The Ecology Of Chytridiomycosis

In Boreal Chorus Frogs (*Pseudacris maculata*)

by

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ABSTRACT

Infectious diseases have emerged as a significant threat to wildlife. Environmental change is often implicated as an underlying factor driving this emergence. With this recent rise in disease emergence and the acceleration of environmental change, it is important to identify the environmental factors that alter host-pathogen dynamics and their underlying mechanisms. The emerging pathogen *Batrachochytrium dendrobatidis* (Bd) is a clear example of the negative effects infectious diseases can have on wildlife. Bd is linked to global declines in amphibian diversity and abundance. However, there is considerable variation in population-level responses to Bd, with some hosts experiencing marked declines while others persist. Environmental factors may play a role in this variation. This research used populations of pond-breeding chorus frogs (*Pseudacris maculata*) in Arizona to test if three rapidly changing environmental factors nitrogen (N), phosphorus (P), and temperature influence the presence, prevalence, and severity of Bd infections.

I evaluated the reliability of a new technique for detecting Bd in water samples and combined this technique with animal sampling to monitor Bd in wild chorus frogs. Monitoring from 20 frog populations found high Bd presence and prevalence during breeding. A laboratory experiment found 85% adult mortality as a result of Bd infection; however, estimated chorus frog densities in wild populations increased significantly over two years of sampling despite high Bd prevalence. Presence, prevalence, and severity of Bd infections were not correlated with aqueous concentrations of N or P. There was, however, support

for an annual temperature-induced reduction in Bd prevalence in newly metamorphosed larvae. A simple mathematical model suggests that this annual temperature-induced reduction of Bd infections in larvae in combination with rapid host maturation may help chorus frog populations persist despite high adult mortality.

These results demonstrate that Bd can persist across a wide range of environmental conditions, providing little support for the influence of N and P on Bd dynamics, and show that water temperature may play an important role in altering Bd dynamics, enabling chorus frogs to persist with this pathogen. These findings demonstrate the importance of environmental context and host life history for the outcome of host-pathogen interactions.

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Chapter 1

INTRODUCTION

BACKGROUND

Emerging infectious diseases (EIDs) present an urgent challenge facing modern science and society. EIDs are infectious diseases that have newly appeared or are rapidly increasing in incidence or geographic range and include some of the world's deadliest diseases such as HIV, malaria, cholera, and tuberculosis (Morse 1995). Although novel diseases have infected new host populations throughout history, some evidence suggests that infectious diseases may be emerging more frequently than before (Harvell et al. 1999, Johnson & Paull 2011, Jones et al. 2008). In fact, since the 1970s, newly emerging diseases have been identified at a rate of one or more per year (World Health Organization, 2007), with 87 diseases that were unknown 25 years ago (Woolhouse & Gaunt 2007). Similar trends have been noted in infectious diseases of wildlife, often resulting in large-scale host population declines and even species extinctions (Daszak et al. 1999, Dobson & Foufopoulos 2001, Harvell et al. 1999, Johnson & Paull 2011, Okamura & Feist 2011, Stuart et al. 2004).

Nearly all of these diseases are united by a common fact: they are regulated by ecological interactions in a rapidly changing world (McKenzie & Townsend 2007). Numerous examples demonstrate how changes in abiotic factors such as land use, climate, and nutrient availability can alter infectious diseases of humans, animals, and plants (Anderson et al. 2004, Daszak et al. 2001, Harvell et

al. 1999, Harvell et al. 2002, Johnson et al. 2007, Johnson et al. 2010, Okamura et al. 2011). With the recent rise in disease emergence and the acceleration of changes in land use, climate, and biogeochemical cycles it will be important to identify which environmental factors can alter host-pathogen dynamics and how they do it.

Temperature and the availability of biologically reactive forms of nutrients (nitrogen and phosphorus) are two rapidly changing environmental factors that are implicated in infectious disease emergence (Harvell et al. 1999, Harvell et al. 2002, Johnson et al. 2007, Okamura & Feist 2011, Okamura et al. 2011, Rohr et al. 2011). Nitrogen (N) and phosphorus (P) are essential and often limiting nutrients in ecosystems throughout the world (Elser et al. 2007), and humans are increasing the amount of biologically available N and P at rates that far exceed levels seen in recent centuries (Carpenter et al. 1998, Vitousek et al. 1997). These changes are known to alter terrestrial and aquatic ecosystems, and evidence is emerging that an increased supply of both N and P to ecosystems may also cause an increased risk of disease in humans and wildlife (Johnson et al. 2011, McKenzie & Townsend 2007). There is also compelling evidence that climate change, and specifically changes in temperature regimes, can affect the dynamics of many diseases (Harvell et al. 1999, Harvell et al. 2002, Pounds et al. 2006). Changes in temperature can influence patterns of disease by shifting host and pathogen ranges, changing pathogen or host replication rates, and altering host–parasite interactions (Okamura et al. 2011). The overall influence of climate change on disease emergence and severity remain unclear (Rohr et al. 2011), but

some studies suggest that global increases in temperature will increase the global distribution and prevalence of infectious diseases (Harvell et al. 2002). In the work undertaken for this thesis I test the hypothesis that nutrients (N and P) and temperature can influence the dynamics chytridiomycosis, an emerging infectious disease linked to global declines in animal diversity and abundance.

CHYTRIDIOMYCOSIS

Amphibian populations on several continents have experienced dramatic decline linked to the emerging infectious disease chytridiomycosis (Collins & Crump 2009). This disease is caused by the pathogenic fungus, *Batrachochytrium dendrobatidis* (hereafter referred to as Bd; Berger et al. 1998). Bd infects almost 300 amphibian species (K. Kriger pers. comm.) and has been found on all continents where amphibians exist (Speare & Berger 2004, Bai et al. 2010). Whether Bd's emergence is the result of recent spread or environmental change has been the subject of considerable debate (Lips et al. 2008, Pounds et al. 2006, Rachowicz et al. 2005, Skerratt et al. 2007). Genetic analyses (Morehouse et al. 2003, Morgan et al. 2007), field surveys (Lips et al. 2003, Lips et al. 2006, Lips et al. 2008), and museum specimens (Ouellet et al. 2005, Weldon 2004) suggest that Bd's emergence is due to its relatively recent spread (Skerratt et al. 2007). Other studies suggest a role for temperature and possibly climate change in the emergence of this disease on a regional scale (Bosch et al. 2007, Kriger et al. 2007, Pounds et al. 2006). There is considerable variation in both individual- and population-level responses of amphibian hosts to Bd with some host populations

experiencing marked declines while others persist (Daszak et al. 2005, Hale et al. 2005, Lips et al. 2006, Lips et al. 2003, Puschendorf et al. 2011, Reeder et al. 2012, Retallick et al. 2004). Although it is likely that several factors contribute to the outcome of Bd introductions into amphibian populations (Blaustein & Kiesecker 2002), outbreaks of chytridiomycosis are often associated with specific environmental conditions (Lips et al. 2003, Pounds et al. 2006, Stuart et al. 2004). These studies demonstrate the potential for abiotic factors to influence Bd host-pathogen dynamics. Many of these studies, however, focus only on the terrestrial environment, largely ignoring the aquatic environment, which may act as a center for Bd transmission (Lips et al. 2006). These studies also concentrated mostly on air temperature and precipitation as the major factors influencing the distribution of Bd and outbreaks of chytridiomycosis, overlooking other factors known to influence Bd reproduction, such as nutrient availability (Piotrowski et al. 2004). This research addresses these gaps using populations of pond breeding frogs from Arizona.

MODEL SYSTEM AND CHAPTERS

Arizona is a microcosm of global amphibian declines. Declines and extirpations are known in all seven native leopard frog species (Clarkson & Rorabaugh 1989, Sredl 1997, U.S. Fish and Wildlife Service 2007, Witte et al. 2008). However, populations of the boreal chorus frog (*Pseudacris maculata*) appear to persist with Bd, despite evidence that this species suffers up to 80% mortality as a result of Bd infection (Retallick & Miera 2007). My research

combines laboratory experiments with detailed descriptions of the dynamics of Bd in wild chorus frog populations to test if abiotic aspects of the aquatic environment (temperature or N and P availability) influence the ability of these populations to persist with Bd.

Chapter Two reports the diagnostic sensitivity of a PCR-based water filtration technique used to detect Bd. This technique has the potential to reduce greatly ethical and logistical constraints of current Bd monitoring techniques by removing the need to capture host species. This study compared the results of four repeated filter sampling events from 20 ponds to those of skin swabs from ~60 boreal chorus frogs from each pond. Filters failed to detect *Bd* in 31-77% of the swab-positive ponds, with the lowest rates of detection late in the season when young-of-the year froglets emerge. However, after three repeated sampling events, filtration of small volumes of water (~600 ml) correctly identified 94% of the ponds that tested Bd positive with swabbing. These results demonstrate the importance of timing and resampling for the detection of an aquatic microbial pathogen from water and aid in the development of cost-effective monitoring regimes for Bd. They also provide evidence that Bd presence and prevalence is reduced during warmer times of the year, when young-of-the-year froglets emerge.

Chapter Three reports a test of how the distribution and pond-level rates of Bd transmission were related to aquatic concentrations of N and P in breeding ponds. Pond water and breeding chorus frogs were sampled at 20 sites over two years. Seventeen sites harbored Bd over both years with high Bd prevalence (up

to 100%) and loads (up to 193,000 zoospore genomic equivalents per frog) at each site. Bd presence, prevalence, short-term changes in prevalence (a proxy for incidence), loads, and aquatic densities of Bd were not related to concentrations of N or P but were associated with the timing of sampling and host densities. Chorus frog densities increased significantly over the two years of sampling despite the presence of Bd. These results demonstrate that Bd can persist across a wide range of aquatic nutrient concentrations and that chorus frog populations persist with Bd at high host and pathogen densities for at least two generations.

Chapter Four summarizes a test of two hypotheses to explain the ability of chorus frog populations to persist with Bd: host tolerance and temperature induced environmental rescue. In laboratory experiments adult chorus frogs with natural Bd infections suffered 85% mortality with no mortality in uninfected controls, providing minimal support for host tolerance of Bd infections playing a role in population persistence. Support was found for a seasonal, temperature induced reduction in Bd prevalence in newly metamorphosed larvae. Bd prevalence in summer-emerging froglets was significantly lower than Bd prevalence in adults, with 11 of 15 ponds testing Bd negative despite these same ponds testing Bd positive when adults were tested earlier in the season. Experimental heat-treatment at naturally relevant temperatures removed Bd infections from developing chorus frog larvae, indicating that warm water temperatures remove Bd infections during tadpole development. Also, intensive weekly surveys from two ponds found that Bd infections were completely absent from the larval cohort in a warmer, ephemeral pond, while Bd persisted in larvae

and newly metamorphosed froglets in a deeper, cooler, perennial pond. These results support the hypothesis that warm water temperatures reduce Bd prevalence in developing larvae and may explain the reduction and frequent absence of Bd from summer emerging froglets. A mathematical model examining the relative importance of juvenile versus adult frog survival found that juvenile survival may be most important for projected chorus frog population growth, supporting the hypothesis that pronounced seasonal reduction in Bd prevalence in young-of-the-year froglets in combination with quick rates of maturation (~1 yr) might enable populations of boreal chorus frogs to persist with Bd, despite high rates of annual adult mortality from chytridiomycosis.

In combination, these results provide an example of how host life history and environment may interact to enable a population of susceptible hosts to persist with a virulent pathogen. My research also demonstrates how subtle environmental differences can alter the dynamics of Bd in chorus frog populations, potentially altering the selective pressures that Bd exerts on chorus frog populations at scales as small as individual ponds. Future studies should examine how pond-level differences in disease dynamics can shape the evolution of Bd in chorus frogs and influence the ability of chorus frog meta-populations to persist with this pathogen.

Chapter 2

EVALUATION OF A FILTRATION-BASED METHOD FOR THE DETECTION OF BATRACHOCHYTRIUM DENDROBATIDIS IN NATURAL BODIES OF WATER

ABSTRACT

Infectious diseases are emerging as a significant threat to wildlife. The resulting increased effort to monitor wildlife diseases is driving the development of innovative pathogen monitoring techniques, including many polymerase chain reaction (PCR)-based diagnostics. Despite the utility of these PCR-based techniques, there is still much to be learned about their ability to accurately detect target pathogens in nature. I assessed the diagnostic sensitivity of a PCR-based water filtration technique to detect the directly transmitted aquatic fungal pathogen *Batrachochytrium dendrobatidis* (Bd) by comparing the results of four repeated filter sampling events from 20 ponds to those of skin swabs from ~60 boreal chorus frogs (*Pseudacris maculata)* from each pond. Filters failed to detect Bd in 31-77% of the swab-positive ponds, depending on the time of sampling. However, after three repeated sampling events, filtration of small volumes of water (~600 ml) correctly identified 94% of the ponds that tested Bd positive with swabbing, with the highest rates of detection occurring after breeding but before larvae reached metamorphosis. These results are a case study demonstrating the importance of timing and resampling for the detection of an aquatic microbial pathogen, Bd, from water. This will be a useful technique for monitoring Bd, but

additional studies are needed to test the degree to which my findings are species- or population-specific. These studies will aid in the development of cost-effective monitoring regimes for Bd and potentially other aquatic pathogens.

INTRODUCTION

Infectious diseases are emerging as a significant threat to wildlife (Daszak et al. 2000). Understanding this threat necessitates the development of innovative monitoring techniques that can increase the accuracy and efficiency of pathogen surveys. Surveys of wildlife pathogens are often limited by time, money, host species abundance, and ethical issues related to invasive animal-sampling techniques (Spalding & Forrester 1993). Environmental detection of pathogens has the potential to greatly reduce these ethical and logistical constraints by removing the need to capture host species (e.g. Kirshtein et al. 2007). Environmental sampling can also enrich our understanding of pathogen dynamics outside of their central hosts (e.g. Epstein 1993, Walker et al. 2007). For these reasons, DNA screening of environmental samples has been used to detect a number of water and soil-borne pathogens of humans (e.g. Loge et al. 2002) and wildlife (e.g. Brinkman et al. 2003, Audemard et al. 2004). Advances in DNA purification and amplification have enabled the development of highly specific and analytically sensitive environmental diagnostics (e.g. Kirshtein et al. 2007). However, without knowledge of the rates at which these assays miss the presence of a target pathogen, disease prevalence estimates can be greatly underestimated, negating the aforementioned advantages and misleading scientists and managers

(e.g. Greer & Collins 2007). It is, therefore, critically important to thoroughly assess the sensitivity of these diagnostics.

