeiana,* with both being positively correlated, amphibian community diversity for *R. draytonii* with a weak negative correlation, and predator diversity index for *P. regilla,* also with a weak positive correlation.

None of my combinations of variables adequately explained tadpole neutrophil to lymphocyte ratios, aside from a positive correlation with *Bd* infection for *L. catesbeiana*. White blood cell counts, however, were negatively correlated with predator diversity for *L. catesbeiana*, positively correlated with amphibian diversity in *R. draytonii* and positively correlated with *Bd* infection in *P. regilla*. These blood metrics provide a unique insight into the stressors that impact amphibian health in complex, real-world conditions.

My findings suggest a complex set of biotic and abiotic conditions acting on tadpole physiology and which varied across species, reinforcing the difficulty in generalizing across species when planning conservation actions.

2.2 Introduction

Species have always had to respond to constantly changing and complex environments. However, rapid anthropogenic changes to environmental conditions pose new challenges that may emerge too quickly to allow successful adaptation (Carey, 2005). Knowledge of an organisms' physiological responses to environmental stress can help us better predict lethal and sub-lethal impacts on

species, and comparison of those impacts among species and locales can enhance our understanding of the scope of these consequences. Biotic and abiotic influences are commonly studied in one species at a time and under controlled conditions. This is an important approach to help clarify complex systems, however, it is also essential to examine influences of multiple factors acting simultaneously in the natural environment. Studies across many sites and species that include a diversity of biotic and abiotic influences may help illuminate dissimilarities and general trends in the multitude of threats these species face. Additionally, they provide a rare glimpse of the impacts of threats under complicated, real-world conditions.

Amphibians appear to be particularly vulnerable to extinctions under present conditions (Stuart et al., 2004), with current rates of extinction 211 times historic background rates (McCallum, 2007). Challenged with severe declines in amphibian populations, it is important to understand the interacting and cumulative impacts of the numerous threats they face. These impacts include degraded water quality (Berrill et al., 1994; Harris et al., 1998; Hayes et al., 2002; Brodman et al., 2003; Houlahan, and Findlay, 2003), habitat fragmentation and loss (Blaustein et al., 1994; Corn, 2000; Green, 1997), non-native competitors and predators (Fisher, and Shaffer, 1996; Lawler et al., 1999; Knapp et al., 2007), and emerging infectious diseases (Daszak et al., 1999; Blaustein, and Kiesecker, 2002), among others. Co-occurring species may respond differently to different stressors. Consequently, investigating impacts across species can help avoid spurious generalizations and identify and

prioritize research directions, potentially targeting more effective conservation actions for species of concern.

A relatively new concern for amphibian populations is emerging infectious disease. *Batrachochytrium dendrobatidis* (*Bd*), which causes chytridiomycosis in amphibians, has been implicated in the decline of many amphibian populations globally (Berger et al., 1998; Daszak et al., 2003; Wake, and Vredenburg, 2008). *Bd* is a water-born fungus that attacks keratin, a protein found in the skin of adult frogs and the mouthparts of amphibian larvae (Marantelli et al., 2004; Olsen et al., 2004; de Castro, and Bolker, 2005; Berger et al., 2005). The likely mechanism of *Bd*'s lethality in adult amphibians comes via disruption of osmoregulation through the epidermis, leading to cardiac arrest (Voyles et al., 2009). Recent research suggests that the disease is widespread in apparently healthy North American populations and that the pathway to lethality in *Bd* infection is complex (Ouellet et al., 2005). For example, *Bd* may become lethal or animals may be more susceptible to the infection when exposed to other stressors such as predators, climate change or pesticides (Davidson et al., 2007; Gabor et al., 2013).

In this study, I aim to understand the relative importance and relationship between amphibian community diversity, predator diversity and infection by *Bd* in three anuran species widespread along the Central Coast of California. My study focuses on two native and one introduced, invasive amphibians. *Rana draytonii* (California red-legged frog) are federally listed as threatened due to extirpation from over 70% of its historic range. Much of its remaining habitat is in agricultural regions

where they are often relegated to agricultural drainages and ponds (Fellers, 2005). *Pseudacris regilla* (Pacific chorus frogs) is a smaller local amphibian, that is highly vagile and thought to form an important prey base for adult *R. draytonii* and adults of other species, including *Lithobates catesbeiana* (American bullfrogs). *L. catesbeiana* is a large, introduced anuran whose adults compete with and prey on post-metamorphic *R. draytonii* (D'Amore et al., 2009). Controlling and removing bullfrogs throughout their introduced range globally is a conservation priority for many native anurans (D'Amore, 2011).

Known effects of *Bd* differ between species and hosts. It has also been suggested that the ubiquitous, widely invasive *L. catesbeiana* are carriers of *Bd,* and they do not experience mortality due to infection (Daszak et al., 2004; Blaustein et al., 2005), although one study suggests that some strains do cause mortality even if they may not be competent long-term carriers (Gervasi et al., 2013). There has been one study investigating the fate of *Bd*-infected *R. draytonii* that found no mortality in captive animals over 18 months (Padgett-Flohr, 2008), but remains unclear whether *R. draytonii* in a wild setting with continuous exposure to the pathogen experience detrimental effects from infection with *Bd*. *P. regilla* have been found to act as a carrier for *Bd,* and their populations may have lower levels of infection as compared to other species in the western United States where they are widespread (Pearl et al., 2007). Despite this, 16% of post-metamorphic *P. regilla* died during experimental infections (Gervasi et al., 2013).

In order to understand how different amphibian species are affected by amphibian diversity, predator presence and *Bd* infection, I chose to work on their larval stage. The growth and development of tadpoles are particularly sensitive to biotic and abiotic stressors (Alford, 1999; Relyea, 2002). The life histories of these three anuran larvae vary significantly, with *L. catesbeiana* generally requiring more than one to three years (Collins, 1979; Bury, 1984) and permanent water habitats to metamorphose and *R. draytonii* and *P. regilla* generally requiring 8-10 weeks (Nussbaum et al., 1983) or 14-28 weeks (Storer, 1925; Wright, and Wright, 1949; Jennings and Hayes, 1989) respectively. *P. regilla* distribution is highly variable across years, reproducing in more ephemeral habitats than can the larger *R. draytonii* (D'Amore et al., 2009). Despite these differences, the three species readily co-occur in the pond systems throughout the Central Coast of California.

In order to quantify larval response to predator diversity, community diversity and *Bd*, I used an established method to gain an in situ snapshot of relative stress for these anurans in their breeding ponds. The white blood cell counts and neutrophil to lymphocyte ratios of amphibians respond to a host of environmental stressors (Turner, 1988; Pottinger, and Pickering, 1992; Bartolomucci, 2007; Davis, and Maerz, 2009; Dhabhar, 2009; Schnüriger et al., 2010; Delehanty, and Boonstra, 2011; Clinchy et al., 2013). These blood metrics are reliable indicators of relative stress levels experienced by the species in real world conditions. I hypothesize that infection with *Bd* (*Bd* incidence) or higher *Bd* infection intensity and increased number of competitor species in a pond would result in decline in body condition of

all three species, whereas tadpoles without *Bd* infection will be more strongly affected by predator diversity. I also hypothesize that white blood cell counts and neutrophil to lymphocyte ratio will be unaffected or depressed with increasing numbers of predator species, whereas neutrophil to lymphocyte ratios will be elevated in tadpoles infected with *Bd* or those with higher *Bd* infection intensity. Understanding the relative impact of these stressors for each species will help direct conservation measures for *R. draytonii* and may help point to indirect methods of control of invasive *L. catesbeiana.* By examining this suite of physiological responses across species, I add to the growing body of work examining the complex interactions of physiological traits within heterogeneous and dynamic environments.

2.3 Methods

Field Data Collection

Study site: Between June and August 2008, I collected data and samples from tadpoles in a total of 37 ponds in the Monterey Bay region of central coastal California. These ponds are located across various properties including the Elkhorn region, Wilder Quarry on the north end of the Bay, and Santa Lucia Conservancy on the south end of the Bay (Figure 1). This region is generally temperate with a Mediterranean climate.

Larval sampling: During up to five hours of sampling in each pond, I attempted to capture 20 tadpoles of each species. If I had not captured 20 tadpoles after five hours of sampling, my sample size consisted of the tadpoles I had caught. Using dipnets I made sweeps through the entire pond, endeavoring to collect tadpoles

from throughout the pond despite their occasional aggregation. I placed tadpoles individually in containers filled three-quarters with pond water.

Disease testing: I collected a *Bd* sample from tadpoles by gently swabbing their mouthparts with a sterile swab (Obendorf, 2005). I noted their Gosner Developmental Stage (GDS)(Gosner, 1960), mass, and length. Finally, to estimate relative amounts of various white blood cells I made blood smears on glass slides from the drop of blood that accumulated at the raw edge of a two-millimeter long tail clip that I collected for another study. Once samples were collected, the tadpoles were released near their point of capture in their natal pond. To minimize the potential spread of pathogens, all field gear was sterilized between pond visits using a standard protocol with chlorine bleach (Speare et al., 2004; U. S. Fish and Wildlife Service, 2005).

Predator presence and index: To document the predator community, I noted the presence or absence of several common aquatic invertebrate predators by sampling with a fine-meshed net at three locations per pond. I also recorded the predatory fish and amphibian species I encountered while sampling for tadpoles and used my historical knowledge of fish and amphibian diversity in these ponds. I made a composite variable representing predator stress on tadpoles called Predator Index, which is equal to the sum of weights given to each species found in the pond (Appendix A). Predators that prey only on small tadpoles were given a weight of 0.5, whereas predators preying on large or all stages of tadpoles were given a weight of one. Predator index ranges from zero to three. To calculate the Amphibian Diversity

Index, each amphibian species found in a given pond was given a score of one (any stage of *L. catesbeiana*, *R. draytonii*, *P. regilla*, *Taricha torosa*, *Taricha granulosa*, *Anaxyrus boreas halophilus*, and *Ambystoma californiense*), and then I summed them for each pond, subtracting one to take into account the dependent variable species. Amphibian Diversity Index ranges from zero to three.

Quantification of *Bd* infection: To test for relationships between tadpole developmental stage and body condition with predator index and amphibian diversity index, I tested the *Bd* swab samples I collected in the lab. I transported *Bd* swab samples to the lab, and I stored them in a freezer at -20C until analysis. To extract the DNA from the swabs in their Eppendorf storage tubes, I added 40µl of PrepMan Ultra to cover the swab tip, heated the samples to between 95 and 100C for two minutes, placed them in a centrifuge for three minutes at 13,000 RPM, and placed the resulting fluid in a sterile Eppendorf tube. I then diluted this sample tenfold with sterile water. I analyzed the resulting diluted samples according to standard procedure (Boyle et al., 2004), running all samples in triplicate using a quantitative PCR (qPCR) assay and including an internal positive control (Hyatt et al., 2007).

With careful handling in the field and the lab, the negative success rate for the swabs in the lab was 100%, with no false positives, thus my tests provided good specificity (Skerratt et al., 2011). This is particularly helpful when deciding how to deal with indeterminate results, those with only one or two of a triplicate returning a positive result. If the likelihood of contamination is very low to null (i.e., no false positives in negative controls), then classifying the indeterminate results as positives

can increase the sensitivity of the test (Hyatt et al., 2007; Skerratt et al., 2011) . Thus, any of my samples that had a single positive out of a triplicate was treated as a positive result. For samples that tested positive, I averaged the positive results of the triplicate run to obtain a single genetic equivalent score.

To obtain a more biologically relevant *Bd* genetic score, I multiplied the genetic equivalent by the dilution factor, the amount the sample had been diluted during extraction and by addition of the quantitative PCR reagents. This returned the corrected zoospore genetic equivalent score, the *Bd* z_{swab} score, also called the *Bd* infection intensity. Typically the dilution factor was 80, but when the internal positive control indicated inhibition, I diluted the sample by a further factor of ten. Thus the dilution factor for those samples was 800. Any sample with a *Bd* z_{swab} score greater than zero was treated as a positive sample (the lowest positive *Bd* z_{swab} score was 0.008, with only nine of the 358 positive samples scoring below one zoospore genetic equivalents).

To normalize *Bd* infection intensity data for analysis, I calculated the natural log of the averaged data plus one. I categorized individual tadpole *Bd* infection incidence by identifying tadpoles with *Bd* infection intensity of zero as zero and any tadpole with *Bd* infection intensity greater than zero as one.

Quantification of blood markers: To estimate total white blood cell counts and neutrophil to lymphocyte ratios, I performed a differential blood cell count on the blood smears taken from the tadpoles. The method of blood collection did not provide a large quantity of blood to work with, but was the least invasive method to

collect blood from the tadpoles as they were already being clipped for another study. Due to the low quantity of blood, only about a third of the blood smears were readable.

Staff from IDEXX Laboratories mounted coverslips on the slides and stained the blood cells with Wright's stain (Fischer Scientific) on a commercial automated stainer (Hema-Tek). They made white blood cell estimates by counting the number of white blood cells per ten different 50 X magnification oil immersion fields (taking the average per field) in areas where blood most approximated a monolayer. Due to clots in many samples, it was difficult to find ten evenly distributed samples, thus the white blood cell estimates should be treated as rough estimates and not absolute quantifications.