A diagnostic assay's sensitivity has two components: analytical sensitivity and diagnostic sensitivity. Analytical sensitivity is the smallest amount of a substance in a sample that can be measured accurately by an assay. Diagnostic sensitivity is the percentage of samples that have the target substance that are properly identified by the assay (Saah & Hoover 1997). The sensitivity of DNA-based diagnostics for soil- and water-borne pathogens is influenced by a number of factors, including the volume of sample processed, the efficiency of target recovery, and the presence of polymerase chain reaction (PCR)-inhibitory compounds (Loge et al. 2002). Variation in these factors will influence the overall diagnostic sensitivity of the assay, potentially resulting in false negatives (lack of detection despite the presence of the pathogen). This, in turn, will influence the interpretation of sampling results, with the potential to underestimate pathogen prevalence and distribution. Here, I assess the diagnostic sensitivity and advantages of a novel PCR-based water filtration technique for the detection of an emerging aquatic fungal pathogen, *Batrachochytrium dendrobatidis* (Bd).

Bd is a member of the fungal phylum Chytridiomycota (chytrids). Like other chytrids, Bd is transmitted by flagellated propagules called zoospores (Berger et al. 1998, 2005). These zoospores travel through water to encyst in the outermost layers of keratinized amphibian host skin cells. Once encysted, the zoospores develop into zoosporangia that eventually open, releasing more infective zoospores into the environment (Berger et al. 2005). Heavy Bd

infections can result in chytridiomycosis, a disease in which amphibians lose their ability to osmoregulate due to the inhibition of cutaneous ion exchange (Voyles et al. 2009).

Chytridiomycosis is linked to declines and extinctions of about 200 species of frogs on several continents (Collins & Crump 2009). In response to this emerging infectious disease, international and governmental agencies on several continents are investing millions of dollars in research and development of amphibian threat abatement plans, which include guidelines to describe the distribution and understand the epidemiology of chytridiomycosis (Skerratt et al. 2007, USFWS 2007). These, along with other Bd-monitoring initiatives, make clear the need for time- and cost-effective tools that can accurately monitor Bd in the field.

One such tool is filtering water to detect Bd in the aquatic environment (Kirshtein et al. 2007, Walker et al. 2007). This technique is designed to capture zoospores or remnant Bd DNA from the water column and then amplify this DNA using PCR. These filtration techniques have the potential to significantly reduce Bd sampling effort and costs by eliminating the need to collect and process DNA samples from the currently accepted standard of 30 to 60 animals per habitat, which can be difficult to obtain when dealing with sites with low amphibian abundance or rare or cryptic species (Skerratt et al. 2008). Recent studies (Kirshtein et al. 2007, Walker et al. 2007) successfully detected Bd from small volumes (<1 l) of field-collected water using a highly specific PCR-based assay (Hyatt et al. 2007). They also found these techniques to have a high

analytical sensitivity, with detection limits as low as 0.1 zoospores (Walker et al. 2007) and 0.06 zoospores (Kirshtein et al. 2007). Yet, we do not know the overall diagnostic sensitivity of these filtration techniques, nor do we clearly understand the factors that enhance the likelihood of Bd detection by filtration.

My goal was to assess the ability of water filters to correctly identify a pond as Bd positive by comparing filter outcomes to those of the currently accepted standard of skin swabs from 60 frogs. Although swabs from 60 animals cannot definitively confirm the absence of Bd from these ponds, this method currently serves as the most accurate way to determine the presence of Bd within a habitat, with 95% certainty if prevalence is ≥ 5% (Skerratt et al. 2008). Using 20 ephemeral ponds with Bd-infected populations of the boreal chorus frog (*Pseudacris maculata*) I addressed the following questions: (1) What percentage of filters fails to detect Bd when present in swabs collected from live animals? (2) How many filters should be taken to maximize the probability of detecting Bd? (3) When is the ideal time to filter water to maximize Bd detection?

METHODS

Field Sites and Focal Amphibian Host Species

Water samples and skin swabs were collected from each of 20 ponds in Coconino National Forest, Coconino County, Arizona, USA. All ponds were lentic bodies of water: ephemeral ponds, perennial ponds, man-made cattle ponds, and one spring. Ponds ranged in elevation from 1700 to 2400 m, with a mean maximum surface area of 2.3 ha (SEM: ±1.2 ha; limits: 0.15 to 24 ha), and a mean

maximum depth of 180 cm (SEM: ±50 cm; limits: 68 to 250 cm).

Skin swabs were collected from breeding adult boreal chorus frogs. This species is an annual spring breeder. In Arizona, adult males aggregate in dense choruses at shallow ends of ponds and conduct mating calls for several weeks following snowmelt in late February to early April. Tadpoles develop over an approximately 2.5 mo period, with metamorphic frogs typically emerging when ponds begin to dry in mid-June. Adults and metamorphic frogs forage near pond edges during the summer and overwinter under leaves, rocks, and logs beneath the snow (Moriarity & Lannoo 2005). Over this lifecycle, chorus frogs may migrate a maximum of 200 m from their natal pond (Kramer 1973). I, therefore, considered my study ponds to be independent populations as they were separated by a minimum of 1 km.

Animal Sampling

From March to May 2010, I swabbed approximately 60 adult frogs from each pond, for a total of 1115 swabs. Approximately 30 adult frogs were collected from each pond during each of 2 time periods: (1) when breeding was initiated (T1) and (2) 1 to 2 wk post-breeding-initiation (T2; Fig. 1). These two time periods were selected to maximize the likelihood of detecting Bd, as Bd prevalence is highest in adult frogs and these are the only times when they are easily obtained (O. Hyman unpubl. data). All animals were swabbed between 19:00 and 01:00 h. Animals were captured by hand, wearing disposable vinyl gloves, which were changed after handling each individual to prevent the spread of Bd. Each frog was swabbed with a wooden toothpick, toe-clipped to prevent

resampling, and released at the site of capture (Retallick et al. 2006). Swabs were placed in individual 2 ml screw-cap microcentrifuge tubes (USA Scientific, No. 1420-9701) containing 70% ethanol. In addition, an unused swab was placed into a tube at each site to act as a negative field control. All vials were kept on ice then stored at −20 °C in the laboratory. All field equipment was rinsed with 20% bleach solution and completely dried between collecting events to prevent contamination across sites. Swabs were extracted in Prepman Ultra™ (Applied Biosystems, Part No. 4318903), diluted 1:10 in sterile water, and stored at −20 °C in preparation for PCR analysis following Retallick et al. (2006). *Bd*-positive and -negative controls were included in each extraction batch.

Water Sampling

Water filters were collected from each pond at four different time points (Fig. 1): T1 and T2 as already described, T3 (3 to 4 wk post-breeding initiation, when tadpoles were present), and T4 (10 wk post-breeding initiation when metamorphosed froglets began to emerge). These time points were selected to maximize the likelihood of Bd detection by matching the times of highest density for each chorus frog life stage. Swabs of larval and newly metamorphosed frogs were not obtained due to monetary constraints and failure to detect Bd infections in these life stages in previous surveys (O. Hyman unpubl. data).

One filter was collected from each pond at each time point, unless ponds had dried, in which case no filters were collected (Table 1). Water was filtered following Kirshtein et al. (2007). Briefly, water from each pond was pushed through an individual Sterivex 0.22 μm filter (Millipore Part No. SVGV01015)

using a sterilized 60 ml Luer-Lok plastic syringe (BD, Part No. 309653). New syringes and filters were used for each pond. After the sample was filtered, 60 ml of phosphate-buffered saline (PBS) was pushed through the filter. Then each filter was drained, labeled, sealed in an individual Ziplok™ bag, and kept on ice for ≤ 5 h until it could be frozen at −20 °C in preparation for DNA extraction. A total of approximately 600 ml of water was filtered from each pond by collecting 20 ml from 30 locations within 3 m of pond edges. Samples were spaced evenly along the entire pond circumference, but taken only from areas where frogs or tadpoles were present. To account for potential heterogeneity in the distribution of Bd in the water column, I sampled water from a variety of within-pond microhabitats including areas of dense/sparse vegetation, detritus, and amphibian densities. Volumes filtered were recorded when filters became clogged prior to 600 ml. DNA was extracted from filters following the protocol developed by Kirshtein et al. (2007).

Real-time TaqMan PCR Assay

Samples were run in duplicate using an Applied Biosystems 7900HT sequence detection system and a modified version of the protocol developed by Boyle et al. (2004) and Garland et al. (2010). To reduce costs, we used 20 µl (rather than 25 µl) reactions containing 10 µl of 2x TaqMan Universal PCR Master Mix (Applied Biosystems, Part No. 43034437), 1 µl of 18 µM primers, 0.5 µl of 5 µM TaqMan probe, 2.5 µl of sterile DNA grade water, 8 µg of bovine serum albumin (BSA), and 5 µl of the previously described sample. This master cocktail was split into two tubes, one of which received TaqMan exogenous internal positive control

(IPC; Applied Biosystems, Part No. 43083283) following Garland et al. (2010). The other tube was left as 'normal' master mix. One duplicate of each sample contained IPC to test for PCR inhibition. In addition, three wells containing IPC and DNA-grade sterile water for template served as IPC controls. Standards of 20.0, 2.0, 0.2, and 0.02 Bd genome equivalents (GE) μl−1 (equivalent to 100, 10, 1, and 0.1 GE in the total 5 μl of template added) and negative controls were included in each 384-well plate.

Analysis

Following Garner et al. (2009), samples were scored positive if both duplicate wells were positive by quantitative PCR (qPCR). If one or none of the duplicate wells tested Bd positive, the sample was rerun in duplicate. If two or more of the four wells tested Bd positive over the course of these runs, the sample was considered Bd positive. If none of the wells tested positive in this rerun and there were no signs of PCR inhibition, the samples were considered Bd negative, with any previous single positive wells considered the result of inadvertent contamination or background fluorescence (Kriger et al. 2006). Following Garner et al. (2009), samples were considered PCR-inhibited if IPC cycle thresholds *(C*T values) were > 2 cycles above IPC *C*T of the sterile water controls. Negative samples with IPC *C*T > 2 *C*T values over the controls were rerun in duplicate. They were counted as negative if they came back with IPC *C*T values ≤ 2 *C*T over the controls. If negative samples still showed signs of inhibition after the rerun, they were rerun in duplicate a third time at a 10-fold dilution and counted as negative or positive following the criteria above.

The amount of Bd DNA in filter extract, in GE units, was calculated as:

Zoospore GE = mean output of all Bd-positive wells x 200

to account for sample dilutions and unit conversions. Results from 10-fold diluted samples were multiplied by an additional factor of 10. Zoospore GE l^{-1} was calculated as:

Zoospore GE l^{-1} = (zoospore GE/volume filtered[ml]) x 1000

Filter sensitivity was calculated within each individual sampling period by dividing the number of ponds testing Bd positive by filtration by those testing positive by swabbing, with the following caveats. Ponds testing Bd negative by swabbing < 60 animals were dropped from calculations of filter sensitivity because they run a > 5% risk of being false negatives (Skerratt et al. 2008). I also assumed that, because amphibian hosts were present in ponds throughout our sampling periods and Bd has persisted in these ponds for multiple years (O. Hyman unpubl. data), it is unlikely that Bd would completely disappear from any Bd-positive pond during the time periods we sampled. Furthermore, a pond testing Bd positive at *any* time point by swabbing, should be considered a Bd-positive location. Therefore, all ponds that tested Bd positive by swabbing by the end of the breeding season (T2) were assumed to be Bd positive throughout the following sampling periods (T3 and T4). I considered any filter testing negative

from these ponds to be a false negative, and calculated the probability of a false negative *(P)* as:

$$P = 1 - (\text{no. of Bd+ ponds by filterT}n/\text{no. of Bd+ ponds by swabT}n)$$

where T*n* = the time period of sampling.

Filter sensitivity relative to swabs was also calculated across all sampling periods by dividing the cumulative number of ponds testing Bd positive with filtration by the number of swab-positive ponds.

Lastly, I calculated the overall sensitivity of filters (i.e. the percent of Bd-positive ponds by swabbing that were correctly identified across each sampling period) for every possible combination of one, two, three, and four sampling events. These percentages were used to determine the mean, minimum, and maximum sensitivity of filters after one, two, three, and four sampling events. These percentages were also used to identify the most time-effective combination of sampling periods to filter water in order to maximize detection while minimizing sampling effort.

Bonferroni-corrected Spearman rank correlations were used to explore the relationship between *Bd* prevalence within a pond and the total number of filters testing *Bd* positive within that pond. Linear regression was used to compare log-transformed zoospore GE from Bd-positive filters to log-transformed volume of pond water filtered. Log-transformed zoospore densities detected by filters were

compared across sampling periods using a non-parametric Kruskal-Wallis test to account for small sample sizes and zero inflation. All statistics were performed in JMP (Ver. 5.0.1.2, SAS).

RESULTS

Swabs

Sixty swabs were collected from all except 7 ponds (Table 1). Two ponds with total sample sizes < 60 (Baker Lake, n = 31, and No Name 13, n = 25) also tested Bd negative with swabs, and were therefore omitted from our calculations comparing filters to swabs (see justification above; Table 1). Swabs detected Bd at 16 of 20 ponds (80%) when breeding began (T1; data not shown). Swabs detected Bd at the same 16 ponds as well as 1 additional pond (Twin) at T2, for a total of 17 of 20 ponds testing Bd positive (85%; Table 1). Bd prevalence at each pond varied from 0 to 98% (mean = 32%, SD = 27%; Table 1). Despite high Bd prevalence, no mass mortalities were observed.

Filters

Filters were collected in all four time periods for all but six ponds, which either dried completely before the final sampling was finished or had lost samples (Table 1). The mean volume of water filtered across all ponds within each time period was significantly lower at T4 ($ANOVA_{3,69}$, $p < 0.0001$; Tukey's honestly significant difference $[HSD]_{\alpha=0.05}$), with mean (±SEM) volumes of 510 (±32), 513 (±31), 545 (±32), and 297 (±36) ml filtered at T1, T2, T3, and T4, respectively. This reduction in filtrate at T4 was due to increased water turbidity as ponds dry during the course of the summer.

PCR inhibition was minimal in my samples. Only 11 negative samples had IPC *C*T > 2 cycles above sterile water controls. All of these samples tested negative for *Bd* after reruns and dilutions that brought IPC *C*T within 2 cycles of controls *(C*T = 33). IPC *C*T values of all other negative samples ranged from −1 to 2 cycles above control IPC *C*T values, with a mean of 1 cycle above controls. Although we cannot rule out inhibition as the reason Bd was not detected in the samples that had IPC *C*T values > 0 cycles above IPC controls (n = 15), the fact that the IPCs in these samples amplified within 2 cycles of controls indicates that, although there were inhibitors present in these samples, they did not significantly reduce or preclude DNA amplification. I, therefore, considered these to be true negatives. All positive and negative extraction and field controls worked properly.

Filters were able to detect Bd in every pond with Bd prevalence ≥ 10% in amphibian hosts (Table 1). Overall, filters produced results similar to swabs, with 17 of 20 ponds (85%) testing Bd positive by filtration (Table 1). However, these were not the exact same 17 ponds that tested positive by swabbing. One Bd-negative pond by swab tested Bd positive by filtration (No Name 13) and vice versa (McClure; Table 1). The pond testing Bd positive by filtering, but not swabbing, had the smallest sample size, with 25 swabbed animals (Table 1). The pond testing Bd positive by swabbing, but not filtering, had the lowest Bd prevalence (7%) of all ponds tested (Table 1). The overall number of filters testing positive within a pond was positively correlated with the prevalence of Bd in adult chorus frogs that bred there (Spearman's rho = 0.48, p = 0.03), indicating that higher prevalence in breeding adults at the start of the season increases the

likelihood of Bd detection by filters throughout chorus frog breeding and development. The mean density of aquatic zoospores across only positive filters (n = 31) was 30.0 zoospore GE l^{-1} (SEM: 10.8; limits: 1.3 to 313 zoospore GE l−1). Mean (±SEM) zoospore densities increased from 5.4 (±2.7) to 20.2 (±15.5) zoospore GE l^{-1} from T1 to T2 then decreased to 14.0 (±5.9) and 10.4 (±8.8) zoospore GE l^{-1} at T3 and T4, respectively (Fig. 2). However, there was no significant difference in the detected zoospore densities across sampling time periods (Kruskal-Wallis $\chi^2_3 = 5.8$, = 0.12). There was no relationship between the number of zoospore GE detected in Bd-positive filters and the volume of water filtered (linear regression adjusted $r^2 = 0.001$, $F_{1,29} = 0.03$, p = 0.86); however, I filtered a narrow range of water volumes (240 to 600 ml), which limits the interpretation of this result.

Two ponds (Baker Lake and No Name 13) were dropped from the sensitivity analyses due to Bd negative results and insufficient sampling of animals at the start of the breeding season (see criteria for dropping above; Table 1). These ponds were excluded from all calculations in the following paragraph. Of the remaining 18 ponds that met the inclusion criteria, 17 tested Bd positive by swabbing (94%; Table 1, Figs. 3 & 4). After four sampling events, filters failed to detect *Bd* from only one of these 17 ponds that tested Bd positive by swabbing (McClure Lake; Table 1, Fig. 4). However, filter-positive ponds did not consistently test positive across all filter-sampling events. In fact, 32 of the 62 (52%) total filters taken from ponds that were known to be Bd positive by swabs (n = 17) tested Bd negative (Table 1). The percentage of Bd positive ponds by

swab (n = 17) that tested Bd positive by filtration was 31 (T1), 65 (T2), 69 (T3), and 23 (T4) within each sampling event (Table 1). This translates to a false negative rate varying from 31 to 77% within a single sampling period, with the most false negatives occurring at T1 and T4 (Fig. 3). The cumulative percent of swab-positive ponds that tested positive by filtration increased from 31 to 71 to 94% after one, two, and three/four sampling events, respectively (Fig. 4). This translates to 69, 29, and 6% of swab-positive ponds falsely testing negative by filtration after one, two, and three/four filter sampling events, respectively.

Including all ponds, regardless of swab sample size, a total of 18 ponds tested Bd positive by either swabbing or filtering. At the two time points when both swabs and filters were taken (T1 and T2) swabbing ca. 30 adult host animals (T1) detected Bd in 16 of the 18 Bd-positive ponds (89%) and swabbing ca. 60 animals (T1 and T2) detected Bd in 17 of the 18 Bd-positive ponds (94%), while filtering detected Bd in only six (33%) and 11 (61%) of the 18 known Bd-positive ponds at T1 and T2, respectively.

Repeated water sampling at key time periods related to the life stages and natural history of our study species corrected this lack of detection. Filtering water at all four time points (T1 to T4) detected Bd at 17 of the 18 ponds that tested Bd positive by swabs or filters (94%; Table 2). Examining every permutation of filter sampling regimes revealed that filtering at only three time points would have detected Bd at 14 to 17 ponds (78 to 94%), filtering at only two time points would have detected Bd at eight to 15 ponds (44 to 83%), and filtering

at only one time point would have detected Bd at three to 11 ponds (17 to 61%) depending on the timing of sampling (Table 2).

DISCUSSION

Detecting pathogens by water filtration has several clear advantages over sampling animals. First, sampling water eliminates many ethical concerns related to the harm and stress caused during the sampling of animal hosts. Second, filtration enables pathogen detection in the absence of a sufficient number of animal hosts. These advantages are especially important when working with cryptic, rare, or endangered species (e.g. USFWS 2007). Third, filtering can reduce the time and money spent to detect a target organism in the field by reducing the number of samples needed to accurately detect a pathogen (Skerratt et al. 2008). However, all of these advantages depend on the sensitivity of the filtration technique.

I found that sampling small volumes of pond water for *Bd* had a low diagnostic sensitivity at individual time points. Overall, 31 to 77% of filters failed to correctly identify *Bd* swab-positive ponds in any single sampling period. This is an unacceptable level of sensitivity to determine the presence or absence of a target pathogen (Pfeiffer 2002). Despite the low sensitivity of filters in individual sampling periods, the 94% (16 of 17 ponds) agreement between filters and swabs after three sampling events and an equal number of *Bd*-positive ponds (n = 17) being detected by 60 swabs and three filtering events demonstrates that, with proper resampling, filters can be a viable Bd monitoring technique in this system.

Matches between sample IPCs and controls indicate the reduced filter sensitivity in individual sampling periods is unlikely to be caused by PCR inhibition. It is more likely that Bd is either not present in the small volume of water that was sampled or present at densities below detection. Increasing the volume of filtrate by prefiltering water with a more coarse filter or switching to slightly larger pore sizes that will still capture zoospores (e.g. 45 μm) may increase the chance of detection.

My finding of 94% (16 of 17 ponds) agreement between filters and swabs after three sampling events demonstrates the importance of sampling a pond multiple times in order to detect Bd by water filtration (Fig. 4). Although sampling all three filters in one time period could greatly reduce effort and expenses related to revisiting sites, the highest percentage of filters detected Bd 1 (T2) to 4 (T3) wk following breeding initiation (Fig. 3), indicating that timing of sampling influences the likelihood of detection. These results suggest that stratifying sampling across times that coincide with periods of potentially high pathogen prevalence (e.g. following breeding; Table 1) will increase the probability of detection. If data are available, surveyors should plan to collect filters in the days and weeks following times that typically display the highest pathogen prevalence. Two ponds tested *Bd* positive by one technique and not the other, indicating that in certain contexts one technique can be superior. The single pond that tested *Bd* positive by swabbing and not filtration (McClure Lake; Table 1) had the lowest *Bd* prevalence (7%; Table 1) of all the ponds I sampled. Also, only three filters (instead of the usual 4) were collected from this pond because it

dried before the final sampling period (Table 1). Reduced sample size combined with low Bd prevalence may explain why this pond did not test Bd positive by filtration. The inability of filters to detect Bd from a low prevalence pond indicates that there may be a threshold Bd prevalence (in this case 7%) at which filters do not successfully detect this pathogen. Thus, swabbing may be superior to filtering in cases where Bd prevalence is low but amphibian hosts are easily captured and/or in high enough abundance to obtain sufficient sample sizes (see Fenichel et al. 2008 and Skerratt et al. 2008 for in-depth treatment of sample sizes and disease detection). On the other hand, filters may be advantageous when amphibian hosts are not abundant. For example, filters identified a pond (No Name 13) as Bd positive even though swabs failed to detect Bd (Table 1). Only 25 animals (the smallest sample size) were collected from this site. This sample size has a < 95% probability of detecting Bd at prevalences ≤ 11% (Fenichel et al. 2008). It is apparent that swabbing 25 animals was not sufficient to detect Bd at the low prevalence at this pond. Thus, filtering water may be superior when a sufficient number of hosts cannot be collected to detect low levels of Bd prevalence by swabbing. Future studies should formally examine the ability of filters to detect Bd in areas of low amphibian abundance/low Bd prevalence.

The increase in Bd detection by filtration following breeding (T2 and T3; Fig. 3) could be a result of the accumulation of Bd zoospores in the water following times of high densities of infected and susceptible amphibian hosts (e.g. Vredenburg et al. 2010), such as breeding. This is supported by the positive correlation between Bd prevalence in breeding adults and filter detection rates.

This is also supported in part by the general increase in detected zoospore densities after the initiation of breeding (T1 to T2) followed by a steady decrease in mean zoospores densities after breeding (T2 to T4; Fig. 2), though these differences were not statistically significant. Yet, I found reduced detection by filtration later in the season, when metamorphs emerged (T4), despite the fact that metamorphs emerge at extremely high densities (O. Hyman pers. obs.). This may indicate a reduced density of aquatic Bd zoospores due to increases in water temperature or other abiotic factors that change as ponds become warmer and dry out. This reduced detection may also be a result of lowered *Bd* susceptibility in tadpoles or metamorphic froglets (Blaustein et al. 2005). If these stages are not susceptible to Bd infection, the number of zoospores released into the environment would be reduced, resulting in decreased rates of detection. Future studies should examine the susceptibility of these life stages to Bd infection and the influence of environmental factors such as pond salinity and temperature on disease pathology and Bd transmission. I cannot rule out the overall reduction in volume of filtrate at T4 as the cause of reduced detection. However, Walker et al. (2007) found that zoospore densities decreased with increasing volume of filtrate, indicating that more turbid waters often contain higher zoospore densities, though we did not find this trend.

The persistence of Bd in the environment can have important implications for host−pathogen dynamics (Mitchell et al. 2008). Although the filtration technique I used cannot distinguish between the presence of live, infectious Bd versus non-living (but detectable) Bd DNA, the repeated detection of Bd in

the water column suggests that Bd zoospores, or perhaps other life stages, persist in the water column throughout chorus frog development. DNA fragments of approximately 400 base pairs (bp) may persist up to 1 wk at 18°C in lake water (Matusi et al. 2001). Also, Dejean et al. (2011) found that bullfrog and sturgeon DNA persist < 1 mo in pond water. This suggests that the detection of Bd throughout our 3 to 4 mo sampling period is from the presence of live zoospores, as opposed to remnant DNA fragments from infected individuals present during the breeding season. However, the reduced detection at T4 does suggest that many of our Bd positive filters could be detecting remnant DNA fragments from infected adults (Dejean et al. 2011). The filtration tests I used cannot determine the ultimate source of these zoospores (environmental or amphibian hosts); however, reduced Bd detection from the water column at the start and the end of the breeding season (Fig. 3) indicates that Bd is either not present in the environment in the absence of infected amphibian hosts, or present at lower densities.

Similar to Walker et al. (2007) and Kirshtein et al. (2007), I found aquatic zoospore densities high enough to cause infections without direct contact with an infected individual (Carey et al. 2006). If these densities represent live infectious zoospores (as opposed to remnant DNA), this could help explain Bd's ability to transmit independent of host density, resulting in local host extinction (Mitchell et al. 2008). Despite these high environmental zoospore densities and high Bd prevalence in breeding adults, I found no evidence of disease induced die-offs or local extinctions in the chorus frog populations we studied. Chorus frogs may be

tolerant of Bd infections or suffering from disease cryptically, after the breeding season, when animals are hidden in the area surrounding ponds.

Filtering small volumes of water (≤ 600 ml) detected Bd in small, ephemeral ponds with high Bd prevalence. However, 52% of filters from ponds known to be Bd positive falsely tested negative, with the percent of false negatives varying from 31 to 77% depending on the season. Repeatedly sampling individual ponds at multiple time points (start of breeding, end of breeding, and when tadpoles were present) maximized detection, correctly identifying 94% of the ponds that swabs designated Bd positive. The ideal time to filter water for the presence of Bd was at the end of the breeding season and during the following 3 wk, when tadpoles were present in ponds. Detection was reduced at the start of breeding and late in the season when metamorphic frogs emerge. These results indicate that filtering water from three key time periods in the life cycle of boreal chorus frogs has similar sensitivity to sampling about 60 animals across two time points. This is encouraging, as there are many advantages to filtering over swabbing. For example, in on-site person-hours, capturing, swabbing, and releasing 60 animals takes significantly longer than filtering 600 ml of water four times (O. Hyman pers. obs.). In addition, I estimate a savings of ~$330 (US) in laboratory fees to process three water filters versus 60 frogs, per a pond (field-related costs to visit sites not included; calculations available from O. Hyman upon request). These savings could be increased by ending resampling after the first positive result. Had I employed this strategy I would have eliminated an

entire sampling event (T4) for all but three ponds, and reduced the number of filters processed by ~50%.