Once IDEXX staff obtained the average number of cells per field, they multiplied the average by the square of the objective (2500 per Olympus America Inc.) for the total number of cells per microliter. As this is an estimate instead of an absolute numerical value, it is convention to report it as plus or minus 1000 per microliter from the result. When cells were concentrated in clots, they believed the estimate to be artifactually high, so they estimated it plus zero and minus 2000. Alternatively, if the only well-distributed areas were close to the thinner edges where estimates may be artifactually low, they estimated by minus zero and plus 2000 for a higher range. I used the mean of this range for my analysis.

Statistical analyses:

Tadpole length to mass: I used linear regression to determine the strength of the relationship between length and mass for each species of tadpole, and whether that relationship differed by *Bd* infection status. According to Fick's Law, the relationship of length to mass in animals can be well approximated as the cube root of mass to the square root of length (Gotelli, and Ellison, 2004), so I transformed the data to reflect this relationship prior to analysis.

Tadpole GDS to mass: I examined the relationship of GDS to mass for tadpoles not infected with *Bd* by performing a one-way ANOVA separately for positive and negative *Bd* results for each species. These results would then help determine if I could use mass to developmental stage ratio as a proxy for tadpole body condition in further analyses.

Tadpole GDS to *Bd* infection: To assess whether *Bd* infection intensity would increase with developmental stage and then drop off at stage 42, I split stage into two categories, less than GDS 42 (prior to emergence of front legs and major changes in keratin distribution in mouthparts) and stage 42 and above. I used a one-tailed student's t-test to examine whether mean *Bd* infection intensity was significantly lower for tadpoles at developmental stage 42 and above than for those below developmental stage 42 for each species. To further evaluate the relationship between developmental stage and *Bd* infection intensity, I used a one-way ANOVA on *Bd* infection intensity and GDS for tadpoles below developmental stage 42 and with *Bd* infection intensity greater than zero.

Body condition to *Bd* infection: To estimate how body condition may vary with *Bd* incidence and *Bd* infection intensity, I examined the data using an ANOVA on the *Bd* incidence and a regression on the continuous *Bd* infection intensity data.

Multivariate analyses**:** To determine which variables best explain variation in tadpole body condition, white blood cell count, and neutrophil to lymphocyte ratio, I constructed and compared a series of alternative explanatory models to predict each dependent variable (Figure 6). Because my samples were taken in discrete ponds, there is a strong potential for lack of independence in these data. I opted to use a mixed modeling approach to account for this non-independence, and also control for data structure and variation due to pond. This allowed me to include all the data in a single, united set of models, which I could then compare, using AICc methods. In this mixed model, I treated pond as a random effect, estimating the variance across the ponds throughout the dataset and taking into account the non-independence of multiple samples collected within each of the 39 ponds. Response variables were body condition, white blood cell count, and neutrophil to lymphocyte ratio (Figure 6). I constructed sets of models for each of these response variables for each of the three amphibian species. Predator index, amphibian diversity index, and *Bd* infection were fixed effects. Models were fit with lmer in the lme4 package (version 1.1-7) in R (R Development Core Team 2014). I then used AICc model selection with the MuMln package in R (version 1.10.5), including a set of models with each combination of fixed effects.

For variables included in the top models, I examined several measures to interpret the parameters of interest. I calculated the relative importance of the variables in models with AICc weights greater than 10% with the predictor weight for each parameter, defined as the summed AICc weights for a given parameter. I also noted the significance of the fit of the linear effects model between the response variable and individual fixed effects in the top models. I noted the correlation coefficients for each variable of interest from the fit of the linear effects model (β-value) to better understand how the explanatory variables relate to the response variables for each species. I included two measures of *Bd* infection in these models. The first is whether a tadpole was infected with *Bd* or not, thus a binary measure of *Bd* infection that I call *Bd* incidence. The second measure is how infected individual tadpoles were, their *Bd* infection intensity.

2.4 Results

Relationship of Tadpole Mass to Length

L. catesbeiana: Mass-length relationships are very strong regardless of *Bd*-infection for *L. catesbeiana* tadpoles (Appendix B, Figures B1, B2, B3 and B4). Thus, I am confident in using mass, rather than length, for this study species.

R. draytonii: Mass-length relationships are very strong regardless of *Bd*-infection for *R. draytonii* tadpoles (Appendix B, Figures B1, B2, B3 and B4). Even the mass-length relationship for *Bd*-positive *R. draytonii* tadpoles is strong, despite a very small sample size due to a loss of length data (Appendix B, Figure B4). Thus, I am confident in using mass, rather than length for this study species.

P. regilla: The mass-length relationship for uninfected *P. regilla* tadpoles, though highly significant, is weaker ($r(142)= 0.53$, $p=<0.0001$, Appendix B, Figure B5). Equipment failure led to a loss of length data, thus there is an insufficient sample size (n=3) for *Bd*-infected *P. regilla* to test the relationship.

Relationship of Tadpole Mass to GDS

To examine how mass relates to GDS, I split the groups into those with and without *Bd* to avoid any influence of *Bd* infection on the relationship.

L. catesbeiana: Mean body mass differs significantly among the GDSs regardless of the absence or presence of *Bd* infection, [ANOVA: $F(13, 122) = 92.7185$, $p\leq 0.0001$], [ANOVA: $F(15, 46) = 3.0742$, $p = 0.0017$].

R. draytonii: Mean body mass differs significantly among the GDSs regardless of the absence or presence of *Bd* infection: [ANOVA: $F(20, 487) = 25.0479$, $p\leq 0.0001$], [ANOVA: $F(18, 143) = 6.8103$, $p\leq 0.0001$],

P. regilla: Mean body mass differs significantly among the GDSs regardless of the absence or presence of *Bd* infection [ANOVA: $F(19, 753) = 17.7786$, $p\leq 0.0001$], [ANOVA: $F(16, 112) = 3.9086$, $p\leq 0.0001$]

All three species exhibit a general increase or hyperbolic relationship between mass and GDS under both conditions, with or without *Bd* (Appendix C, Figures C1 – C6).

Relationship of *Bd* Infection and GDS

Mean *Bd* infection intensity is significantly greater for GDSs below 42 compared to stage 42 and above for all three tadpole species (Figure 2):

L. catesbeiana: (Mean (± SD)= 33.32 (131.01) versus 11.45 (13.62); t(196)= -1.948, p= 0.029) (Figure 2A)

R. draytonii: (Mean (± SD)= 124.6 (586.23) versus 2.71 (7.77); t(668)= -5.24, p≤ 0.0001) (Figure 2B)

P. regilla: (Mean (± SD)= 68.03 (392.8) versus 0.965 (2.03); t(928)= -5.16, p≤ 0.0001) (Figure 2C)

Within the broad category of GDS of tadpoles below stage 42, differences among stages in *Bd* infection intensity are mixed among the three species.

L. catesbeiana: *Bd* infection intensity varies significantly among stages (ANOVA: F(14, 178)= 1.8097, p= 0.0402) with a slightly positive relationship between GDS and *Bd* infection intensity (Figure 3A).

R. draytonii: I do not detect significant differences in *Bd* infection intensity among Gosner stages less than stage 42 (ANOVA: F(16, 620)= 0.7916, p= 0.70) (Figure 3B)

P. regilla: I do not detect significant difference in *Bd* infection intensity among Gosner stages less than stage 42 (ANOVA: F(17, 870)= 0.6658, p= 0.84) (Figure 3C).

Relationship of Body Condition and *Bd* Infection

L. catesbeiana: Body condition between *Bd* incidence levels for *L. catesbeiana* has the largest effect size (n = 53, Mean (± SD)= 0.69 (0.33) versus n = 141, Mean (± SD)= 0.26 (0.29); ANOVA $F(1, 191) = 74.50$, $p \leq 0.0001$), respectively (Figure 4A). Body condition and *Bd* infection intensity exhibit a significant positive relationship ($r(191)= 0.18$, $p \leq 0.0001$).

R. draytonii: With and without *Bd* incidence for *R. draytonii* are (n = 148, Mean (± SD)= 0.22 (0.092) versus n= 492, Mean (± SD)= 0.19 (0.11); ANOVA $F(1, 638)= 13.12$, $p= 0.0003$) (Figure 4B). Body condition and *Bd* infection intensity exhibit a significant positive relationship ($r(636)= 0.02$, $p = 0.0016$).

P. regilla: *P. regilla* (n= 119, Mean (± SD)= 0.02 (0.01) versus n= 768, Mean (± SD)= 0.02 (0.01); ANOVA $F(1, 885)= 13.62$, $p= 0.0002$), the effect sizes are negligible (Figure 4C). Body condition and *Bd* infection intensity exhibit a significant negative relationship. ($r(885)= 0.01$, $p= 0.0007$)

Which biotic factors best predict body condition?

Prior to testing for the influence of amphibian and predator diversity on tadpole body condition with mixed effects models, I tested for independence of these two factors across surveyed ponds. Amphibian and predator diversity are not significantly correlated (Pearson's chi-square: $X^2(9,37)= 15.151$, $p= 0.087$). Therefore, I include both of these variables in the set of models. For body condition

and each blood indicator, I conducted two sets of *Bd* models – those including *Bd* incidence and those including *Bd* infection intensity.

Models including *Bd* incidence

Of the set of mixed effects models I generated to explain variation in body condition that include tadpole *Bd* incidence, predator index, and amphibian diversity index as fixed factors and pond as a random factor, those factors that contribute to the top models vary among the three study species (Table 1A).

L. catesbeiana: The most parsimonious model includes *Bd* incidence and predator index (96% support), and the second best model adds amphibian diversity index (Table 1A). The correlation coefficients for the relationship between body condition and *Bd* incidence and predator index from the fit of the linear effects model for *L. catesbeiana* reveal a significant positive relationship for both [(β-value=0.2660, p= <0.0005), (β-value= 0.7567, p= <0.0005)] respectively.

R. draytonii: The top model includes the fixed effect amphibian diversity index with 92% support and the second model adds *Bd* incidence (Table 1A). Body condition of *R. draytonii* tadpoles is significantly negatively related to amphibian diversity index (β-value=-0.0408, p= <0.0005). Interestingly, ANOVAS examining the relationship between *R. draytonii* body condition and presence or absence of amphibian species singly shows significantly higher body condition when in the presence of three of these species and no significant effect on *R. draytonii* body condition in the presence of the other amphibian species: without and with *L. catesbeiana* (n = 492, Mean (± SD)= 0.18 (0.099) versus n= 194, Mean (± SD)= 0.22

(0.11); ANOVA F(1, 685)= 16.09, p= <0.0001), without and with *T. torosa* and *T. granulosa* (n = 488, Mean (± SD)= 0.18 (0.005) versus n= 198, Mean (± SD)= 0.23 (0.007); ANOVA F(1, 685)= 39.57, p= <0.0001), and without and with *A. boreas halophilus* (n = 648, Mean (± SD)= 0.18 (0.10) versus n= 38, Mean (± SD)= 0.28 (0.06); ANOVA F(1, 685)= 29.88, p= <0.0001).

P. regilla: The top model for body condition includes predator index with 96% support and the second top model adds amphibian diversity index (Table 1A). Body condition of *P. regilla* tadpoles is significantly positively related to predator index (β-value=0.0064, p= <0.0005).

Models including *Bd* infection intensity

Of the set of mixed effects models generated to explain variation in body condition that include *Bd* infection intensity, predator index, and the amphibian diversity index as fixed factors and pond as a random factor, those factors that contribute to the top models also vary among the three study species (Table 1B).

L. catesbeiana: The model with the greatest support for *L. catesbeiana* tadpole body condition includes *Bd* infection intensity and predator index with 94% support, and the second model adds amphibian diversity index (Table 1B). Body condition of *L. catesbeiana* tadpoles is significantly positively related to *Bd* infection intensity and predator index [(β-value=0.0552, p= <0.0005), (β-value= 0.7565, p= <0.0005)], respectively.

R. draytonii: The top models that explain tadpole body condition include amphibian diversity index with 98% support, and the second model adds *Bd* infection

intensity (Table 1B). As with the models explaining body condition for *R. draytonii* including *Bd* incidence, amphibian diversity index is significantly negatively related to body condition (β-value=-0.0408, p= <0.0005).

P. regilla: The top models that explain tadpole body condition include predator index with 96% support, and the second model adds amphibian diversity index (Table 1B). And like the models explaining body condition for *P. regilla* including *Bd* incidence, predator index is significantly positively related to body condition (β-value=0.0064, p= <0.0005), while it was negatively correlated to amphibian diversity index (β-value=-0.0032, p= 0.0058).

Which biotic factors best predict white blood cells counts?

Models including *Bd* incidence

L. catesbeiana: Of models that examine the relationship between white blood cell counts and *Bd* incidence, predator index, and amphibian diversity index, four models have AICc weights greater than 10% (Table 2A). Although *Bd* incidence and amphibian diversity index are in two of the four top models, predator index is a component of all four top models. Further, the predictor weight for *Bd* incidence is 0.46, whereas it is 0.73 for amphibian diversity index and 0.80 for predator index. Predator index has a very marginally significant negative correlation with white blood cell counts in the third and fourth models [(β-value= -2.6309, p= 0.1521), (β-value= -2.6104, p= 0.1591)], respectively.