However, these results are likely to be host species and even population specific. Researchers will want to conduct similar studies to confirm the sensitivity of water filters in their own system before they incorporate this technique into Bd monitoring programs. Given the high percentage of false negatives for filters at any single time point, I do not recommend using this technique for the purpose of determining the presence or absence of Bd from areas where little is known about filter sensitivity. I instead recommend a combination of filtering with the swabbing survey recommendations of Skerratt et al. (2008). Combining filtering with animal sampling may be especially worthwhile when amphibian hosts are difficult to capture or in low abundance, as was demonstrated in No Name 13 pond in this study.

Filtering will be most useful for larger scale studies of systems where the dynamics of Bd are already relatively well understood and the sensitivity of this technique already assessed. Once the system-specific sensitivity of this technique is known, then filtering can be used to determine the spatial and temporal distribution of Bd on larger scales (e.g. landscapes/ years) with much finer resolution at greatly reduced costs. Given the low costs/effort and high potential sensitivity, I recommend this technique be considered for incorporation into long-term Bd monitoring initiatives.

CONCLUSIONS

Sampling small volumes of water can be a viable technique to detect aquatic pathogens such as Bd. This has now been demonstrated in several systems including ponds (Walker et al. 2007, present study), lakes (Kirshtein et al. 2007), and streams and bromeliads (Cossel & Lindquist 2009). However, I found that particular attention must be paid to timing and resampling to improve its likelihood of Bd detection. It should be emphasized that these results represent a case study of environmental detection of Bd in a metapopulation of a single, seasonally breeding, chorusing amphibian species, with larvae that develop quickly (approximately 2.5 mo) in small, mostly ephemeral ponds. Given the considerable variation in the habitats, ecology, and natural history of Bd's amphibian hosts, future studies need to examine how the sensitivity of this technique varies across systems with different hosts (e.g. long-lived larvae, continuous breeders, non-seasonal species, non-chorusing species) and habitats (e.g. streams, lakes, rivers, wetlands, bromeliads) before using it as a definitive diagnostic.

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Table 1. Results of swabs of boreal chorus frogs (*Pseudacris maculata*) and filters for the detection of *Batrachochytrium dendrobatidis* (Bd) taken from 20 ponds on the Mogollon rim, Arizona, USA. Percent of swab-positive ponds (n = 17) testing Bd positive by filtration when ponds with potential false negative results were removed from analyses (i.e. Baker Lake and No Name 13); only 16 ponds tested Bd positive by swabs at T1 (data not shown). BS: breeding starts; BE: breeding ends; Tad: tadpoles; Meta: metamorphs. T1: initiation of breeding; T2: 1 to 2 wk post-breeding; T3: 3 to 4 wk post-breeding; T4: 10 wk post-breeding; na: missing samples

Pond	Swabs			Filters					
	Bd status	N	% infected	T1 (BS)	T2 (BE)	T3 (Tad)	T4 (Meta)	Filters overall	Total no. of positive filters
27 Mile Lake	+	60	67	+	+	–	–	+	2
Alder Lake	+	60	38	–	+	+	–	+	2
Aspen Lake	+	60	98	+	+	+	–	+	3
Baker Lake[a]	–	31	0	–	–	–	na	–	0
Bar D Tank	+	60	58	+	+	–	–	+	2
Brolliar Park Tank	+	60	22	–	+	+	+	+	3
Brolliar Wetland	+	60	35	na	+	na	–	+	1
Calloway Lake	+	60	43	+	+	+	–	+	3
Clints Lake	+	60	12	+	–	–	+	+	2
Mahan Park	+	47	15	–	–	+	+	+	2
McClure Lake	+	57	7	–	–	–	na	–	0
Mud Tank	–	60	0	–	–	–	–	–	0
No Name 13[a]	–	25	0	+	–	–	–	+	1
No Name 7	+	51	59	–	+	–	na	+	1
Salmon Lake	+	55	75	–	–	+	–	+	1
Tbar 2	+	60	32	–	–	+	–	+	1
Tinny Tank	+	60	30	–	+	+	na	+	2
Twin Ponds	+	70	10	–	–	+	–	+	1
Van Deren Spring	+	60	25	–	+	+	na	+	2
Allen Park Tank	+	59	22	–	+	+	–	+	2
Total	17	1115	–	6	11	11	3	17	31
% *Bd* positive$_{\text{all ponds}}$	85	34	–	32	55	58	20	85	42
% of swab-positive ponds	100	–	–	0.31[b]	0.65	0.69[b]	0.23[b]	94	48

[a]These ponds were dropped from filter sensitivity analyses because their small swab sample size and failure to detect *Bd* cannot be distinguished from false negatives with >95% certainty (Skerratt et al. 2008)
[b]Ponds that were not sampled (na) within the given time period were removed from calculation of this percentage

Table 2. Evaluation of the role of resampling in the detection of *Batrachochytrium dendrobatidis* (Bd) using filters of water from boreal chorus frog (*Pseudacris maculata*) breeding ponds. Every possible combination of sampling was examined to identify the survey regime that maximizes the likelihood of Bd detection (ideal resampling schedule). Increasing the number of sampling events was vital for increasing the number of ponds where Bd was detected by water filtration. The maximum percent of ponds testing falsely negative was calculated by subtracting the minimum percent of Bd+ ponds (n = 18) detected by each sampling regime from 100. T1–T4 defined in Table 1

No. of filter sampling events	Mean no. *Bd*+ ponds detected by filters (min.–max.)	% *Bd*+ ponds detected by filters[a] (min.–max.)	Max. % of ponds testing falsely negative	Ideal resampling schedule
1	8 (3–11)	17–61	83	T2 or T3
2	13 (8–15)	44–83	56	T2+T3 or T1+T3
3	16 (14–17)	78–94	22	T1,T2,T3
4	17	94	6	T1, T2, T3, T4

[a]Out of 18 total ponds that tested *Bd*+ by either filtration or swabbing 47+ animals

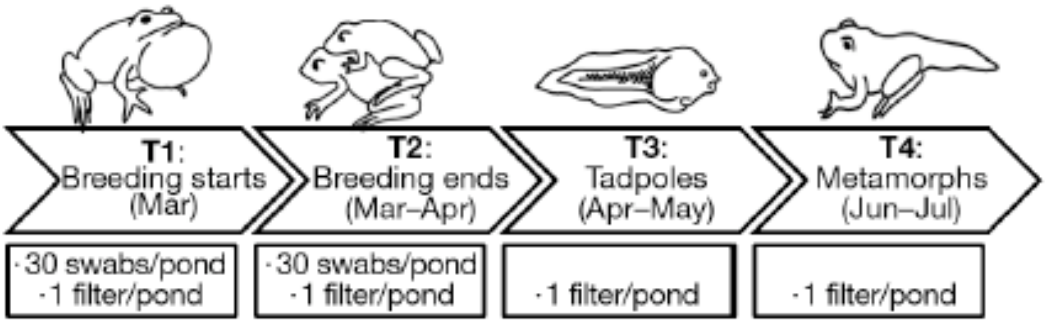

Figure 1. Timeline of pathogen sampling strategy from 20 ponds in relationship to the lifecycle stages of the boreal chorus frog (*Pseudacris maculata)*

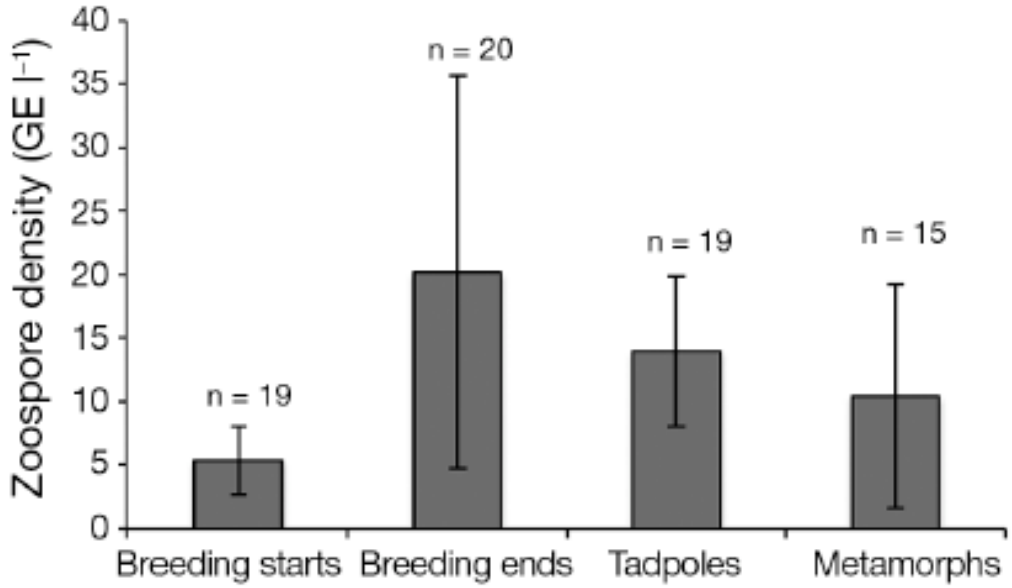

Figure 2. Mean (± SEM) density of *Batrachochytrium dendrobatidis* (Bd*)* zoospore genome equivalents (GE) l^{-1} detected in water filters taken from 20 chorus frog (*Pseudacris maculata*) breeding ponds at four different time points related to frog breeding and development. No significant differences were found (Kruskal-Wallis $\chi^2_3 = 5.8$, = 0.12)

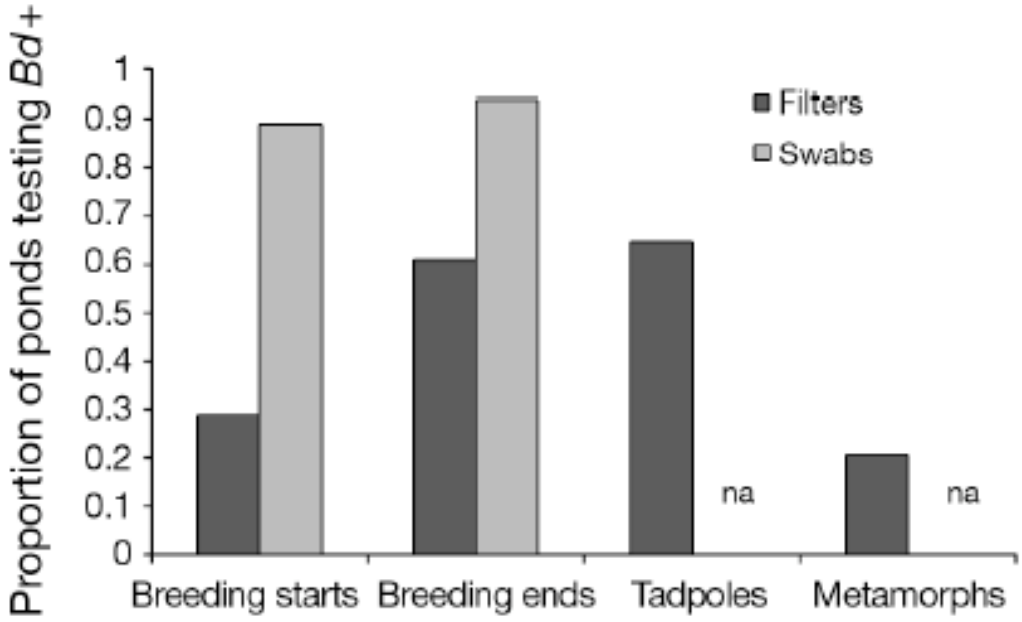

Figure 3. Proportion of ponds (n = 18) testing positive for the presence of *Batrachochytrium dendrobatidis* (Bd) at four different time points in the life cycle of boreal chorus frogs (*Pseudacris maculata)* by two sampling methods: filtration of 600 ml of pond water and swabbing ≥ 47 animals. (Two ponds were dropped from these analyses because an insufficient number of animals were swabbed from these ponds to have 95% confidence in their disease status.) When swabs were collected, they consistently detected Bd in a greater number of ponds than filters (swabs were not collected from tadpoles or metamorphs). na: not applicable

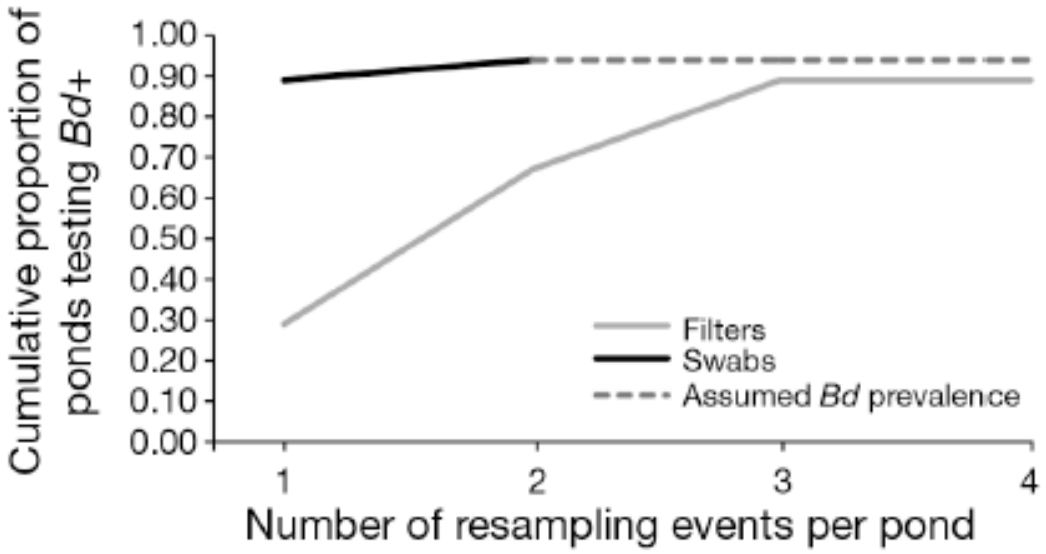

Figure 4. Cumulative proportion of ponds (n = 18) testing positive for the presence of *Batrachochytrium dendrobatidis* (Bd*)* by water filtration and swabbing of boreal chorus frogs (*Pseudacris maculata*). (Two ponds were dropped from these analyses because an insufficient number of animals were swabbed from these ponds to have 95% confidence in their disease status.) Ponds that tested positive by swabbing were considered Bd positive throughout the course of sampling; 95% of ponds tested Bd positive after two swabbing events. After three resampling events, filters were able to detect Bd in 16 of the 17 ponds (94%) testing Bd positive by swabbing ~ 60 animals

Chapter 3

DYNAMICS OF BATRACHOCHYTRIUM DENDROBATIDIS IN A SEASONAL, POND-BREEDING HOST AND THEIR RELATIONSHIP TO AQUATIC NUTRIENTS

ABSTRACT

Nitrogen (N) and phosphorus (P) are essential and often limiting nutrients in many ecosystems. Humans are increasing the amount of biologically available N and P at rates that far exceed levels seen in recent centuries, altering ecosystem patterns and processes. New evidence is emerging that these changes in N and P availability may increase the risk of infectious disease, especially for aquatic pathogens.

Batrachochytrium dendrobatidis (Bd) is a well-documented aquatic pathogen linked to the global decline of amphibian populations. Abiotic factors have been shown to play a role in Bd dynamics, but the influence of N and P on this pathogen have not been explored. This study examines the presence, prevalence, and intensity of Bd infections in populations of pond breeding chorus frogs (*Pseudacris maculata*) and whether these measures are correlated with aquatic concentrations of N and P.

Chorus frog densities, Bd presence, prevalence, intensity of infection, and aquatic zoospore densities were estimated along with aquatic concentrations of N and P from 20 independent frog breeding sites in each of two years. A mean of 57 adult frogs were sampled per pond during breeding in each year (2,251 total). Bd

was detected in frogs from 19 of 20 ponds. Seventeen ponds harbored Bd for both years of sampling. Individual chorus frog populations had high Bd prevalence (up to 100%) and loads (up to 193,000 zoospore genomic equivalents per frog). Despite this, estimated chorus frog densities increased significantly over the two years of sampling. Individual ponds showed wide variation in mean concentrations of total P (18.1 - 381.1 $\mu g\ l^{-1}$) and total N (20 - 1,460 $\mu g\ l^{-1}$), but N and P concentrations were not correlated with any measures of Bd presence, prevalence, or infection severity. These results did not support the hypothesis that nutrients influence Bd dynamics, but instead demonstrate that Bd can persist across a wide range of aquatic nutrient concentrations.

Given the strong negative effects Bd has had on populations of susceptible amphibian species, it is unexpected that populations of chorus frogs persist with Bd at the high host and pathogen densities observed in this study. Yet, this finding parallels those of others documenting the persistence of susceptible species with high Bd prevalence. Future work should examine the mechanisms that enable these populations to persist with this pathogen.

INTRODUCTION

Nitrogen (N) and phosphorus (P) are essential and often limiting nutrients in ecosystems throughout the world (Elser et al. 2007). Humans are increasing the amount of biologically available N and P at rates that far exceed levels seen in recent centuries (Carpenter et al. 1998, Vitousek et al. 1997). These changes are altering terrestrial and aquatic ecosystem patterns and processes (Smith et al.

1999, Elser et al. 2007). Evidence is emerging that increased supplies of N and P to ecosystems may also cause increased risk of disease in humans and wildlife, especially aquatic diseases (Bruno et al. 2003, Bruning 1991, Epstein 1993, Frost et al. 2008; Harvell et al. 1999, Johnson et al. 2007, Johnson et al. 2010, King et al. 2010, Schotthoefer et al. 2011, Smith 2007).

McKenzie & Townsend (2007) reviewed 34 studies of N, P, and infectious diseases that included a variety of human and wildlife pathogens. Most observations (93%) from these studies showed a positive correlation between increased nutrient availability and various measures of disease severity. In contrast, Marcogliese (2001) argues that while increases in nutrient availability may initially increase parasite abundance, high nutrient inputs and resulting eutrophication are most likely to decrease overall parasite abundance and diversity in favor of only a few, generalist species. Johnson et al. (2010) concede that the effects of nutrients will often vary according to the idiosyncrasies of the system being studied, but maintain that increased nutrient availability is still likely to exacerbate disease, especially for diseases caused by generalist pathogens with simple life cycles and direct transmission. The goal of this study was to test how the dynamics of an aquatic, directly transmitted, generalist pathogen with a simple life cycle, the emerging infectious parasite *Batrachochytrium dendrobatidis* (Bd), are related to nutrient concentrations in pond water.

Bd is the etiological agent of chytridiomycosis (Berger et al. 1998), a disease linked to the global decline and extinction of numerous amphibian species (Collins & Crump 2009). Bd is directly transmitted via flagellated cells called

zoospores, which encyst in adult amphibian skin where they develop into zoosporangia that release new zoospores into the environment (Berger et al. 2005). Zoospores may die, infect a new host, or reinfect their original host (Berger et al. 2005). As pathogen burdens (loads) increase on individual hosts, those susceptible to chytridiomycosis die from a reduced ability to osmoregulate (Voyles et al. 2009). Therefore, determining the factors that influence the ability of Bd to grow, reproduce, infect, or reinfect hosts is important for understanding Bd's transmission dynamics and onset of chytridiomycosis.

Despite the clear links between Bd and amphibian decline, there is considerable variation in the responses of amphibian hosts to Bd, with some host populations experiencing marked declines while others persist (Daszak et al. 2005, Hale et al. 2005, Lips et al. 2003, Lips et al. 2006, Rachowicz et al. 2006, Retallick et al. 2004). Although factors such as pathogen strain (Retallick & Miera 2007), host microbial communities (Harris et al. 2009), host immunity (Savage & Zamudio 2011, Woodhams et al. 2006), and population density (Briggs et al. 2010) contribute to the outcome of Bd introductions into amphibian populations, outbreaks of chytridiomycosis are often associated with specific environmental conditions, especially those related to water availability and temperature (Bosch et al. 2007, Bradley et al. 2002, Forrest & Schlaepfer 2011, Kriger et al. 2007, Kriger & Hero 2007a,b, Lips et al. 2003, Pounds et al. 2006, Raffel et al. 2010, Ron 2005, Rowley & Alford 2007, Savage et al. 2011, Schlaepfer et al. 2007, Woodhams et al. 2003). However, the focus on temperature and moisture as the major abiotic factors influencing the spread of Bd

and chytridiomycosis has overlooked other abiotic factors that could influence Bd dynamics, such as nutrient availability.

Nutrient inputs can influence the transmission of a directly transmitted pathogen such as Bd by indirectly altering host densities, altering host susceptibility to infection through behavioral or immunological shifts, or changing pathogen growth and vigor by providing additional resources directly to the pathogen or its competitors (Johnson et al. 2010). Nutrients can influence the survival, health, abundance, and behavior of amphibian hosts (Bishop et al. 1999, Wood & Richardson 2009, Ficetola et al. 2011, Marco et al. 1999), which could alter rates of Bd transmission. The availability and form of nitrogen used in media influence Bd growth in culture (Piotrowski et al. 2004), indicating that variation in the availability of N and potentially P within amphibian or currently unknown hosts could influence disease dynamics by altering Bd growth rates and longevity. N and P availability could also indirectly influence Bd dynamics by altering abundance and feeding rates (DeMott & Gulati 1999, Makino et al. 2002, Sterner & Hessen 1994) of zoospore predators, such as zooplankton (Buck et al. 2011). This suggests another mechanism by which nutrient availability could influence Bd dynamics; namely, by altering zoospore densities in the aquatic environment, thereby reducing rates of environmental transmission (Woodhams et al. 2011).

In light of the rising evidence for the role of nutrients in the emergence of aquatic infectious diseases and the importance of understanding the abiotic factors that influence chytridiomycosis, I tested whether Bd dynamics are related to aquatic nutrient concentrations using populations of pond breeding boreal chorus

frogs (*Pseudacris maculata)*. Boreal chorus frogs were chosen because 1) they use a variety of wetland habitats capturing much of the natural variation in N and P concentrations, 2) they harbor Bd infections in the wild, 3) they suffer high rates of Bd-induced mortality (up to 80%) in the laboratory, and 4) very little is known about the distribution or dynamics of Bd in wild chorus frog populations. The goals of this study were first to gain a detailed understanding of the distribution and dynamics of Bd in chorus frog populations. Secondly, I tested how chorus frog densities and the presence, prevalence, short-term changes in prevalence (a proxy for incidence), aquatic density of Bd zoospores and intensity of Bd infections in amphibian hosts correlated with pond-level concentrations of two major nutrients, N and P.

METHODS

Field Sites and Study Species

Water chemistry and disease data were collected from 20 ponds with breeding populations of boreal chorus frogs located over approximately 75 km^2 within Sitgreaves-Apache and Coconino National Forest in north-central Arizona (Table S1). This region is a matrix of forest (Petran Montane Conifer Forest and Great Basin Conifer Woodland) and livestock rangeland (Brown 1994). These forests are among the driest in North America, with annual rainfall varying from 460-760 mm, so ranchers have constructed ponds to collect and hold natural run-off to provide water for livestock. Amphibians often use these ponds to breed.

Sites in this study included ephemeral ponds, perennial ponds, man-made cattle ponds, and one spring (Table S1). Ponds were selected based on two criteria: (1) presence of breeding chorus frogs to ensure that studies were conducted within a range of N and P concentrations that the host species can tolerate, and (2) a minimum distance of 1 km from other ponds to ensure little to no mixing of frogs from each site (Kramer 1973). This distance enabled me to consider each pond as an independent data point. It also ensured that water chemistry measures were relevant to adults across years since there is little to no immigration or emigration by frogs to or from different ponds. Ponds had a mean maximum surface area of 1.59 ± 0.59 ha in 2009 and 2.3 ha ± 1.2 ha in 2010.

Immediately following snowmelt in late February to mid-March, boreal chorus frogs in Arizona aggregate in shallow areas of ponds for one to seven weeks to breed (O. Hyman, pers. obs.). Tadpoles develop over about 2.5 mo, with metamorphic frogs typically emerging when ponds begin to dry in mid-June and July. Adults and metamorphic frogs forage over the summer in upland forest surrounding ponds, overwinter under leaves, rocks, logs, etc. beneath the snow, and then return to ponds the following spring to breed. This study focused on Bd dynamics in adults during breeding, as this is likely to be a key time for Bd transmission.

Host and Pathogen Sampling

Swabs. Approximately 30 to 60 adult breeding chorus frogs were sampled for Bd infections in each pond by skin swab sampling (described in detail below)

from late February to early April in 2009 and late March to early May in 2010 to determine Bd prevalence at each pond where:

Bd prevalence = (# Bd positive animals/total # of animals sampled)

These large sample sizes within a single life stage (adult) during one season (spring breeding) provide confidence in the accuracy of our Bd presence and prevalence data at the time of sampling (Skerratt et al. 2008). Sampling over two years also enabled me to test if Bd persisted in chorus frog populations over two breeding events and whether Bd infected populations showed evidence of decline over this period.

In 2009, Bd prevalence at each pond was estimated by swabbing about 60 frogs at one time point coinciding with the initiation of breeding. In 2010, short-term-changes in Bd prevalence in each pond were estimated as a proxy for Bd incidence by swabbing about 30 frogs at each of two time points: 1) when *P. maculata* breeding started (T1) and 2) about 1 week after the initial sampling (T2). Mean daily change in Bd prevalence (Δ prevalence) was used as a proxy for Bd incidence and calculated as:

Δ Bd prevalence = (Bd prevalence at T2 – Bd prevalence at T1)/# days between sampling dates

All adult animals were swabbed between 1900 and 0100 hours to control for any diel variation. Animals were captured by hand, wearing disposable nitrile gloves, which were changed after handling each individual to prevent the spread of Bd. All animals were collected from aquatic sites in the pond. The hands, feet, thighs, and vent of each frog were uniformly swabbed with a wooden toothpick (Retallick et al. 2006, Retallick & Miera 2007). Following swabbing, animals were toe-clipped to prevent resampling and released at the capture site. Swabs were placed in individual 2-ml screw-cap microcentrifuge tubes (USA Scientific, No.1420-9701) containing 70% ethanol. In addition, an unused swab was placed into a tube after finishing sampling at each site to act as a negative field control. All vials were immediately put on ice and stored at -20°C within 3 hours. All field equipment was rinsed with a 20% bleach solution and dried completely between collecting events to prevent contamination across sites.

Host density estimation. Relative host densities at each pond were assessed by calculating the animal capture rate at each pond, where:

Animal capture rate = total number of animals captured/total sampling time.

All times were calculated from capture rates of a single sampler (OJH) to control for any biases in experience.

Water filters. In addition to skin swabs, in 2009, water filtration (Hyman & Collins 2012) was used to estimate the densities of zoospores in the water column of each pond. Five separate 400 ml samples (2 l of total filtrate pond^{-1})

were collected from each pond on the day following animal swabbing by forcing water through individual 0.22-μm Sterivex™ filters (Millipore Part No. SVGV01015). Water samples were collected from four locations along pond edges, where frogs were present, and the fifth sample was collected from the bottom of the deepest part of the pond accessible by wading. A negative "field" control filter of 60 ml of phosphate buffered saline solution was included for each pond to ensure filters were not contaminated with Bd during the sampling process. In addition, individual 400 ml water samples from each pond were kept on ice, brought back to the laboratory, inoculated with 0, 10, 100, or 1000 zoospores, filtered, extracted, and used to construct curves to standardize zoospore densities across ponds to account for pond level differences in DNA recovery during DNA extractions from filters. Following Kirshtein et al. (2007), each filter was drained, labeled, sealed in an individual Ziploc® baggie, and kept on ice for ≤5 hours until it could be frozen at -20°C in preparation for DNA extraction.

DNA extraction. Swabs were extracted using the PrepMan Ultra™ protocol (Boyle et al. 2004) with modifications of Retallick et al. (2006). Filters were defrosted and DNA extracted using a modified version of the Puregene protocol developed by Kirshtein et al. (2007) as described in Hyman & Collins (2012). Bd negative and positive controls were used in all extractions. Extracted samples were stored at -20 °C for quantitative PCR analysis.

Real-time Taqman PCR assay. Samples were amplified using an Applied Biosystems 7900HT Sequence Detection System and a modified version

of the protocol developed by Boyle et al. (2004) and Garland et al. (2009) as described in Hyman & Collins (2012). Samples were run in duplicate with bovine serum albumin and exogenous internal positive controls (Exo IPC, Applied Biosystems Part No. 4308328). Each 384-well plate also had a negative control. Bd status (positive/negative) and infection loads (zoospore genomic equivalents (GE)) were calculated as described in detail in Hyman & Collins (2012).