R. draytonii: Of models examining the relationship between white blood cell counts and *Bd* incidence, predator index, and amphibian diversity index, four models

have AICc weights greater than 10%, with the full model three times more likely than the next model, *Bd* incidence and amphibian diversity index (Table 2A). In the top four models, each variable is represented an equal number of times (Table 2A), and the predictor weights are fairly similar, amphibian diversity index is 0.86, and *Bd* incidence and predator index each score 0.77. In the top model, amphibian diversity index is positively correlated with white blood cell counts, although the correlation is marginally significant (β-value=1.7260, p= 0.0692). In the second model, the correlation between white blood cell count and amphibian diversity index is strongly significant (p= 0.0002), as it is in the third model (p= 0.0218). *Bd* incidence and predator diversity are also significantly positively correlated with white blood cell count in the fourth top model [(β-value= 1.9785, p= 0.0323) (β-value= 2.6719, p= 0.0017)], respectively. These results suggest that although amphibian diversity index is most strongly supported, all three parameters are likely important in explaining white blood cell counts in *R. draytonii*.

P. regilla: Of models that examine the relationship between white blood cell counts and *Bd* incidence, predator index, and amphibian diversity index, four models have AICc weights greater than 10% (Table 2A). Of those models, all of them contain *Bd* incidence, whereas only two of the four contain either amphibian diversity index or predator index. The top-ranked model, which includes all three effects, is only 1.4 times more likely than the next model, the single-parameter model containing *Bd* incidence. Following, the predictor weight for *Bd* incidence is 0.92, and the predictor weights for amphibian diversity index and predator index are 0.57

and 0.56 respectively. White blood cell counts and *Bd* incidence are significantly positively correlated in both the top model (β-value= 2.7445, p= 0.0504) and in the second model (β-value= 2.851, p= 0.0400).

Models including *Bd* infection intensity

L. catesbeiana: Of models that contain white blood cell counts and *Bd* infection intensity, predator index, and amphibian diversity index, three models rank in the top candidate set with the top AICc weight only 3.3 times the weight of lowest top-ranked model (Table 2B). Predator index and amphibian diversity index are the only components of these models; *Bd* infection intensity is not a component of any of the top-ranked models. The predictor weight for amphibian diversity index is 0.74, and it is 0.82 for predator index. The second model is the only one containing a significant correlation; in this case white blood cell count and predator diversity is very marginally significantly and negatively correlated (β-value= -2.6309, p= 0.1521).

R. draytonii: Of models that contain white blood cell counts and *Bd* infection intensity, predator index, and amphibian diversity index, three models rank in the top candidate set. The top model, predator index and amphibian diversity index, is just 2.2 times more likely than the next top model containing amphibian diversity index (Table 2B). Amphibian diversity index is a component of all three top models and its predictor weight is also highest at 0.90 compared to 0.73 for predator index and 0.24 for *Bd* infection intensity. The positive relationship between white blood cell count and amphibian diversity in the top model is marginally significant (β-value= 1.7470,

p= 0.0781), but in the second model they are very strongly correlated (β-value= 2.9895, p=<0.001). Although the predictor weight for predator diversity is high, it occurred in two of the three models and is not significantly correlated with white blood cell count.

P. regilla: Of models that contain white blood cell counts and *Bd* infection intensity, predator index, and amphibian diversity index, all seven models rank in the top candidate set with AICc weights ranging from 11-20% (Table 2B). All effects are equally represented throughout the models, and predictor weights are similar for *Bd* infection intensity (0.57), predator index (0.62), and amphibian diversity index (0.64). Although white blood cell counts are not significantly correlated with predator index or amphibian diversity index for any of the linear effect models, it is marginally positively significant for *Bd* infection intensity in the second, third, fourth, and sixth models [(β-value= 0.4693, p= 0.0931), (β-value= 0.4940, p= 0.0738), (β-value= 0.4826, p= 0.0828), (β-value= 0.4907, p= 0.0763)], respectively.

Which biotic factors best predict neutrophil to lymphocyte ratios?

Models including *Bd* incidence

L. catesbeiana: Of the models that examine the relationship between neutrophil to lymphocyte ratio and *Bd* incidence, predator index, and amphibian diversity index, three models have AICc weights greater than 10%, and they contain only *Bd* incidence and predator index (Table 3A). Predictor weight for *Bd* incidence is 0.78 whereas it is a mere 0.42 for predator index. Although predator index is not significantly correlated with neutrophil to lymphocyte ratio in any of the linear fixed

effects models, *Bd* incidence is significantly positively correlated in first and second models [(β-value= 0.3997, p= 0.0436), (β-value= 0.4255, p= 0.0295)], respectively.

R. draytonii: Models for *R. draytonii* have little support for our suite of factors in explaining neutrophil to lymphocyte ratios; AICc weights are very similar across all the single-parameter top models (Tables 3A and 3B). Of these, predictor weights for their effects are all less than 0.42, and none have a significant correlation with neutrophil to lymphocyte ratio in any of the linear fixed effects models.

P. regilla: Models for *P. regilla* have little support for our suite of factors in explaining neutrophil to lymphocyte ratios; AICc weights are very similar across all the single-parameter top models (Tables 3A and 3B). Of these, predictor weights for their effects are all less than 0.42, and none have a significant correlation with neutrophil to lymphocyte ratio in any of the linear fixed effects models.

Models including *Bd* infection intensity

L. catesbeiana: Of the models that examine the relationship between neutrophil to lymphocyte ratio and *Bd* infection intensity, predator index, and amphibian diversity index, two models have AICc weights greater than 10%. These models are single-parameter models, and they contain amphibian diversity index and predator index (Table 3B). Although predictor weights favor predator index (0.63) over amphibian diversity index (0.38), neither effect is significantly correlated with neutrophil to lymphocyte ratio for either of the linear fixed effects models.

R. draytonii: Models for *R. draytonii* have little support for my suite of factors in explaining neutrophil to lymphocyte ratios; AICc weights are very similar across

all the single-parameter top models (Tables 3A and 3B). Of these, predictor weights for their effects are all less than 0.42, and none have a significant correlation with neutrophil to lymphocyte ratio in any of the linear fixed effects models.

P. regilla: Models for *P. regilla* have little support for my suite of factors in explaining neutrophil to lymphocyte ratios; AICc weights are very similar across all the single-parameter top models (Tables 3A and 3B). Of these, predictor weights for their effects are all less than 0.42, and none have a significant correlation with neutrophil to lymphocyte ratio in any of the linear fixed effects models.

2.5 Discussion

Implications for larval sampling and field study of *Bd*

Several of the findings of this study have useful application for further field studies of these species specifically and for studying *Bd* in amphibian larvae generally. For example, understanding the strength of mass to length relationships in anuran larvae is useful in potentially streamlining future field collection efforts. The extra step of measuring length or weighing individual larvae can add a minute or more to the processing time for each individual handled. Being able to use one as a proxy for the other can save significant amounts of time in the field. *L. catesbeiana* and *R. draytonii* follow a predictable pattern of increasing mass with length, regardless of *Bd* infection status. This does not follow for *P. regilla*, the smallest of these tadpole species. To check whether decreased accuracy in my measuring devices at lower masses may be responsible for this result, I tested the relationship for a subset of *L. catesbeiana* and *R. draytonii* tadpoles in the same size range as *P.*

regilla tadpoles. The small *L. catesbeiana* and *R. draytonii* tadpoles maintain their strong positive correlation. Thus my results likely reflect that unlike *L. catesbeiana* and *R. draytonii* tadpoles, length and mass are not strongly correlated in *P. regilla* tadpoles, likely due to their large, protruding abdomens and relatively thin tail. This is important for my study as near the end of my field data collection I had an equipment failure resulting in the loss of much of the tadpole length data. Length is commonly used in studies of tadpole metrics (Altig, and McDiarmid, 2000), but my study indicates that tadpole mass may be substituted in studies of metrics for some anuran larval species.

As I predicted, tadpole mass generally increases with GDS. However, except for uninfected *L. catesbeiana,* (Appendix C, Figure C1), there is a large amount of variation around the mean for each of the species I tested. This large variation between GDS and mass highlights that some factor or set of factors besides GDS impact mass in tadpoles, regardless of *Bd* infection status. GDS is essentially an indicator of a tadpole's development towards metamorphosis, and it is well known that environmental triggers like pond dry-down may accelerate or decelerate an individual's progression, regardless of chronological age. Various pressures may play a role in tadpole body condition, including predation pressure, competition, temperature, food quality and quantity (Venesky et al., 2012), and infection. Thus I caution other researchers from using GDS alone as a proxy for mass.

I found a marked drop in *Bd* infection intensity when tadpoles reached GDS 42. This can be explained by mortality of infected tadpoles at stage 42, although this

is an unlikely explanation for *R. draytonii* given 100% survivorship of *Bd*-positive tadpoles held through metamorphosis in a field caging study (Hemingway, unpublished study). A more likely explanation is that keratin distribution changes as tadpoles develop. Throughout their development, tadpoles have keratin in their mouthparts until they are shed between GDS 41 and 42 (Marantelli et al., 2004). This seems the most likely reason *Bd* infection intensity in all three species drops precipitously between stage 41 and 42. This has important implications for scientists surveying for *Bd* infection, suggesting that when looking for presence or absence of *Bd*, efforts should be made to sample tadpoles below GDS 42.

Impacts of *Bd* on body condition

My study found generally greater variation in body condition for tadpoles infected with *Bd* (Appendix C, Figures C2-C6). The greater variation in mass of tadpoles with *Bd* makes intuitive sense, as larvae may exhibit emaciation, little to no symptoms or increased susceptibility to secondary infections that cause edema. In addition, if tadpoles are large for their GDS, it could suggest that progress towards metamorphosis may be hindered by *Bd* infection, i.e. that tadpoles are not growing limbs or otherwise progressing to metamorphosis, despite having reached the mass at which they would generally do so (Parris, and Baud, 2004). Other researchers have suggested individuals gaining extra mass in order to metamorphose sooner could also explain this phenomenon. By metamorphosing and exiting the pond environment, these individuals are potentially escaping further infection (Searle et al., 2014).

Although I expected that *Bd* load would increase in tadpoles as they develop and spend more time in the pond with other potentially infected amphibians as others have found (Smith et al., 2007), *Bd* infection intensity is only correlated with developmental stage in *L. catesbeiana*. *L. catesbeiana* is often characterized as a carrier of *Bd* that is not strongly impacted by infection (Daszak et al., 2004), so it may be that as tadpoles develop in infected waters, they have a greater chance of becoming infected and living to be assessed. The prolonged larval stage of *L. catesbeiana* means that it spends much longer in the ponds, thereby increasing possibility of infection in each developmental stage.

The lack of correlation between GDS and *Bd* infection intensity in *R. draytonii* and *P. regilla* is unexpected at first glance, but may be explained by the fact that these animals may move through the different developmental stages quite rapidly when in-pond conditions are not ideal due to drying or predator pressure. Simply put, GDS is not as closely related to time spent in the pond for these quicker-developing species as may be for the slower-developing *L. catesbeiana.*

Equally surprising is the lack of a clear relationship between body condition and *Bd* infection in most of my study species. I expected some of the variation in body condition to be correlated with *Bd* incidence or infection intensity as other researchers have found (Parris, and Cornelius, 2004; Parris, 2004). There is considerable evidence in the literature for large differences in the virulence of different strains of *Bd.* It is possible that the lack of relationship between body condition and infection is a result of the particular strain of chytrid in my study

system being a relatively benign variety, or that those larvae it kills are killed swiftly before I had the opportunity to capture and assess them.

This is only the case for the relationship of *L. catesbeiana* body condition and *Bd* incidence; mass is greater for developmental stage in infected tadpoles. *L. catesbeiana* body condition is not altered by the intensity of their infection, lending further support for categorizing these animals as carriers of *Bd*.