Water Chemistry

Water samples were collected from each pond on the day following the first frog-swabbing period. For each pond, five water samples were collected in new 50-ml copolymer centrifuge tubes (USA Scientific part no. 1500). One sample was collected about 1 m from shore at mid-depth from each of the N, S, E, and W compass points (4 samples total) and one additional sample was collected from the deepest part of the pond accessible by a 2 m-length tube connected to a handheld pump. In addition, a negative control of 50-ml of Nanopure^{TM} water (poured from a carboy after pond sampling) and positive controls of 49 ml of pond water spiked with 1-ml of 50-µM P solution or 750-µM N solution were created at each pond. All vials were stored on ice and frozen at -20 °C within 3 hours of collection. Total nitrogen (TN) and total phosphorus (TP) concentrations of each unfiltered sample from 2009 and 2010 were estimated following standard methods after digestion (persulfate method; APHA 1998). Nutrient analyses were conducted using a Bran-Luebbe TrAAcs 800 system (Bran and Luebbe, Delavan, Wisconsin, USA). All chemical analyses were conducted within thirty days of sample collection by technicians at the Goldwater Environmental Laboratory at

Arizona State University. Quality assurance for nutrient analyses included Nanopure™ field controls, sample splits, spike recovery, and routine evaluation of external standards and blanks.

Data Analysis

The nutrient concentrations ([TP], [TN]) of each pond were estimated by calculating the mean of the five water samples. Aquatic zoospore densities were calculated as the mean of all five filters taken from each pond adjusted according to the standardized curves developed using spiked water from each individual water body. Distributions of all variables were assessed for normality using Shapiro-Wilk W-tests for goodness of fit to a normal distribution. Bd prevalence data were arcsin-transformed. Nutrient and host density data were log-transformed to approximate normality. The number of filters testing positive within a pond, the mean density of aquatic zoospores detected within a pond, and the mean Bd load at individual ponds in 2010 (T1 and T2) were all non-normally distributed despite transformations. Therefore, non-parametric Spearman's rank correlations were used to compare these response variables to nutrient and host density data.

Univariate analyses were used to compare pond-level explanatory variables ([TN], [TP]) to pond-level response variables (host densities, Bd prevalence, Bd load, and Δ Bd prevalence) assuming linear responses. Quadratic responses were also fit to univariate analyses with [TP] to account for potential non-linear responses across the large range in [TP]. Host densities were also used as an explanatory variable in univariate analyses with disease measures. Mean Bd

prevalence and mean Bd load within each pond were compared across 2009, 2010 T1, and 2010 T2 using paired t-tests. Chorus frog density within each pond was compared across years using paired t-tests. Simple linear correlations were used to compare Bd load and Bd prevalence within each sampling period as well as Bd prevalence within ponds across years.

Mean [TP], [TN], host density, and Bd prevalence was compared between ponds testing Bd-positive versus Bd-negative by water filtration using t-tests. Nonparametric Spearman's Rho correlations were used to compare the number of filters testing Bd-positive within an individual pond to [TP], [TN], host densities, Bd prevalence, and Bd loads in amphibian hosts. Nonparametric Spearman's rank correlations were also used to compare pond-corrected mean zoospore densities to [TP], [TN], and host densities in each locality. Non-parametric Wilcoxon signed-rank tests were used to compare [TP], [TN], and host densities between ponds that tested Bd-positive and Bd–negative by swabbing animals in 2010. All statistics were calculated using JMP (Ver. 5.0.1.2, SAS) statistical software.

RESULTS

Swabs

A total of 2,251 individual adult chorus frogs were swabbed over two years, with a mean of 57 animals swabbed per pond annually. In 2009, ≥ 60 individual frogs were collected from all except 5 ponds, and no fewer than 30 animals were sampled from any individual pond (Fig. 1, Table S2). In 2010, fewer than 30 animals were sampled from only four ponds at T1 and only six

ponds at T2 (Fig. 2, Table S3). No fewer than 25 animals were collected from any individual pond, with the exception of one pond from which no frogs were sampled at T2 (No Name 13), which was dropped from all subsequent analyses requiring T2 data (Fig. 2, Table S3). Seven ponds had > 7 but ≤ 13 days between sampling events T1 and T2 (Table S3). All other ponds had 7 days between sampling (Table S3).

2009 swabs. In 2009, all but one pond (No Name 13) tested positive for Bd (Fig. 3). This pond had the fourth lowest frog density of all ponds sampled that year (Table S4). Bd prevalence in positive ponds varied from 2-93% (mean 27 ± 6%; Fig. 1, Table S2). Mean Bd load at each site in 2009 varied from 4 - 2,749 zoospore (zsp) genome equivalents (GE) with an overall mean (± SE) of 568 ± 151 zsp GE across all sites (Fig. 1, Table S2).

2010 swabs. Three ponds tested Bd negative in 2010 (Fig. 2, Fig. 3, Table S2). These three sites had significantly lower frog densities than Bd positive sites (Wilcoxon, p = 0.01; Fig. 4). Bd prevalence in positive ponds ranged from 6-96% (mean 24 ± 6%) at T1 and 7-100% (mean 40 ± 7%) at T2 in 2010 (Fig. 2, Table S3). Mean daily change in Bd prevalence (Δ Bd prevalence) at individual ponds ranged from a -2.9 – 7.3% daily increase (mean 2.3 ± 0.6%) in Bd prevalence over the time period between T1 and T2 (Fig. 2, Table S3). Mean Bd loads in 2010 ranged from 20 to approximately 43,000 zsp GE with an overall mean of 1320 ± 629 zsp GE at T1 and 4738 ± 2069 zsp GE at T2 (Fig. 2, Table S3).

Across time comparisons. Mean Bd prevalence was significantly higher at the second sampling point (T2) in 2010 as compared to T1 (paired t-test$_{df = 18}$, t-

ratio = 3.85, p = 0.001) and 2009 (paired t-test$_{df = 18}$, t-ratio = 2.58, p = 0.02; Fig. 5). Mean Bd prevalence at sampling point 1 (T1) in 2010 was not significantly different from 2009 (paired t-test$_{df = 19}$, t-ratio = -0.7, p = 0.49; Fig. 5). Mean Bd loads showed the same trend, with significantly higher loads at T2 in 2010 as compared to T1 (paired t-test$_{df = 18}$, t-ratio = 3.02, p = 0.007) and 2009 (paired t-test$_{df = 18}$, t-ratio = 3.12, p = 0.006; Fig. 5), but mean Bd load at T1 in 2010 was not significantly different from 2009 (paired t-test$_{df = 19}$, t-ratio = -0.68, p = 0.52; Fig. 5). Mean Bd load was positively correlated with Bd prevalence within ponds in 2009 (ANOVA$_{df = 19}$, p < 0.0001; Fig. S1), and mean load in 2010 was correlated with Bd prevalence at T1 (ANOVA$_{df = 19}$, p < 0.0001) and T2 (ANOVA$_{df = 18}$, p < 0.0001; Fig. S1). Bd prevalence in 2009 was correlated with prevalence at T1 (ANOVA$_{df = 19}$, p = 0.0001) and T2 (ANOVA$_{df = 18}$, p = 0.0003) in 2010 (Fig. 6), indicating ponds have consistently high or low prevalence each year. Despite high Bd prevalence and loads in many of the chorus frog populations I surveyed, no mass-mortalities were observed in either year. In fact, relative frog densities increased from 2009 to 2010 (paired t-test$_{df = 19}$, p = 0.0003; Fig. 7).

Water Filters

Twenty of the 100 water filters sampled tested positive for the presence of Bd (Table 1). Ten ponds had at least one filter test Bd positive (Table 1). The remaining 10 ponds did not have a single Bd positive filter, despite animals from nine of these ponds testing Bd positive. Overall, filters detected Bd in only 53% (10 of 19) of the ponds that tested Bd positive by swabbing, and 79% of

individual filters failed to detect Bd in ponds where at least one frog tested Bd positive. Zoospore densities in the water column ranged from ca. 6-1123 zsp l^{-1} (mean: 50 ± 26 zsp l^{-1}; Table 1). The number of filters testing Bd positive within a pond was positively correlated with Bd prevalence in breeding adult chorus frogs (Spearman's *Rho* = 0.70, p = 0.0006; Fig. 8) and the mean concentration of aquatic zoospores detected within a pond (Spearman's *Rho* = 0.95, p < 0.0001; Fig. 8). Mean aquatic zoospore density was also significantly positively correlated with Bd prevalence in frogs (Spearman's *Rho* = 0.48, p = 0.05). Ponds testing Bd positive by filtration had a significantly higher prevalence of Bd in host animals ($t\text{-test}_{df = 18}$, p = 0.006; Fig. 9).

Disease and Nutrient Analyses

Water chemistry. Mean concentrations of TP in individual ponds ranged from 18.1-381.1 μg-P l^{-1} with an overall mean concentration of 103.3 ± 18.9 μg-P l^{-1} and 76.3 ± 8.5 μg-P l^{-1} across all ponds in 2009 and 2010 respectively (TableS4). Mean TN concentrations in individual ponds ranged from 20 – 1,460 μg-N l^{-1} with an overall mean concentration of 650 ± 60 μg-N l^{-1} and 380 ± 70 μg-N l^{-1} across all ponds in 2009 and 2010 respectively (Table S4). A recent survey of TP and TN in Canadian wetlands found concentrations of 43.4 ± 12.4 μg-P l^{-1} and 117 ± 24 μg-N l^{-1} in unfiltered water taken from low human impact wetlands and 114.7 ± 12.4 μg-P l^{-1} and 862 ± 53 μg-N l^{-1} in wetlands with high agricultural and livestock impact (King et. al 2010). Johnson et al. (2007) cite similar TP and slightly higher TN concentrations in unfiltered water taken from forested (43 μg-P l^{-1} and 1235 μg-N l^{-1}) and agricultural (348 μg-P l^{-1} and 2859

μg-N l^{-1}) amphibian habitats in Wisconsin. Total P in 2009 and 2010 and TN concentrations in 2009 and 2010 were positively correlated across years ($ANOVA_{df = 19,}$ p = 0.004 and p = 0.01, respectively), indicating that TN and TP concentrations stayed consistently higher or lower within individual ponds in both years.

Host densities. Frog capture rates within individual ponds decreased significantly as mean pond TP concentration increased in both 2009 ($ANOVA_{df=19}$, p = 0.04) and 2010 ($ANOVA_{df=19}$, p = 0.01; Fig. 10, Table S5). Total N did not show any consistent relationship with host densities in either 2009 ($ANOVA_{df=19}$, p = 0.23) or 2010 ($ANOVA_{df=19}$, p = 0.58; Fig 10, Table S5).

Animal swabs. The single Bd negative pond in 2009 had the second lowest [TN] (350 μg N l^{-1}) and the third lowest [TP] (31.5 μg-P l^{-1}) of all ponds sampled that year (Table S4). Bd prevalence and load in 2009 were not significantly related to [TP], [TN], or amphibian density in univariate analyses using both linear and, in the case of [TP], quadratic fits (Table 2). Mean [TP] and [TN] at the three Bd negative sites in 2010 were not significantly different from Bd positive sites (Wilcoxon, p = 0.20 and 0.75, respectively). Delta Bd prevalence in 2010 was not related to [TP], [TN], or host densities in 2009 or 2010 (Table 3) nor was Bd prevalence or load at T1 and T2, with the exception of mean host load at T2 being positively correlated with host density (Spearman's *Rho* = 0.55, p = 0.02; Table S6).

Water filters. The number of filters testing Bd positive within a pond and mean zsp density in the water column were not correlated with nutrient

concentrations (Table 4). Ponds with filters testing Bd positive did not have significantly different [TP], [TN], or frog densities than ponds that tested Bd negative by filtration (t-test$_{df\text{-}18}$, p = 0.8, 0.4, and 0.8, respectively; Fig. 9).

DISCUSSION

Bd Dynamics in Chorus Frogs

Breeding plays an important role in the transmission of Bd between adult chorus frogs. As breeding progresses, Bd loads increase and the pathogen spreads, likely through direct contact with infected animals and environmental transmission from free-swimming zoospores. During breeding, Bd prevalence increased daily by a mean of approximately 2%, including an increase as high as 7% per day. At a 7% daily increase, Bd could spread through an entire population in as little as two weeks. High Bd prevalence (up to 100%) in many study ponds indicates that Bd indeed can infect the entire breeding adult population.

In 2009, 19 of 20 populations tested Bd positive, 17 of which tested Bd positive the following year. Despite this pathogen's ubiquity, there is clearly a high level of variation in the prevalence, Δ Bd prevalence, and intensity of Bd infections across individual ponds. The significant increase in prevalence and infection intensity over a one-week period during breeding in 2010 suggests that much of this variation is a function of time: as breeding progresses the pathogen spreads. Yet variation in Bd prevalence is not explained by time alone. For example, Bd prevalence within each pond was correlated between years. High prevalence ponds remained high while low prevalence ponds remained low,

suggesting that there are pond-specific factors that regulate Bd transmission rates. Also, ponds had very different rates of Bd spread over similar time periods, ranging from ca -2.9 to 7.3% per day. If Bd prevalence increases non-linearly, these differences could be related to timing of sampling. Alternatively, these pond-level differences in prevalence and Δ prevalence could be a result of pond-specific factors.

Chorus frog densities may be one of these factors. The three ponds that tested Bd negative in 2010 had the lowest chorus frog densities of all the ponds sampled that year. The absence of Bd from these populations is not for lack of introduction as Bd was detected (though at very low prevalence) in two of these ponds in the previous year and in water from the third pond in 2010 (Hyman & Collins 2012). These observations indicate that there may be a threshold density below which Bd is not able to invade or perhaps persist in chorus frog populations. This is surprising, as a multi-host pathogen, such as Bd, should be able to spread and persist independent of any central host's density as long as there is a sufficient density of reservoir hosts to maintain transmission (de Castro & Bolker 2005). Other amphibians known to harbor Bd, including tiger salamanders (*Ambystoma tigrinum,* Davidson et al. 2003), have been captured at these Bd-negative sites during chorus frog breeding, so these are not single-host systems. However, it may be possible that the overall density of amphibians is below some critical threshold, preventing Bd from invading and persisting.

I found mixed evidence in support of the hypothesis that chorus frog densities influence Bd transmission once it has invaded a population. Host density

was consistently positively correlated with the prevalence, intensity, and Δ prevalence of Bd infections in both years. However, with the exception of the correlation between chorus frog density and mean Bd load at the second sampling period in 2010, none of these correlations were statistically significant. These mixed results suggest that Bd may spread through a combination of density-dependent (host contacts) and density-independent (environmental transmission through aquatic zoospores from alternate hosts) mechanisms.

Chorus Frogs Persist with Bd

Despite high Bd presence (up to 95% of sites), prevalence (up to 100%), and pathogen loads (up to 193,000 zoospore GE), chorus frog populations persisted at high population densities with Bd for at least two generations. There was no evidence of Bd related, mass die-offs in any populations I surveyed. In fact, chorus frog abundance increased significantly over the two years of this study.

The mechanisms that enable these populations to persist with Bd remain unclear. Chorus frogs taken from the same sites suffered high levels of Bd-induced mortality in the laboratory (Retallick & Miera 2007), so it is unlikely that these frogs are innately resistant to chytridiomycosis. Bd strains in these populations may have attenuated in virulence (Retallick & Miera 2007), or there may be environmental factors that enable these frogs to tolerate or remove Bd infections (e.g. Forrest & Schlaepfer 2011, Retallick & Miera 2007). Alternatively, given their high reproductive capacity and quick rates of development (Smith 1987), chorus frog populations may be robust to high rates of

Bd-related adult mortality. Chorus frogs collected from ponds used in this survey did not suffer mortality until 20-30 days post Bd exposure in laboratory experiments (Retallick & Miera 2007), sufficient time for animals to breed and leave a pond. If young of the year are able to survive and reproduce the following year (Smith 1987), the population could persist. Future experiments should examine the Bd-related mortality rates of adults and juveniles in the wild to clarify the mechanisms that enable these populations of apparently susceptible animals to resist Bd-related decline.

Aqueous Nutrient Concentrations and Bd Dynamics

Our results do not support the hypothesis that increasing nutrient availability increases pathogen transmission or disease severity in this directly transmitted, multi-host pathogen, Bd. Indeed, I found no evidence that nutrient concentrations play any role, positive or negative, in Bd transmission during breeding. Sites demonstrated wide variation in [TP] and [TN], representative of undisturbed and nutrient-rich wetlands (King et al. 2010, Johnson et al. 2007). Despite this wide variation in nutrient concentration, I found no association between [TN] and [TP] concentrations and any measures of pathogen transmission or disease severity, including Bd prevalence in amphibian hosts, intensity of Bd infections, rates of Bd transmission (as measured by Δ Bd prevalence), densities of aquatic zoospores, and frequency of Bd detection from water samples. These results clearly indicate that in habitats where amphibians are present, Bd can spread and persist across a wide range of nutrient concentrations.

These findings run counter to those that have linked nutrient concentrations to the abundance and diversity of a variety of pathogens (Johnson et al. 2011), especially of amphibians (e.g. Johnson et al. 2007, King et al. 2010, Schotthoefer et al. 2011). My results do not preclude the ability of nutrients to influence Bd dynamics. Assuming Bd is not able to reproduce outside of amphibian hosts (but see Kilburn et al. 2011), extreme eutrophication that eliminates amphibians from a habitat (e.g. Bishop 1998) would obviously eliminate Bd as well. Thus, it is possible that N and P concentrations beyond the levels found in this study may influence Bd dynamics. In addition, I only tested for evidence that nutrients influence Bd transmission and growth in adult frogs during breeding. Nutrients may instead influence these rates during larval development or metamorphosis. I did not directly measure Bd presence, prevalence, or intensity of infection in these life stages. However, I found no evidence that N or P concentrations in the previous year have any influence on Bd presence, prevalence, or transmission in adults in the next year. Thus, if N and P do influence Bd dynamics in pre-metamorphic life stages, these effects do not appear to carry over to breeding adults in the subsequent generation. Future studies should examine how nutrient availability may influence Bd dynamics in these life stages.

Although nutrient concentrations had no apparent effect on Bd presence, transmission, or aquatic zoospore densities, they were related to host density. Chorus frog densities were negatively correlated with [TP] in both 2009 and 2010. Phosphate has been shown to have direct toxic effects on some amphibians

(Earl & Whiteman 2010, Hamer et al. 2004) but only at extremely high concentrations (15 – 200 mg-P L^{-1}) not observed in these ponds. It is therefore unlikely that this reduction in chorus frog density resulted from direct toxic effects at the TP concentrations I encountered. This correlation could be the result of an indirect negative influence of P on amphibian survival, as demonstrated in mesocosm experiments with *Bufo boreas* (Wood & Richardson 2009). It is also possible that factors that correlate with P, such as landscape alteration (Allan 2004) or livestock use (Knutson et al. 2004), are the causal mechanisms underlying this trend. For example, Ficetola et al. (2011) found a negative relationship between phosphate concentration and fire salamander (*Salamandra salamandra)* distribution. In their study, landscape-level analyses found that lower [P] were correlated with greater natural land cover and increased stream permanence, factors vital to fire salamander reproduction. In the case of my study, ;oss of chorus frog habitat, such as forest and grasslands, from areas surrounding ponds could similarly result in higher [TP] and lower frog densities. Alternatively, Knutson et al. (2004) found that cattle use was positively correlated with TP concentrations in wetland habitats. Cattle could be raising [TP] and lowering chorus frog abundance at our sites by altering pond habitat or trampling amphibians (Schmutzer et al. 2008). Clarifying the mechanisms that link TP concentrations to amphibian density in this and other studies may be important in the face of landscape change, rising P loading, and global amphibian decline (Allan 2004, Carpenter et al. 1998, Stuart et al. 2004).

Implications for Bd Monitoring and Management

These results have several important implications for Bd monitoring and management initiatives. First, my data show that detecting Bd is time sensitive. Numerous other studies have documented seasonality in Bd prevalence (Savage et al. 2011, Retallick et al. 2004) but my findings demonstrate that Bd prevalence can vary significantly even within a season. Bd prevalence and loads increased significantly over a single week, and in one case a pond initially testing Bd negative, tested Bd positive only a few days later, despite sampling ≥ 30 animals at both time points. Bd loads increased in a similar manner. This will be important for the design of field studies such as this one and others that use single sample presence, prevalence, and load data to determine environmental correlates of disease dynamics (e.g., Raffel et al. 2010, Piovia-Scott et al. 2011, Walker et al. 2010). It will be even more important for monitoring initiatives looking for "Bd-free" sites for reintroductions of captively reared species (see USFWS 2007). My findings indicate that, in the case of seasonally breeding/chorusing frogs, it is best to monitor animals late in the breeding season when Bd prevalence peaks. I also found that, despite its advantages, water filtration is not a highly sensitive technique, detecting only 53% of ponds testing Bd positive by swabbing. Timing will be key for detecting Bd using this method as well (see Hyman & Collins 2012 for a detailed discussion).

The nearly ubiquitous presence of Bd in Arizona chorus frog populations is also relevant for the management of two locally threatened amphibian species:

the Northern Leopard Frog (*Rana [Lithobates] pipiens)* and the Chiricahua Leopard Frog (*Rana [Lithobates] chiricahuensis)*. Once locally abundant, Northern Leopard Frogs (NLF*)* and Chiricahua Leopard Frogs (CLF*)* have declined markedly in Arizona (Clarkson & Rorabaugh 1989, Theimer et al. 2011). As a result, the NLF is being considered for federal listing in the western portion of its range (Smith & Keinath 2007). Likewise, the CLF is federally protected under the U.S. endangered species act and is being captively reared and reintroduced to areas surrounding our study sites (USFWS 2007). Bd may have played a role in the declines of these species (USFWS 2007), so managers will want to know the distribution of Bd to inform management and reintroduction decisions. The nearly ubiquitous and persistent presence of Bd in chorus frogs implicates this species as a reservoir host for Bd. Thus, leopard frog reintroductions should avoid areas with chorus frogs. Also, when possible, more effort should be invested in monitoring leopard frog populations that overlap with chorus frogs, as these populations may be at higher risk of disease-related decline and local extinction (de Castro & Bolker 2005).

The emergence of chytridiomycosis has given rise to amphibian "arks" as a means of conservation (Gewin 2008). Under this model, amphibians that are likely to disappear as a result of imminent Bd introductions are reared in captivity until they can be successfully released back into the wild (Gewin 2008). One issue with this technique is that, once Bd has arrived, it is extremely hard if not impossible to eradicate (Lubick 2010), making reintroductions difficult. The absence of Bd from low-density chorus frog populations, despite its detection in

previous years, provides encouraging evidence that Bd can be lost from a system after its introduction. Unfortunately, maintaining low amphibian densities to prevent disease outbreaks runs counter to the goals of repatriation, which strive to establish robust populations that can maintain genetic diversity and tolerate stochastic events (USFWS 2007). Furthermore, this strategy will be extremely difficult in systems with multiple hosts that can act as pathogen reservoirs, such as the tropics (Lubick 2010).

CONCLUSIONS

I found no evidence that Bd transmission was influenced by nutrient availability. This runs counter to other studies that found clear links between nutrients and aquatic infectious diseases (Johnson et al. 2010). Although I cannot reject the hypothesis that nutrients can influence Bd dynamics, I found no evidence to suggest this. These findings contribute to a growing body of literature examining the role of nutrients in the emergence and transmission of infectious disease (Johnson et al. 2010, McKenzie & Townsend 2007), which may suffer from an under-reporting of negative results (McKenzie & Townsend 2007). Our findings also add new information to the suite of abiotic conditions that Bd is able to tolerate.

Given the strong negative effects Bd has had on populations of susceptible amphibian species (Bosch et al. 2001, Lips et al. 2006, Muths et al. 2003, Rachowicz et al. 2006), the ability of chorus frog populations to persist with Bd at high host densities despite suffering from high rates of chytridiomycosis in the

laboratory (Retallick & Miera 2007) is unexpected, though not without precedent (Murphy et al. 2009, Pearl et al. 2009, Pilliod et al. 2010, Piovia-Scott et al. 2010, Savage & Zamudio 2011). The consistent detection of Bd across years at many of our sites suggests that Bd has become endemic in chorus frog populations and implicate this species a reservoir host to Bd (Reeder et al 2012). Clarifying the mechanisms that enable chorus frogs and other susceptible species to persist with Bd will add to our understanding of how host populations respond to the introduction of potentially virulent pathogens and aid in the development of management strategies for Bd and other infectious diseases.

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Table 1. Detection rates and densities of *Batrachochytrium dendrobatidis (Bd)* zoospores (zsp) based on five water filters collected from each of 20 ponds. GE = genome equivalents

Pond	# filters Bd + (n=5)	Max zsp l^{-1} (GE)	Mean zsp l^{-1} (GE)	% Bd prevalence from swabs
Baker	0	0	0	2
Bar D	0	0	0	24
Brolliar Park	0	0	0	13
Calloway	0	0	0	10
Clints	0	0	0	2
Mahan	0	0	0	25
McClure	0	0	0	5
No Name 13	0	0	0	0
Twin	0	0	0	15
Van Deren	0	0	0	13
Brolliar Wet	1	30.34	6.07	16
Mud	1	231.59	46.32	2
No Name 7	1	8.3	1.67	62
Tinny	1	5.79	1.16	25
ZZ	1	66.3	13.3	20
Alder	2	40.04	13.47	55
Salmon	2	19.57	4.54	16
27 mi	3	23.15	8.76	66
Aspen	4	1122.5	249.68	93
T bar 2	4	226.5	150.6	59
Total Bd+ Ponds	10	-	-	19
Mean	-	177.4	49.6	27
S.E.M.	-	108.4	26.5	6

Table 2. Fit of linear and quadratic responses (total phosphorus only) of *Batrachochytrium dendrobatidis* (Bd) prevalence and load to nutrients (TP = total phosphorus, TN = total nitrogen) and host densities (capture rate) of adult chorus frogs ($n \geq 30$ per pond) collected during breeding from 20 ponds on Arizona's Mogollon Rim in 2009

Response var	Explanatory var	Relationship	N	Adjusted R^2 (linear, quadratic)	p (linear, quadratic)
Arcsin Bd Prevalence	log TP	-	20	-0.04, -0.1	0.60, 0.87
Arcsin Bd Prevalence	TN	Flat	20	-0.05, NA	0.90, NA
Arcsin Bd Prevalence	capture rate	+	20	-0.02, NA	0.42, NA
log mean Bd load	log TP	-	20	-0.04, -0.08	0.67, 0.80
log mean Bd load	TN	+	20	-0.05, NA	0.75, NA
log mean Bd load	capture rate	+	20	0.13, NA	0.06, NA

Table 3. Fit of linear and quadratic responses of 2010 **Δ** *Batrachochytrium dendrobatidis* (Bd) prevalence to nutrients (TP = total phosphorus, TN = total nitrogen) and host densities of adult chorus frogs (n ≥ 30 per pond) collected during breeding from 20 ponds on Arizona's Mogollon Rim

Response variable	Explanatory variable	Direction	N	Adjusted R^2 (linear, quadratic)	p (linear, quadratic)
Δ Bd prevalence	log TP (2009)	flat	19*	-0.06, 0.05	0.99, 0.25
Δ Bd prevalence	TN (2009)	-	19*	-0.03, NA	0.56, NA
Δ Bd prevalence	Capture rate (2009)	+	19*	0.03, NA	0.21, NA
Δ Bd prevalence	log TP (2010)	+	19*	-0.04, -0.08	0.46, 0.74
Δ Bd prevalence	log TN (2010)	+	19*	-0.04, NA	0.55, NA
Δ Bd prevalence	Capture rate (2010)	+	19*	0.02, NA	0.25, NA