Relative importance of factors affecting body condition of tadpoles

Consistent with the results of body condition and *Bd* infection above, the mixed effects models ranking factors explaining body condition do not rank *Bd* infection as a top model for *R. draytonii* and *P. regilla*. Additionally, for *L. catesbeiana* the top model containing predator index and *Bd* infection favors predator index as the stronger influence on body condition. While it is surprising that *Bd* infection was not a primary explanation in tadpole body condition for any of the three species I studied, given the relationships of growth to *Bd* infection in other species (Parris, and Beaudoin, 2004; Garner et al., 2009), my results emphasize the complex ecological interactions that play a role in plastic phenotypic traits (Morin, 1986; Relyea, 2003). In this case a subset of biotic factors represented by predator index and amphibian community diversity index appear to better explain tadpole body condition in these three species

Amphibian species can act both as competitors and predators on larval amphibians. *L. catesbeiana*, *R. draytonii, P. regilla,* and *Anaxyrus boreas halophilus* tadpoles use similar food and refuge patches for which they likely compete,

potentially limiting tadpole density and growth (Savage, 1952; Leonard et al., 1993; Kupferberg, 1997; Smith, 2005). Many studies have found that high densities of amphibian larvae can reduce growth (Bardsley, and Beebee, 2000; Dash, and Hota, 1980; Relyea, 2003). One study in particular found high densities of *L. catesbeiana* larvae have a strong negative impact on growth in *R. boylii,* a species closely related to *R. draytonii*, although greater larval density had a much smaller impact on *P. regilla* and *L. catesbeiana* larval mass (Kupferberg, 1997). Alternatively, adult *R. draytonii* and adult and larval *L. catesbeiana*, *Taricha torosa*, *Taricha granulosa*, and *Ambystoma californiense* are likely to be at least occasional predators on tadpoles and have indirect effects on tadpole behavior such as decreasing consumption of higher quality food resources, decreasing activity, and seeking refuge sites or they can decrease intraspecific competition leading to increased body size (Pimentel, 1952; Anderson, 1968; Neish, 1971; Ehrlich, 1979; Hayes, 1985; Baldwin, 1987; Lawler, 1989; Kiesecker, 1997), potentially causing pressure on tadpoles to metamorphose sooner (Morin, 1986).

Increasing predator diversity is hypothesized to have three potential impacts on herbivore density, competition, and behavior: A) different predator species may complement each other in consuming herbivores, thus leading to decreased herbivore density thus decreased competition, B) predator species experience interspecific interference thus reducing predation on herbivores and increasing likelihood of interspecific competition, and C) increasing numbers of predators in a system increases the likelihood that one predator species will be a keystone species that will

have an inordinate impact on herbivore prey density and behavior (Byrnes et al., 2006).

In several studies, tadpoles exposed to predators were larger (Werner, 1986; McCollum, and Leimberger, 1997; Caldwell et al., 1980). It is hypothesized that larger tadpole body size when exposed to predators is adaptive; larger tadpoles exceed the size that some of their predators are capable of consuming, can swim faster to avoid predation, and they can metamorphose sooner (Caldwell et al., 1980; Travis et al., 1985; WASSERSUG, and HOFF, 1985; Werner, 1986; McCollum, and Leimberger, 1997). The mechanism for this may be via a decrease in intraspecific competition, potentially leading to decreased competition for food resources and an increase in tadpole growth (Ullyett, 1950; Morin, 1986; Wilbur, 1972). Alternatively, tadpole prey species may respond to predators by decreasing foraging activity or eating in lower-quality patches thus reducing growth (Sih, 1987; Lima, and Dill, 1990; Kotler et al., 1991; Diehl, and Eklov, 1995; Kupferberg, 1997), although this may be temporary with smaller tadpoles taking this tactic and then increasing foraging activity and diet quality later in development after predators have decreased competition or they are able to escape (Fauth, 1990).

My study found both *L. catesbeiana* and *P. regilla* have a larger mean mass for their developmental stage in the presence of a greater diversity of predators. This lends support to either a decrease in competition for resources for these tadpole species in sites that contain greater predator diversity or a keystone predator that is present in sites with greater predator diversity. When examined singly, *R. draytonii*

body condition was positively correlated with *L. catesbeiana, T. torosa*, *T. granulosa*, *A. californiense* and *A. boreas halophilus.* This relationship may support the explanation of a predatory relationship between *R. draytonii* and *L. catesbeiana, T. torosa*, *T. granulosa*; when these predators are in a site with *R. draytonii,* they may decrease intraspecific competition via predation, thus increasing *R. draytonii* access to higher quality resource patches, ultimately increasing body condition of the remaining animals. If these species were acting primarily as competitors, the body condition would more likely be negatively correlated due to competition for the same resources throughout tadpole development. The positive relationship between *R. draytonii* and *A. boreas halophilus* likely reflects the habitat where these tadpoles breed rather than competition or predation; *A. boreas halophilus* larvae are found in shallow, warm ephemeral ponds that favor quick growth and development of tadpoles.

Seemingly contrary to results of single relationships between *R. draytonii* and the co-occurring amphibians, in the united model, *R. draytonii* body condition is negatively correlated with increasing amphibian diversity. This relationship likely reflects the cumulative impacts of multiple predator and competitor species acting in various ways and on various sizes of *R. draytonii* tadpoles in pond sites; different pressures on *R. draytonii* from many sources may tip the balance to decreased body condition. Importantly, if the amphibians in my study system exert strong predation pressure on *R. draytonii* tadpoles, I would have expected strong support for the model with both predator index and amphibian diversity index. The lack of support for the

combined model in explaining body condition for *L. catesbeiana, P. regilla,* or *R. draytonii* provides a clue that the amphibians in this community may not be exerting strong predation pressure, but instead are decreasing *R. draytonii* access to high quality resource patches. Alternatively, as noted above, another study found that *L. catesbeiana* and *P. regilla* mass was not as strongly impacted by interspecific competition as a species closely related to *R. draytonii*. Thus, it may be that *R. draytonii* is particularly sensitive to competition when compared to the other two species.

Larval blood metrices -- a window into larval stress

Relative white blood cell counts: Many studies have highlighted a relationship between abiotic and biotic environmental factors present in natal ponds and tadpole leukocyte levels. Some environmental factors such as presence of predators, breeding, or interspecific competition can cause stress leading to decreased white blood cell counts (Delehanty, and Boonstra, 2011; Clinchy et al., 2013). Alternatively, trauma and active infection are associated with elevated white blood cell counts (Turner, 1988; Schnüriger et al., 2010). Consistent with these studies, I find that white blood cell counts are positively correlated with both *Bd* incidence and *Bd* infection intensity in *P. regilla* tadpoles. Leukocyte counts in *L. catesbeiana* are marginally negatively associated with predator index; long-term exposure to predators can cause stress in animals, leading to elevated glucorticoid stress hormones thus lowering leukocyte counts (Delehanty, and Boonstra, 2011). Somewhat surprisingly I find *Bd* incidence and *Bd* infection intensity to be positively associated

with predator index in *R. draytonii*, whereas other studies indicate that continual stress can lead to decreased leukocyte counts in other species (Delehanty, and Boonstra, 2011; Clinchy et al., 2013). I noticed that in pond sites with many predators, tadpoles often had lacerations characteristic of invertebrate predator damage (Semlitsch, 1990), particularly on their tailfins (Hemingway, personal observation), thus elevated leukocyte levels may be a response to trauma and potential infection due to predator attacks rather than to stress caused solely by presence of predators.

White blood cell counts in *R. draytonii* are strongly positively correlated with amphibian diversity index. Studies of conspecific and interspecific competition have noted decreased white blood cell counts associated with increased densities and social stress (Pottinger, and Pickering, 1992; Bartolomucci, 2007). Further, elevated white blood cell counts are associated with acute, transient stress rather than the chronic stress that is traditionally associated with competition for resources (Dhabhar, 2009). Thus the positive correlation between leukocyte levels and amphibian diversity index in *R. draytonii* may reflect a response to infection that is more likely in the presence of more amphibian species or predatory interactions between these species rather than a depressed immune system caused by long-term stress (Dhabhar, 2009). Looking at the raw data for species occupancy, this second explanation makes sense. *P. regilla* are found in all but one pond that *R. draytonii* occupy, offering little contribution to explaining variation in *R. draytonii* leukocytes. The next two most common co-

occurring species are *L. catesbeiana* and *T. tarosa*, both of which are know predators on *R. draytonii* tadpoles.

Neutrophil to Lymphocyte Ratios: The nonspecific immune system is a primary defense against invaders, and the proportions of those leukocytes give a snapshot of the health status of an animal, in particular giving clues about whether they are currently fighting infection. Elevated neutrophils and decreased lymphocytes in *Bd*-infected anurans may be indicative of potential for mortality due to infection with a pathogen. Or, in reverse, an animal that is infected with *Bd*, which displays normal to high neutrophils and normal lymphocyte levels may be less likely to experience mortality due to *Bd* infection than an *Bd*-infected animal with high neutrophils and lower lymphocytes.

Although one study found no evidence of leukocytes in the skin of infected amphibians (Berger et al., 2005), other studies have found evidence of changes in proportions of neutrophils in *Bd*-infected amphibians (Davis et al., 2010) and apoptosis in lymphocyte cells exposed to *Bd* (Fites et al., 2013). Researchers found elevated levels of lymphocytes in a study of white blood cell responses to *Bd* infection in post-metamorphic *P. regilla* (Gervasi et al., 2013). In a study comparing differences between two species of anurans, researchers found that neutrophil levels varied whereas lymphocyte levels decreased in *P. regilla*, a species that experienced some mortality due to *Bd* infection (Gervasi et al., 2013). In contrast neutrophils increased and proportion of lymphocytes generally remained steady in *R. cascadae,* a species that did not experience mortality due to *Bd* infection (Gervasi et al., 2013).

In a study of blood cell differentials of *L. catesbeiana*, *Bd*-infected tadpoles had elevated levels of neutrophils compared to uninfected animals whereas lymphocyte levels were statistically insignificant (Davis et al., 2010). Mortality in *L. catesbeiana* exposed to *Bd* varied based on strain from zero mortality to 75% in a small study (Gervasi et al., 2013). Further, as mentioned previously, salamanders raised under crowded conditions had higher neutrophil to lymphocyte ratios (Davis, and Maerz, 2009).

In my study, *L. catesbeiana* have a strong positive correlation between neutrophil to lymphocyte ratio and *Bd* incidence, suggesting an active immune response to *Bd* infection. This active immune response may partially explain why *L. catesbeiana* are often depicted as carriers of the infection that do not succumb to the illness. Although not significant for *L. catesbeiana*, the percent of neutrophils are slightly positively correlated with *Bd* infection whereas percent of lymphocytes were slightly negatively correlated with *Bd* incidence, signifying they may be at risk of mortality due to *Bd* infection. Neither *R. draytonii* nor *P. regilla* have clear correlations between their neutrophil to lymphocyte ratios and the effects I tested; it is possible their neutrophil to lymphocyte ratios were not elevated or were all elevated or all depressed, or that I did not include an effector that was significantly related to their neutrophil to lymphocyte ratios.

2.6 Conclusion

My results drive home the point that it is important to place studies of physiology into a broad ecological context in order to understand causation and rank

of the threats to species. In my study I find some aspects of the species' biotic conditions play an important role in explaining body condition in all three species, but that those top-ranked conditions vary between species. The variables that play an important role in explaining physiological stress levels vary with species and with the blood metric used. My results point to a complex set of influences and interactions on leukocyte levels and body condition in these three species.

When conducting work on threatened species, a primary goal is to gain information that is useful in the management and conservation of the species. The approach of investigating several metrics of tadpole health allowed for a ranking of threats across species. The findings of this paper suggest a powerful role for amphibian community composition governing various indices of tadpole health and stress levels. Threatened *R. draytonii* demonstrate elevated stress responses with increased amphibian diversity. Thus, for this species of concern, removing invasive species and creating multiple ponds, each catered to native species (D'Amore et al., 2009) in order to decrease amphibian diversity within ponds but maintain it across the landscape may be an effective conservation approach to improve *R. draytonii* health and resilience in the face *Bd* and other threats. This approach, using health metrics to rank the relative importance of possible threats and guide management for multiple species can be applied more broadly to other systems to inform and guide conservation actions.

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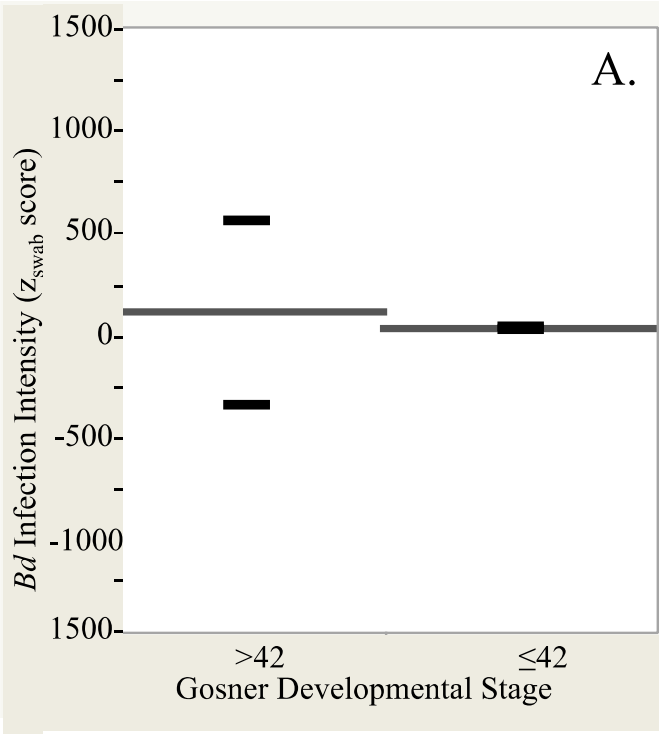
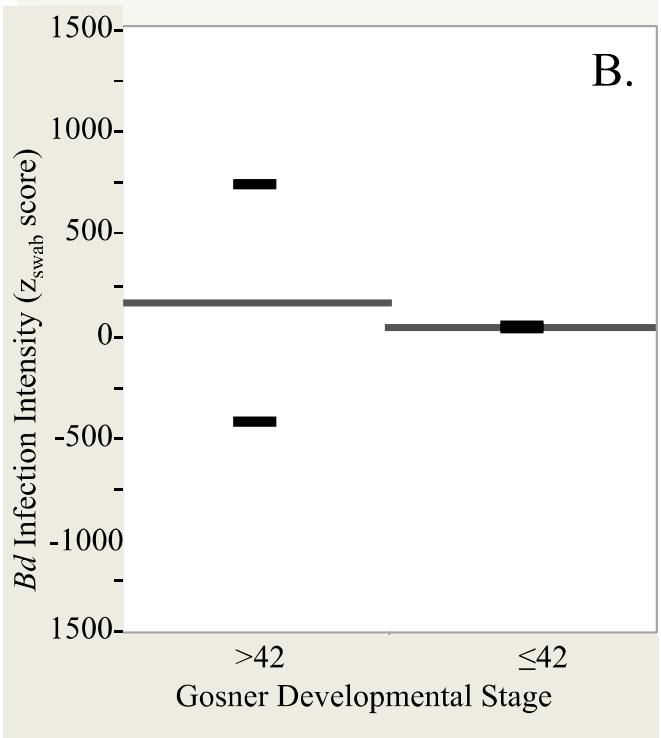
2.8 Figures

Figure 1. Map of pond sites used in the study.


A.
1500
1000
500
0
-500
-1000
1500
Bd Infection Intensity (zswab score)
>42
≤42
Gosner Developmental Stage


B.
1500
1000
500
0
-500
-1000
1500
Bd Infection Intensity (zswab score)
>42
≤42
Gosner Developmental Stage

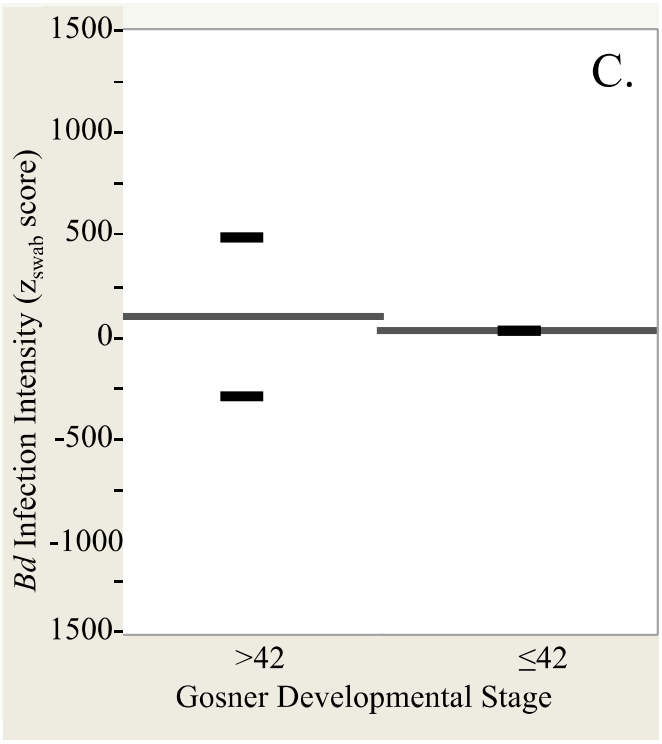


Figure 2. Decline in *Bd* infection intensity for tadpoles below GDS 42 versus at and above 42 for (A) *L. catesbeiana*, (B) *R. draytonii*, and (C) *P. regilla.* Grey lines indicate the mean and black lines indicate standard deviation.

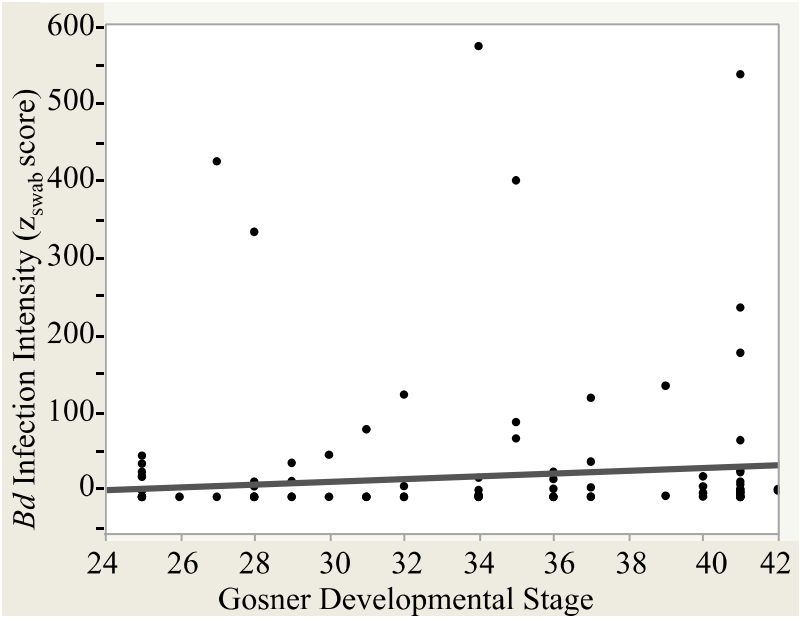
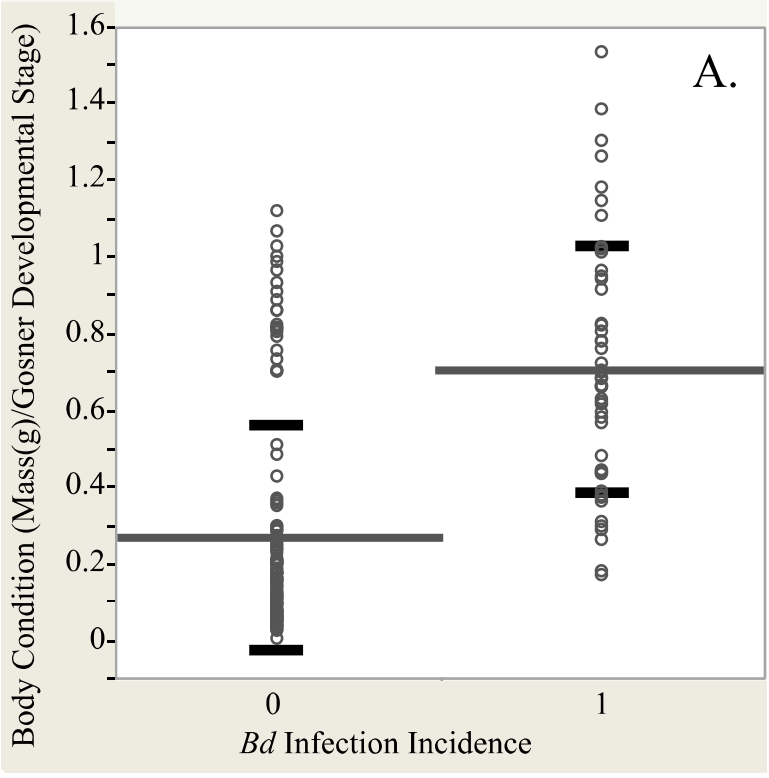


Figure 3. Increase in *Bd* infection intensity with increase in GDS for *L. catesbeiana* tadpoles.



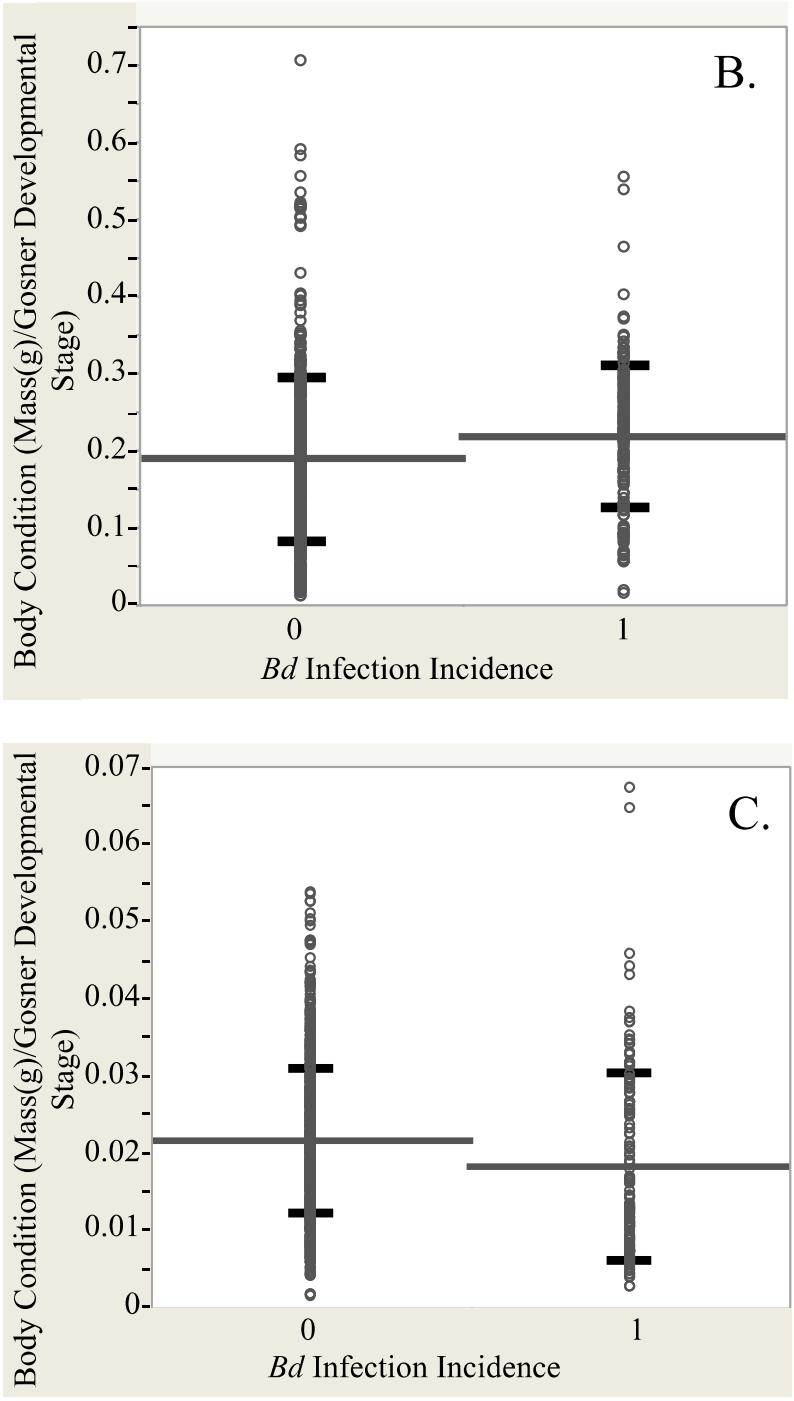
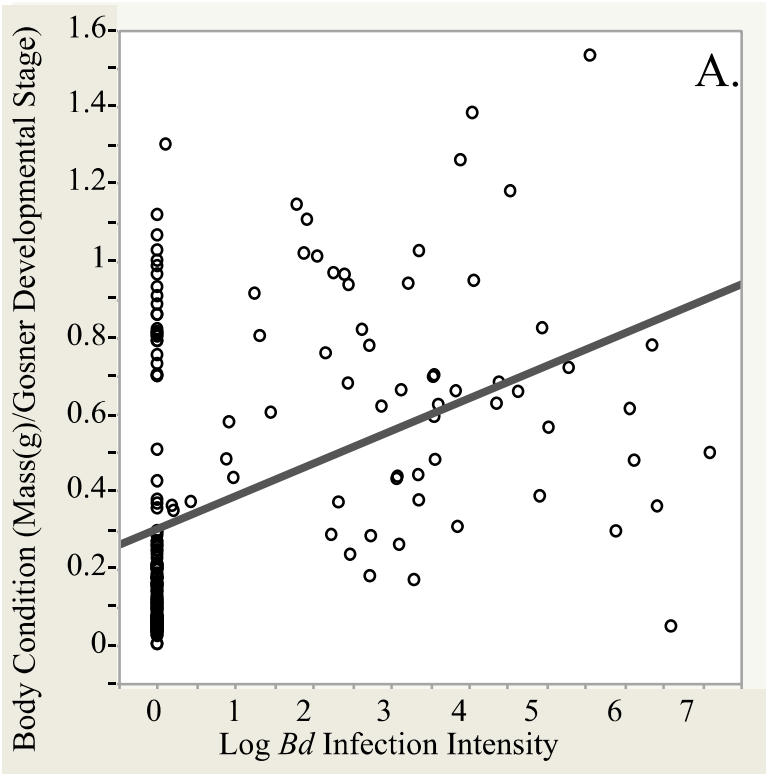
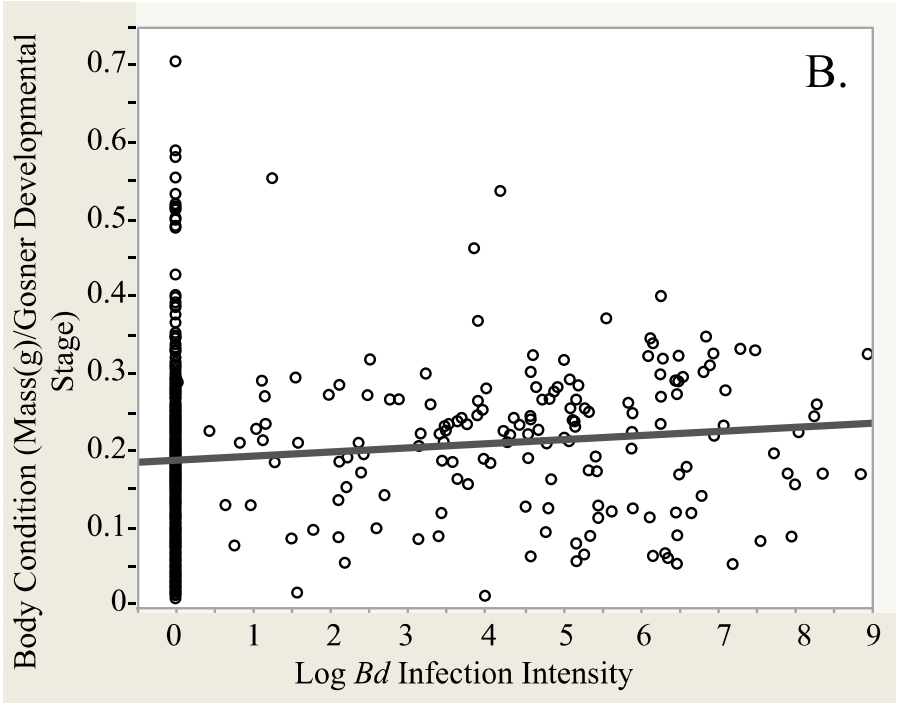


Figure 4. Relationship of body condition (mass(g)/GDS) and *Bd* incidence (0=uninfected, 1=infected) for (A) *L. catesbeiana,* (B) *R. draytonii, and (C) P. regilla* tadpoles. Grey lines indicate the mean and black indicate standard deviation.


A.
Body Condition (Mass(g)/Gosner Developmental Stage)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0 1 2 3 4 5 6 7
Log *Bd* Infection Intensity


B.
Body Condition (Mass(g)/Gosner Developmental Stage)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0 1 2 3 4 5 6 7 8 9
Log *Bd* Infection Intensity

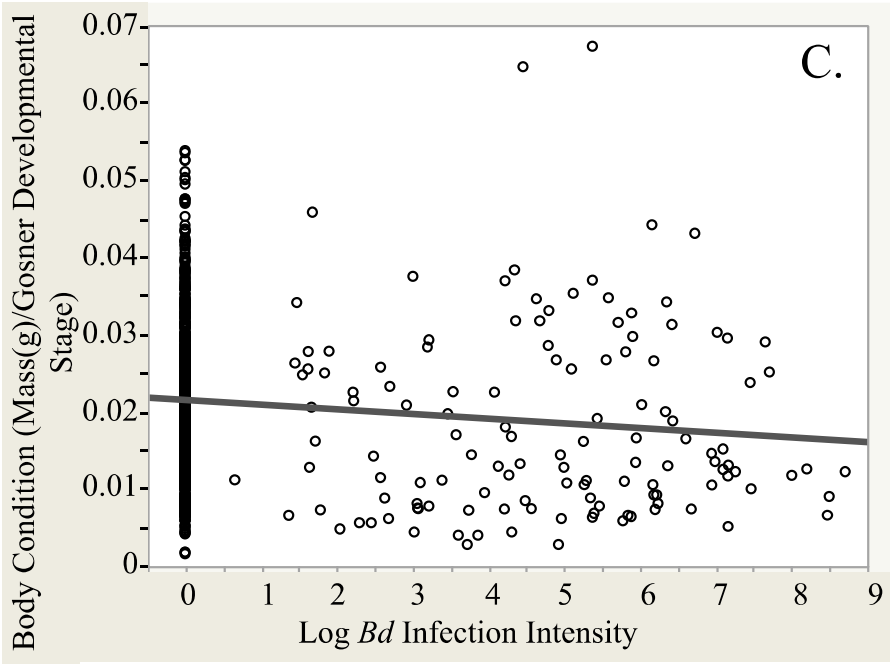


Figure 5. Relationship tadpole body condition and log *Bd* infection intensity for (A) *L. catesbeiana*, (B) *R. draytonii* and (C) *P. regilla*.

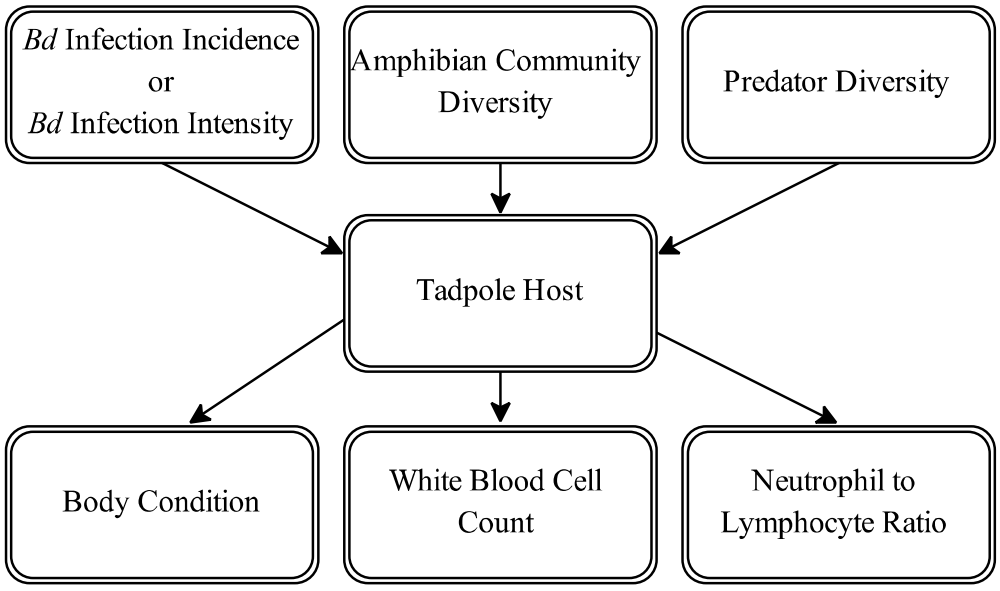


Figure 6. Simplified graphic of study design for parameters included in models for each species of tadpole.

2.9 Tables

A) Species/ Covariates	n	p	ΔAICc	AICc weight
***L. catesbeiana* Body Condition**				
Bd incidence (+), Predator Index (+)	198	5	0	0.9565
Bd incidence, Predator Index, Amphibian Diversity Index	198	6	6.1832	0.0435
***R. draytonii* Body Condition**				
Amphibian Diversity Index	671	4	0	0.9241
Bd incidence (+), Amphibian Diversity Index	671	5	5.3050	0.0651
***P. regilla* Body Condition**				
Predator Index	901	4	0	0.9553
Predator Index, Amphibian Diversity Index	901	5	6.1680	0.0437

B) Species/ Covariates	n	p	ΔAICc	AICc weight
***L. catesbeiana* Body Condition**				
Bd Infection Intensity, Predator Index	198	5	0	0.9432
Bd Infection Intensity, Predator Index, Amphibian Diversity Index	198	6	5.6204	0.0568
***R. draytonii* Body Condition**				
Amphibian Diversity Index (-)	671	4	0	0.9758
Bd Infection Intensity, Amphibian Diversity Index (-)	671	5	8.4820	0.0140
***P. regilla* Body Condition**				
Predator Index (+)	901	4	0	0.9561
Predator Index (+), Amphibian Diversity Index	901	5	6.1680	0.0438

Table 1: Top two AICc candidate mixed effects models for tadpole body condition for each species (n=number of tadpoles sampled for that species included in model, p=number of parameters in model). Parameters include fixed effects of *Bd* infection, an index of tadpole predators at each pond site, an index of amphibian diversity for each pond site, and a random effect of individual pond names. Table A includes *Bd* incidence and Table B includes *Bd* infection intensity.

A) Species/ Covariates	n	p	ΔAICc	AICc weight
***L. catesbeiana* White Blood Cell Count**				
Predator Index, Amphibian Diversity Index	96	5	0	0.3072
Bd incidence, Predator Index, Amphibian Diversity Index	96	6	0.4215	0.2488
Predator Index	96	4	1.5706	0.1401
Bd incidence, Predator Index	96	5	2.1309	0.1059
***R. draytonii* White Blood Cell Count**				
Bd incidence, Predator Index, Amphibian Diversity Index	173	6	0	0.4880
Bd incidence, Amphibian Diversity Index	173	5	2.2430	0.1590
Predator Index, Amphibian Diversity Index	173	5	2.442	0.1439
Bd incidence, Predator Index	173	5	2.71	0.1259
***P. regilla* White Blood Cell Count**				
Bd incidence, Predator Index, Amphibian Diversity Index	130	6	0	0.3030
Bd incidence, Predator Index	130	5	0.8739	0.1958
Bd incidence, Amphibian Diversity Index	130	5	0.7864	0.2045
Bd incidence	130	4	0.6982	0.2137

B) Species/ Covariates	n	p	ΔAICc	AICc weight
***L. catesbeiana* White Blood Cell Count**				
Predator Index, Amphibian Diversity Index	96	5	0	0.4753
Predator Index	96	4	1.5706	0.2167
Amphibian Diversity Index	96	4	2.3945	0.1436
***R. draytonii* White Blood Cell Count**				
Predator Index, Amphibian Diversity Index	173	5	0	0.4856
Amphibian Diversity Index	173	4	1.5830	0.2200
Bd Infection Intensity, Predator Index, Amphibian Diversity Index	173	6	2.4260	0.1444
***P. regilla* White Blood Cell Count**				
Predator Index, Amphibian Diversity Index	130	5	0	0.2002
Bd Infection Intensity, Predator Index, Amphibian Diversity Index	130	6	0.1160	0.1889
Bd Infection Intensity	130	4	0.8318	0.1321
Bd Infection Intensity, Amphibian Diversity Index	130	5	0.8931	0.1281
Amphibian Diversity Index	130	4	0.9858	0.1223
Bd Infection Intensity, Predator Index	130	5	1.0189	0.1203
Predator Index	130	4	1.2297	0.1082

Table 2. Top AICc candidate models for mixed effects models for tadpole white blood cell count for each species (n=number of tadpoles sampled for that species included in model, p=number of parameters in model). Parameters include fixed effects of *Bd* infection, an index of tadpole predators at each pond site, an index of amphibian diversity for each pond site, and a random effect of individual pond names. Table A includes *Bd* incidence and Table B includes *Bd* infection intensity.

A) Species/ Covariates	n	p	ΔAICc	AICc weight
***L. catesbeiana* Neutrophil/ Lymphocyte Ratio**				
Bd incidence	96	4	0	0.4190
Bd incidence, Predator Index	96	5	1.3391	0.2145
Predator Index	96	4	2.3592	0.1288
***R. draytonii* Neutrophil/ Lymphocyte Ratio**				
Bd incidence	166	4	0	0.3474
Predator Index	166	4	0.5002	0.2705
Amphibian Diversity Index	166	4	0.5154	0.2685
***P. regilla* Neutrophil/ Lymphocyte Ratio**				
Bd incidence	122	4	0	0.4190
Amphibian Diversity Index	122	4	1.1285	0.2383
Predator Index	122	4	1.1355	0.2375

B) Species/ Covariates	n	p	ΔAICc	AICc weight
***L. catesbeiana* Neutrophil/ Lymphocyte Ratio**				
Predator Index	96	4	0	0.4795
Amphibian Diversity Index	96	4	1.2481	0.2569
***R. draytonii* Neutrophil/ Lymphocyte Ratio**				
Predator Index	166	4	0	0.4145
Amphibian Diversity Index	166	4	0.0152	0.4114
***P. regilla* Neutrophil/ Lymphocyte Ratio**				
Amphibian Diversity Index	122	4	0	0.3948
Predator Index	122	4	0.0070	0.3934
Bd Infection Intensity	122	4	2.1022	0.1380

Table 3. Top AICc candidate mixed effects models for tadpole neutrophil to lymphocyte ratio for each species (n=number of tadpoles sampled for that species included in model, p=number of parameters in model). Parameters include fixed effects of *Bd* infection, an index of tadpole predators at each pond site, and an index of amphibian diversity for each pond site, and a random effect of individual pond names. Table A includes *Bd* incidence and Table B includes *Bd* infection intensity.

3.1 Appendix A. Prey size categorization

	Tadpole Prey Size		
Predator	**Small**	**All Sizes**	**Source**
Belastoma sp., adult	X		(Brodie et al., 1983)
Coleoptera dytiscidae, adult	X		(Smith, 1983)
Coleoptera dytiscidae, larvae		X	(Licht, 1974; Brodie et al., 1983)
Hemiptera notonectidae	X		(Licht, 1974; Cronin, and Travis, 1986)
Hirudinea	X		(Licht, 1974; Berven et al., 2001)
Lethocerus sp., adult		X	(Licht, 1974; Brodie et al., 1983)
Lethocerus sp., nymphal	X		(Brodie et al., 1983)
Odonata sp., dragonfly naiads	X		(Brodie et al., 1983; Smith, 1983)
Ranatra sp.	X		(Licht, 1974)
Procambarus sp.		X	(Axelsson et al., 1997; Gherardi et al., 2001; Renai, and Gherardi, 2004)
Lithobates catesbeiana		X	(Lawler et al., 1999)
Ambystoma californiense	X		(Burger, 1950; Anderson, 1968; Webb, and Roueche, 1971; Wilbur, 1972)
Taricha sp.	X		(Chandler, 1918; Neish, 1971)
Large Fish*: Lepomis macrochirus, Amiurus sp., Micropterus sp.*		X	(Hoyle, and Keast, 1987; Eklöv, and Werner, 2000; Towey, 2007)
Gambusia affinis holbrooki	X		(Lawler et al., 1999; Komak, and Crossland, 2000)
Gasterosteus aculeatus		X	(Laurila et al., 2006)

Table A1: Literature used to categorize prey size preference of various aquatic predators to estimate Predator Index.

4.1 Appendix B. Mass-length relationships of tadpoles of the three study species with and without *Bd*-infection.

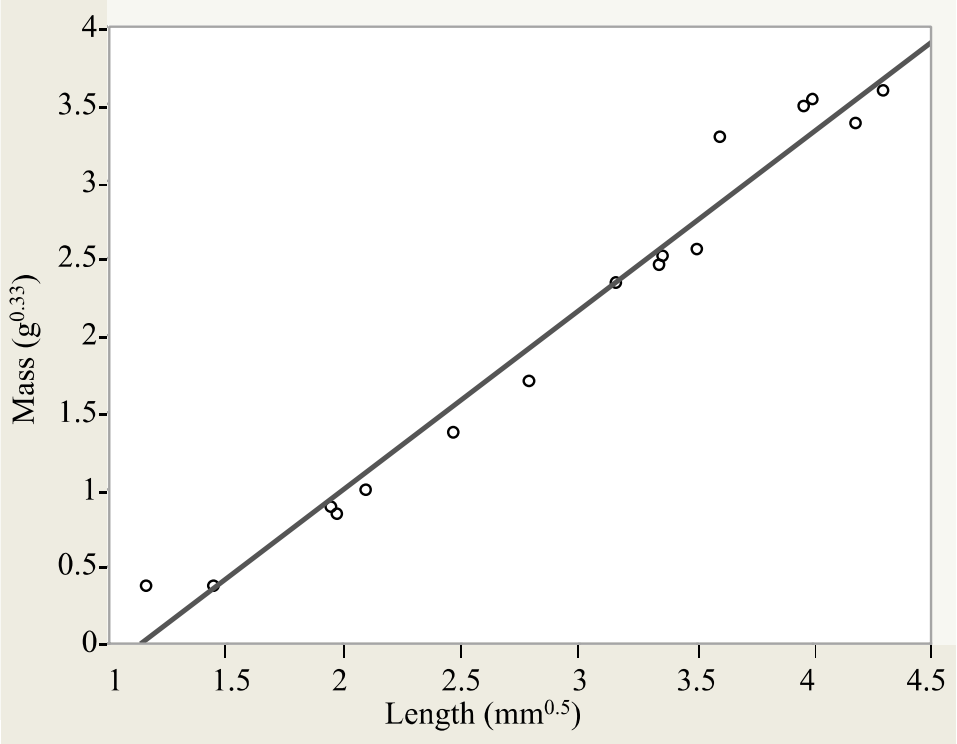


Figure B1. Length-mass relationship of *Bd*-negative *L. catesbeiana.* Length is square root and mass is cube root transformed. Linear regression: $r(15) = 0.97$, $p \leq 0.0001$.

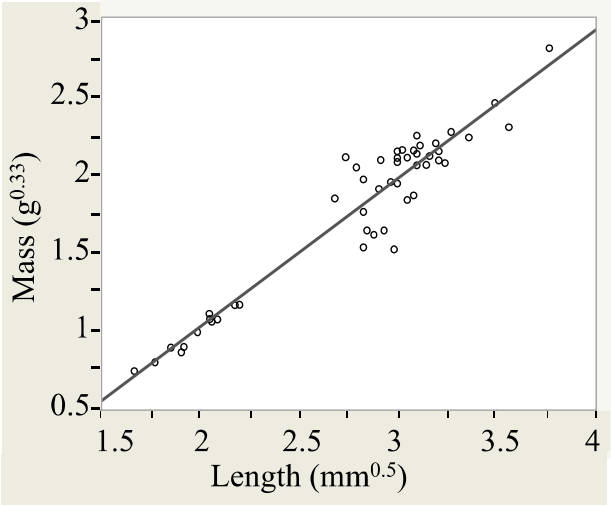


Figure B2. Length-mass relationship of *Bd*-negative *R. draytonii.* Length is square root and mass is cube root transformed. Linear regression: $r(49) = 0.92$, $p \leq 0.0001$.

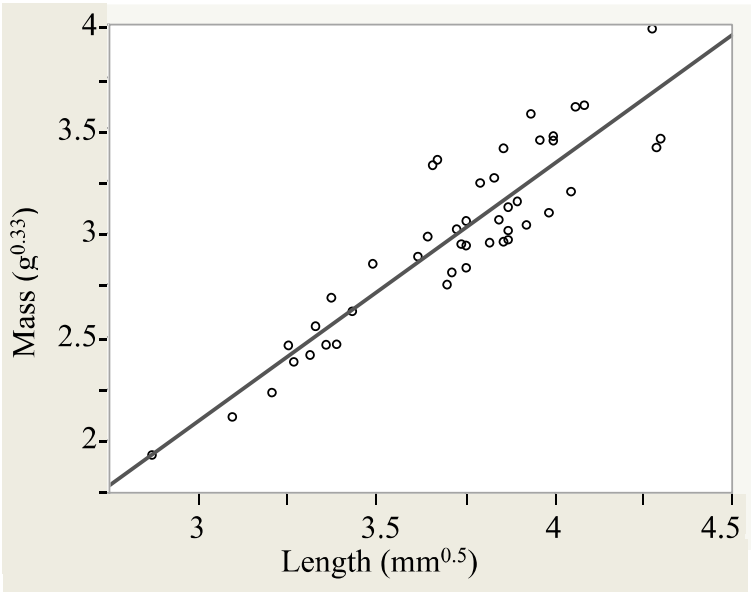


Figure B3. Length-mass relationship of *Bd*-positive *L. catesbeiana.* Length is square root and mass is cube root transformed. Linear regression: r(45)= 0.83, p=<0.0001.

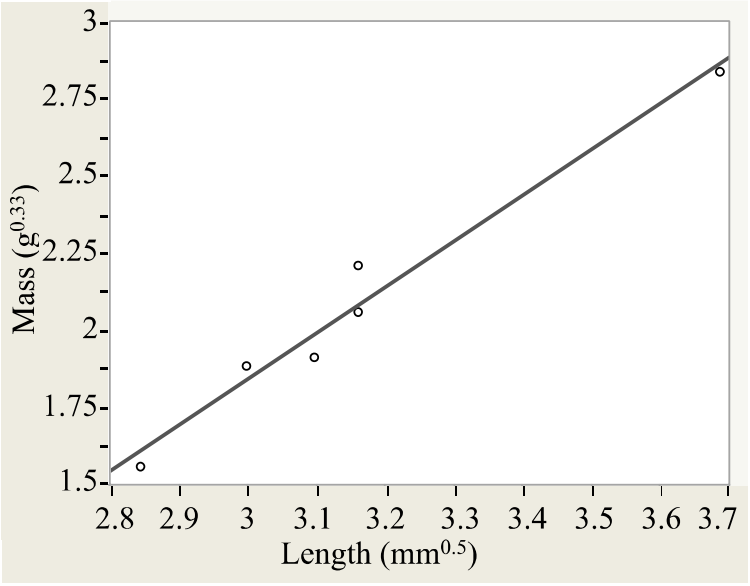


Figure B4. Length-mass relationship of *Bd*-positive *R. draytonii.* Length is square root and mass is cube root transformed. Linear regression: r(5)= 0.97, p= 0.0003.

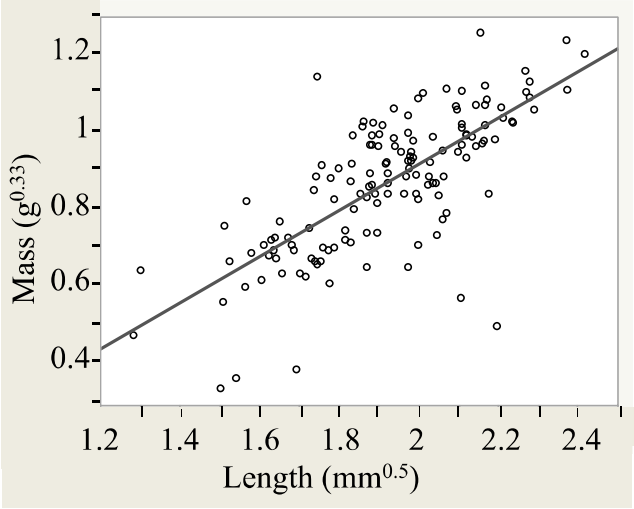


Figure B5. Length-mass relationship of *Bd*-negative *P. regilla.* Length is square root and mass is cube root transformed. Linear regression: r(144)=0.53, p=<0.0001.

5.1 Appendix C. Relationships between mean mass and GDSs for each of the three study species with and without *Bd* infection.

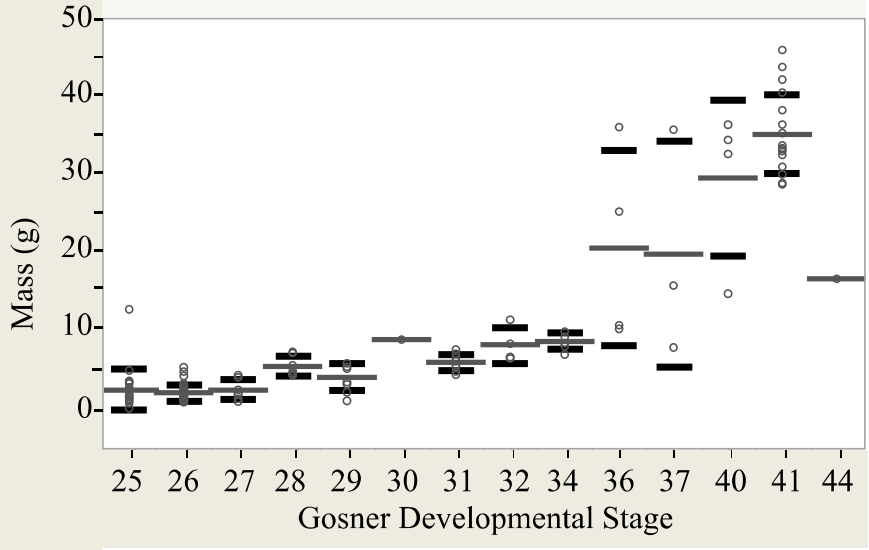


Figure C1. Relationship between mean (±1 std dev) mass (g) and GDS for *L. catesbeiana* tadpoles without *Bd*. Grey lines indicate the mean and black lines indicate standard deviation.

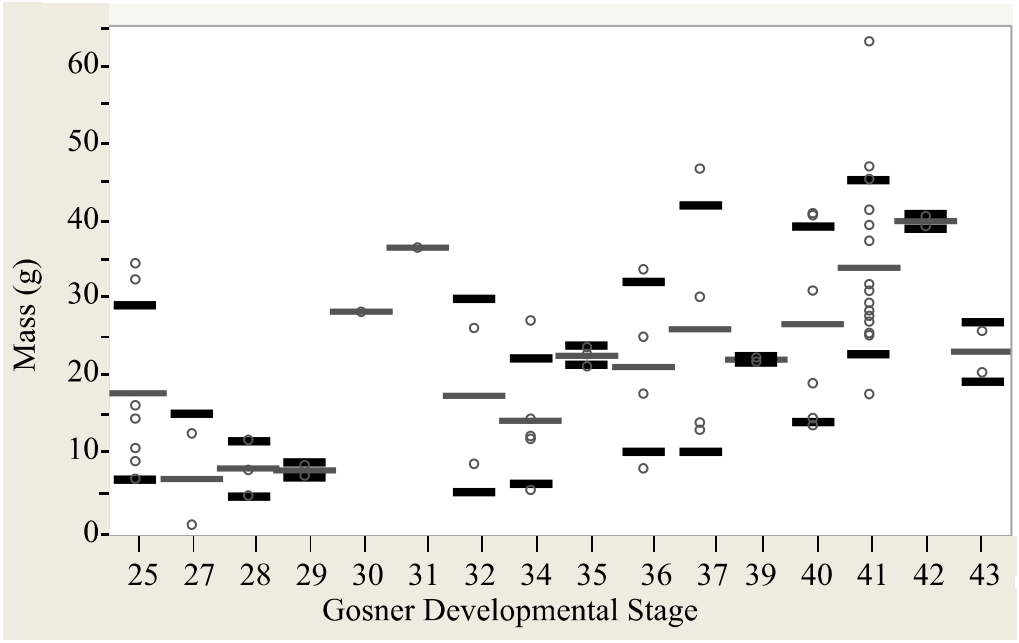


Figure C2. Relationship between mean (±1 std dev) mass (g) and GDS for *L. catesbeiana* tadpoles with *Bd*. Grey lines indicate the mean and black lines indicate standard deviation.

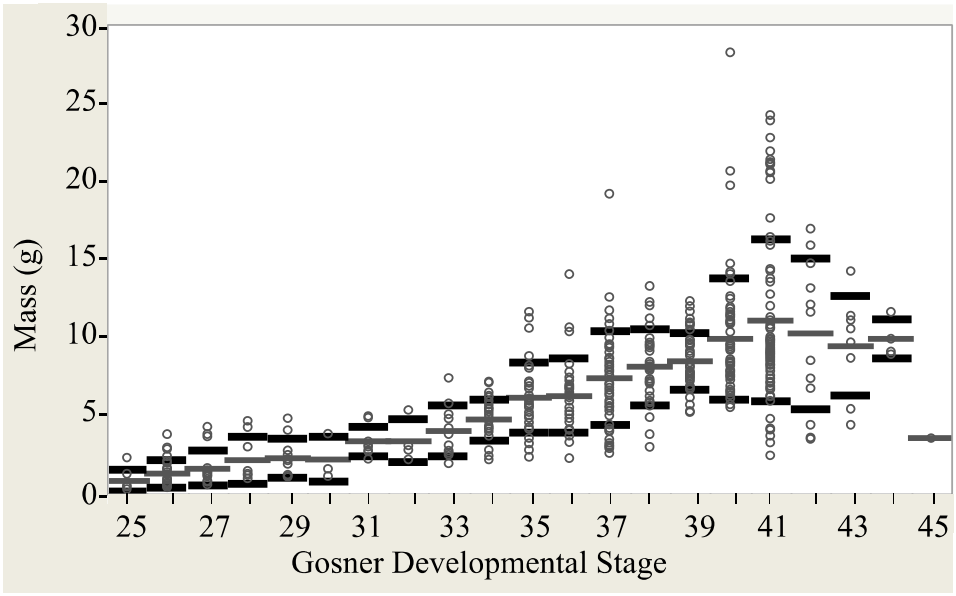


Figure C3. Relationship between mean (±1 std dev) mass (g) and GDS for *R. draytonii* tadpoles without *Bd*. Grey lines indicate the mean and black lines indicate standard deviation.

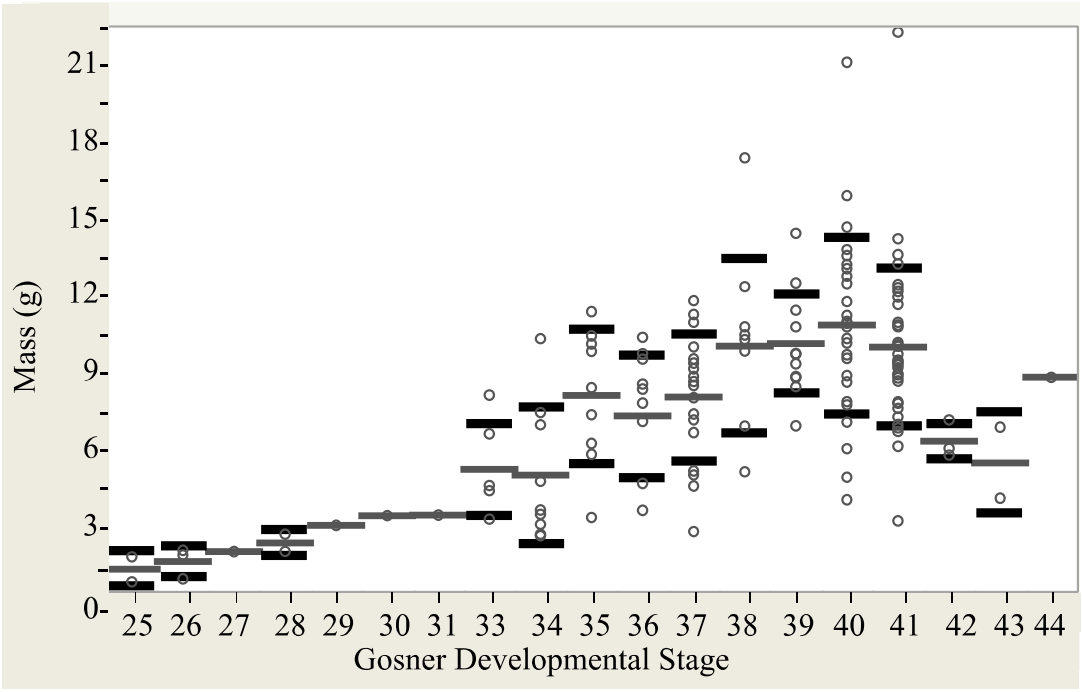


Figure C4. Relationship between mean (±1 std dev) mass (g) and GDS for *R. draytonii* tadpoles with *Bd*. Grey lines indicate the mean and black lines indicate standard deviation.

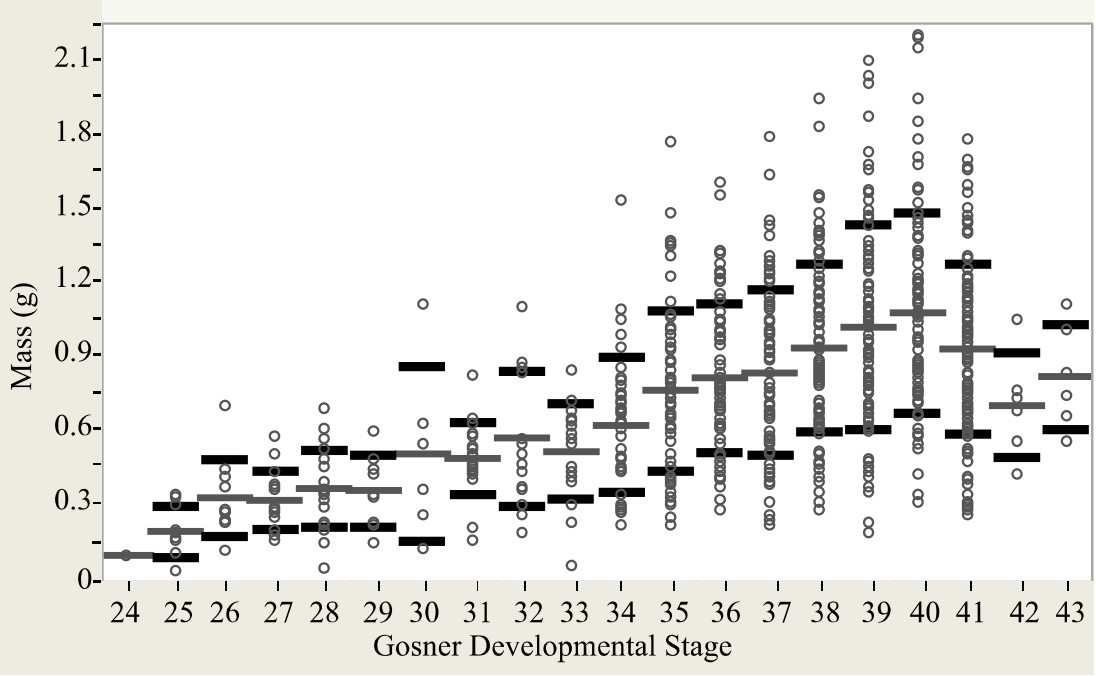


Figure C5. Relationship between mean (±1 std dev) mass (g) and GDS for *P. regilla* tadpoles without *Bd*. Grey lines indicate the mean and black lines indicate standard deviation.

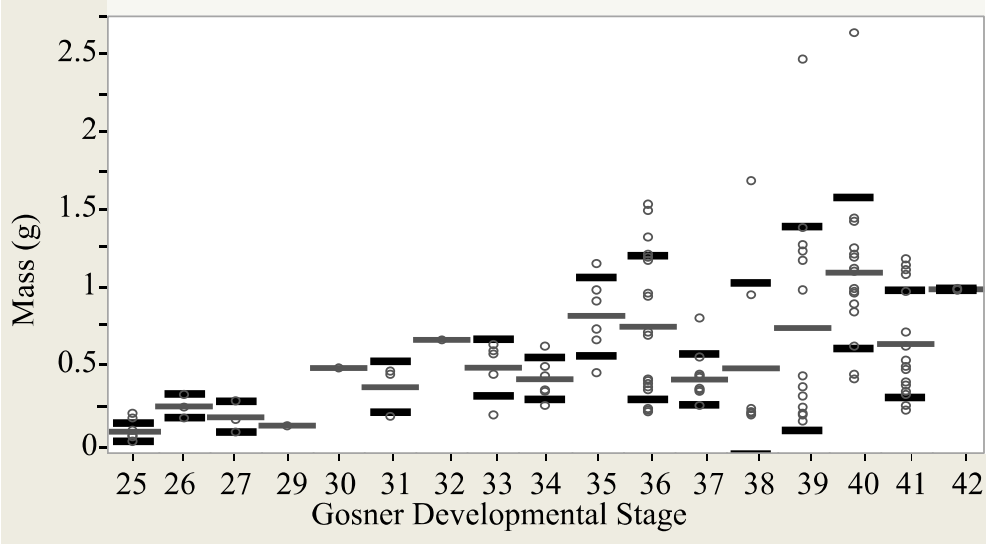


Figure C6. Relationship between mean (±1 std dev) mass (g) and GDS for *P. regilla* tadpoles with *Bd*. Grey lines indicate the mean and black lines indicate standard deviation.

Discussion

As changes in the environment continue to plague amphibians and other taxa, deciphering the most effective conservation actions given the broad suite of threats can be challenging at best (Blaustein et al., 2011). My research demonstrates the importance of taking a comprehensive approach by exploring ecological relationships from several perspectives, incorporating pertinent biotic and abiotic conditions that may influence those relationships, and ranking both the components of the ecological relationship and the threats. This approach can help to avoid making conservation decisions based on partial and hence potentially misleading analyses. Specifically, I demonstrated that investigating a host-pathogen relationship from the perspective of pathogen success in the host given a range of biotic and abiotic conditions could enhance our understanding of the complex relationship across host species and narrow the field of important, interacting stressors. This approach was complimented by my second tactic, which furthered the understanding of this system from the host perspective. By measuring host physiological responses to several biotic stressors, including the pathogen, and ranking them, I was able to expand the understanding of this system and support my assertion that the most effective approach to conservation of native species in this system would not be to control the pathogen, but instead to control the introduced conspecific and provide specialized pond habitats for natives within sites to decrease within-pond amphibian diversity while maintaining it across the landscape.

This dissertation suggests several important directions for species management and future research. In Chapter 1, I found that while other studies had established strong links between *Bd* infection intensity or prevalence and abiotic factors such as pond temperature, humidity, elevation, size, and substrate, biotic factors including pond vegetation, amphibian species diversity, and *Bd* infection levels in co-occurring species were particularly important in explaining *Bd* infection success. In Chapter 2 I found that tadpole body condition of each species was most strongly influenced by different explanatory factors, but that the two proposed carriers of *Bd* in the system had positively correlated leukocyte measures while the declining native did not. Further the declining native had a negative correlation between body condition and amphibian diversity, and amphibian diversity was positively correlated with leukocyte levels. Future studies attempting to understand species interaction and how environmental variables influence them would be improved by including a suite of likely biotic and abiotic factors, multiple species or avoid generalizing across species, and by studying those interactions from several perspectives. My approach could be utilized by those looking to understand a complex spate of threats to sensitive species or in a complex systems of interactions, helping to improve their understanding of the system by exploring how both species are impacted by multiple interacting stressors, ranking them, and examining the results to help guide more effective conservation actions.

Species worldwide are experiencing declines in the face of many anthropogenic changes (Stuart et al., 2004; Blaustein et al., 2011; Jackson et al.,

2001; IUCN 2014). Amphibians face a broad variety of potentially interacting threats, such as habitat loss and fragmentation (Blaustein et al., 1994; Corn, 2000; Green, 1997), compromised water quality (Berrill et al., 1994; Harris et al., 1998; Hayes et al., 2002; Brodman et al., 2003; Houlahan, and Findlay, 2003), introduced species (Fisher, and Shaffer, 1996; Lawler et al., 1999; Knapp et al., 2007), and emerging infectious diseases (Daszak et al., 1999; Blaustein, and Kiesecker, 2002). While it is important to explore how these threats to impact species singly to help clarify their impact on species, we cannot understand how these threats impact species of concern, how they interact, and their importance to species' metrics of health or survival without conducting studies across species in complex ecological habitats, taking into account various biotic and abiotic variables. This dissertation adds to our understanding of how different variables play a role in the success of a pathogen and body condition of three different species, and, in turn, can assist in planning actions to increase the success of threatened species.

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