** The pond with only 1 disease sampling point (No Name 13) could not be included in analyses*

Table 4. Spearman correlations comparing total phosphorus (TP), total nitrogen (TN), frog capture rate, and prevalence of *Batrachochytrium dendrobatidis* (Bd) infections to two different measures of aquatic zoospore densities: (1) number of water filters testing Bd positive within a pond (# Bd positive filters) and (2) mean density of aquatic zoospores (zsp l^{-1}) detected in each pond. Ponds with higher Bd prevalence in hosts had higher densities of aquatic zoospores, and number of filters testing Bd positive within a pond was positively correlated with aquatic zoospore density. Nutrients and host densities were not related to aquatic zoospore densities

Response var	Explanatory var	N	Rho	p
# Bd+ filters	TP 2009	20	-0.07	0.76
# Bd+ filters	TN 2009	20	-0.18	0.45
# Bd+ filters	Capture Rate 2009	20	0.06	0.81
# Bd+ filters	**Bd prevalence 2009**	**20**	**0.7**	**0.0006**
# Bd+ filters	**mean zsp l^{-1}**	**20**	**0.95**	**< 0.0001**
mean zsp l^{-1}	TP 2009	20	-0.11	0.64
mean zsp l^{-1}	TN 2009	20	-0.3	0.21
mean zsp l^{-1}	Capture Rate 2009	20	-0.003	0.99
mean zsp l^{-1}	**Bd prev 2009**	**20**	**0.55**	**0.01**

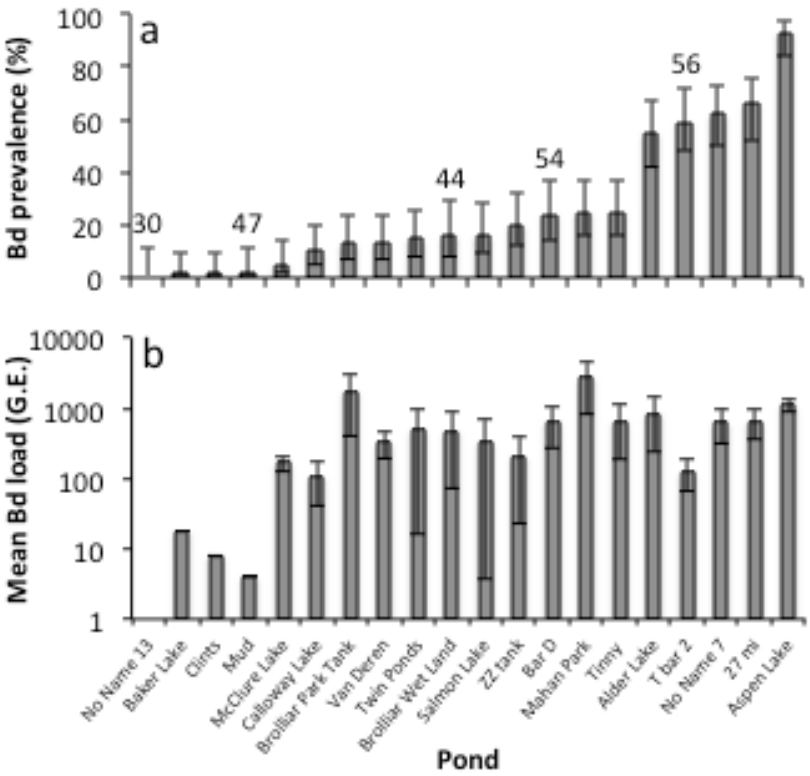

Figure 1. *Batrachochytrium dendrobatidis* (Bd) prevalence and mean infection intensity (load) in 20 separate populations of adult boreal chorus frogs during breeding on Arizona's Mogollon rim in 2009. (a) Bd prevalence at each of 20 ponds. Numbers above bars represent sample size if n < 60. Error bars represent Bayesian 95% central confidence intervals. (b) Mean Bd load (G.E. = genome equivalents) from all individual frogs testing Bd positive within a pond. Note logarithmic scale. Error bars represent ± 1 S.E.M.

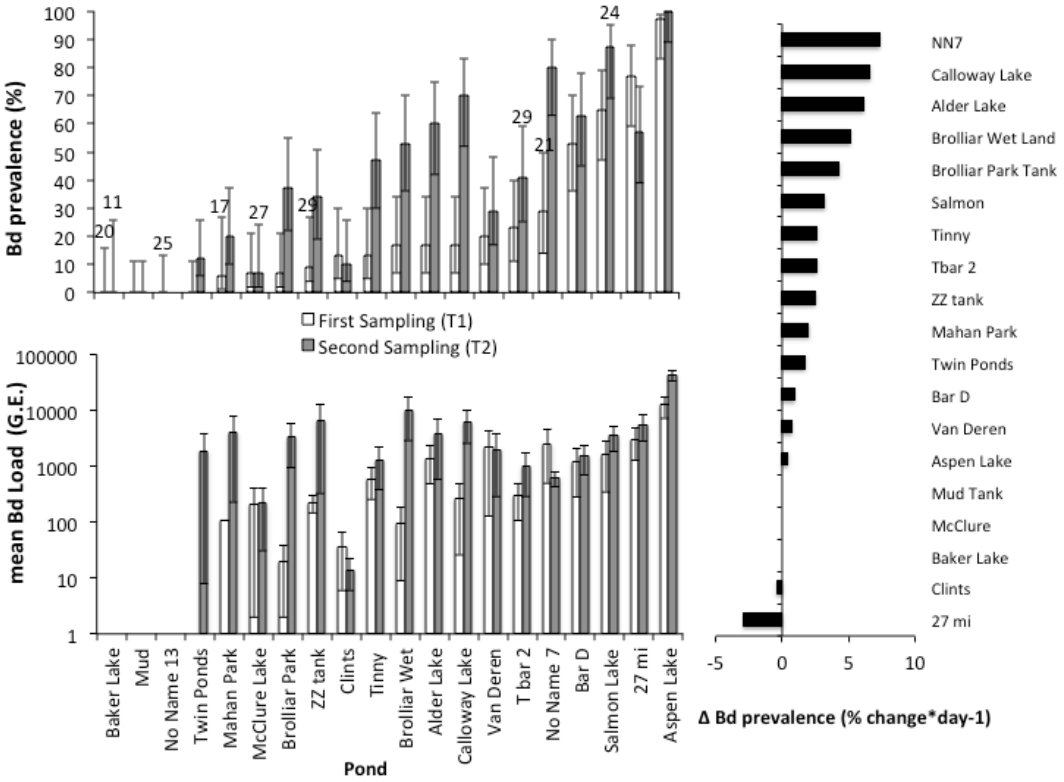

Figure 2. *Batrachochytrium dendrobatidis* (Bd) prevalence (a), mean load (b), and Δ Bd prevalence (c) in 20 populations of boreal chorus frogs sampled during breeding at two time points (T1 and T2) separated by about one week on Arizona's Mogollon Rim in 2010. Error bars in (a) represent 95% confidence intervals and ± 1 S.E.M in (b)

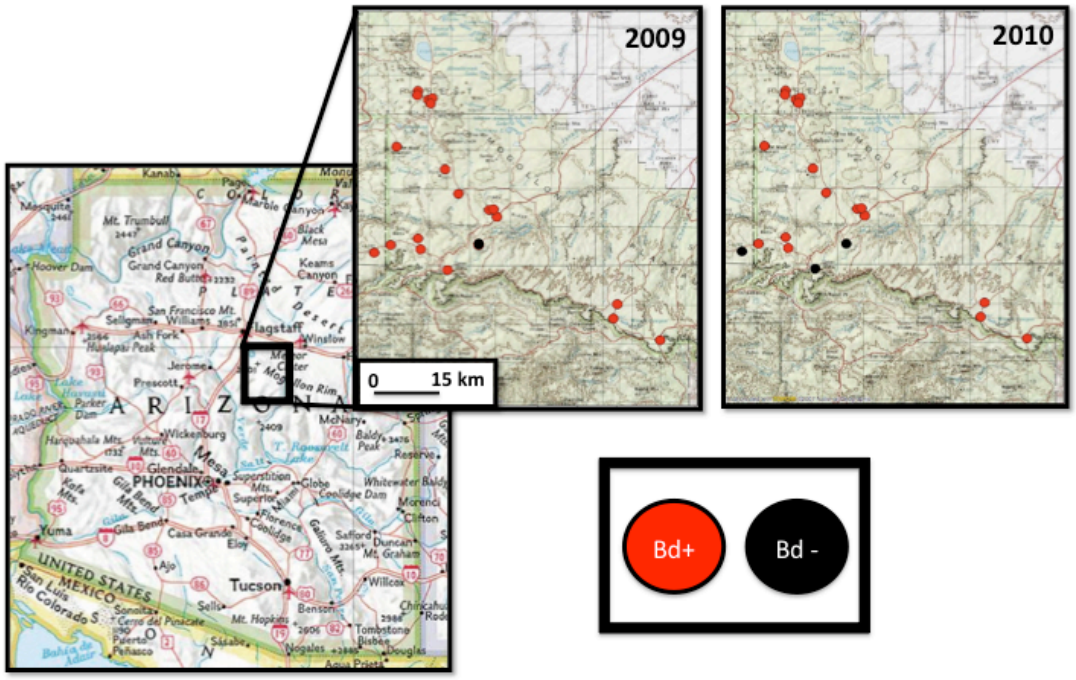

Figure 3. Map of ponds where chorus frogs tested positive or negative for the presence of *Batrachochytrium dendrobatidis* (Bd) in 2009 and 2010 ($n \geq 25$ animals pond^{-1})

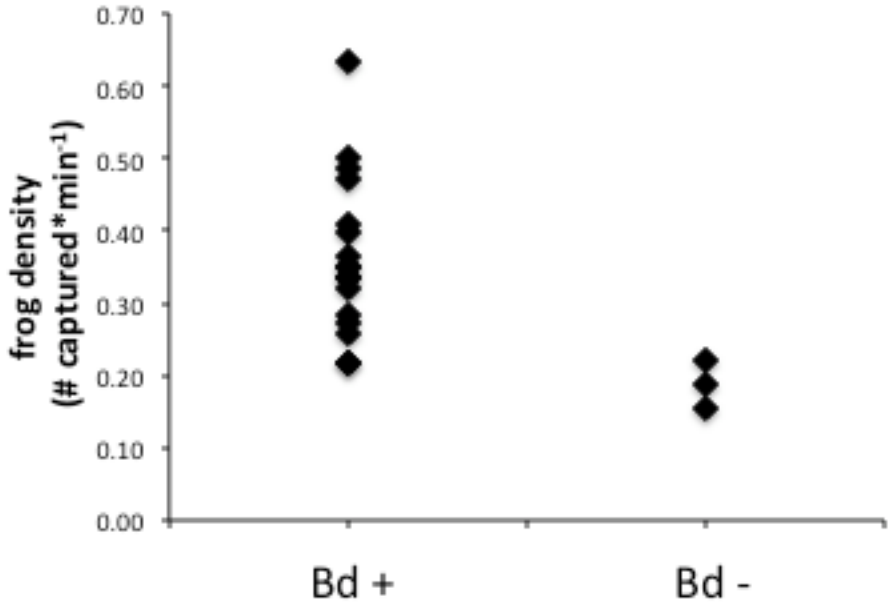

Figure 4. Densities of adult boreal chorus frogs were significantly higher at sites testing positive for the presence of *Batrachochytrium dendrobatidis* (Bd) by swabs in 2010 (Wilcoxon, $p = 0.01$)

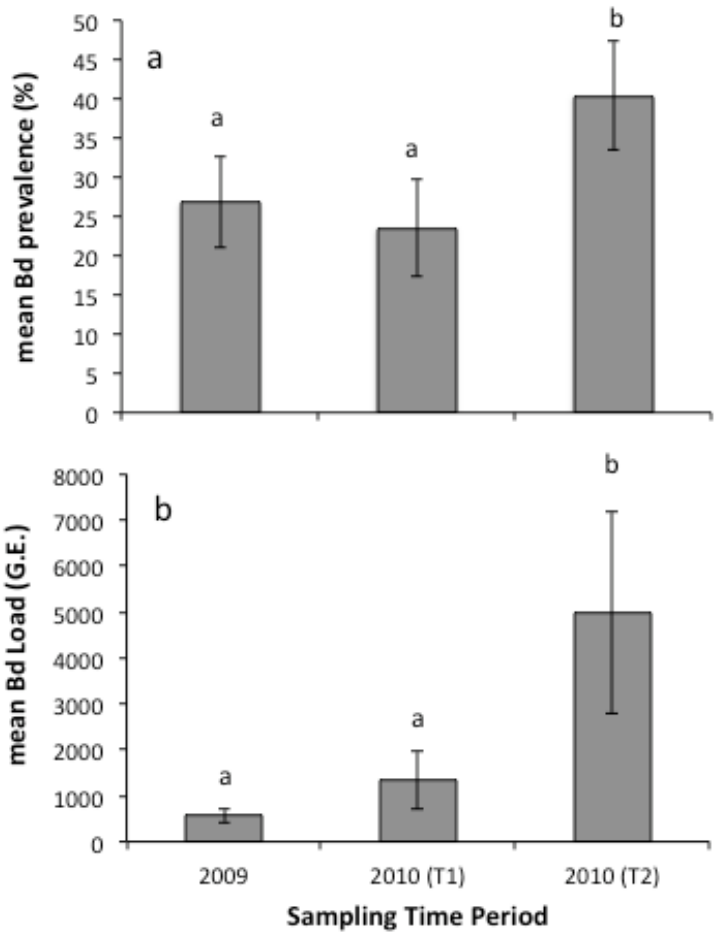

Figure 5. Mean *Batrachochytrium dendrobatidis* (Bd) prevalence (a) and (b) intensity of infection (load) from boreal chorus frogs ($n \geq 25$ pond^{-1}) collected once in 2009 and at two time points (T1 and T2) in 2010 from each of 20 ponds in Arizona. Bd prevalence and load increased significantly at the second sampling point in 2010 (paired t-test$_{df = 19,18}$, t-ratio = 3.85 and 3.02, p = 0.001 and 0.007, respectively). Letters above columns represent statistical differences. Error bars represent ± 1 S.E.M. T1: swabs collected at the beginning of breeding season; T2: swabs collected 1-2 weeks later at the end of breeding

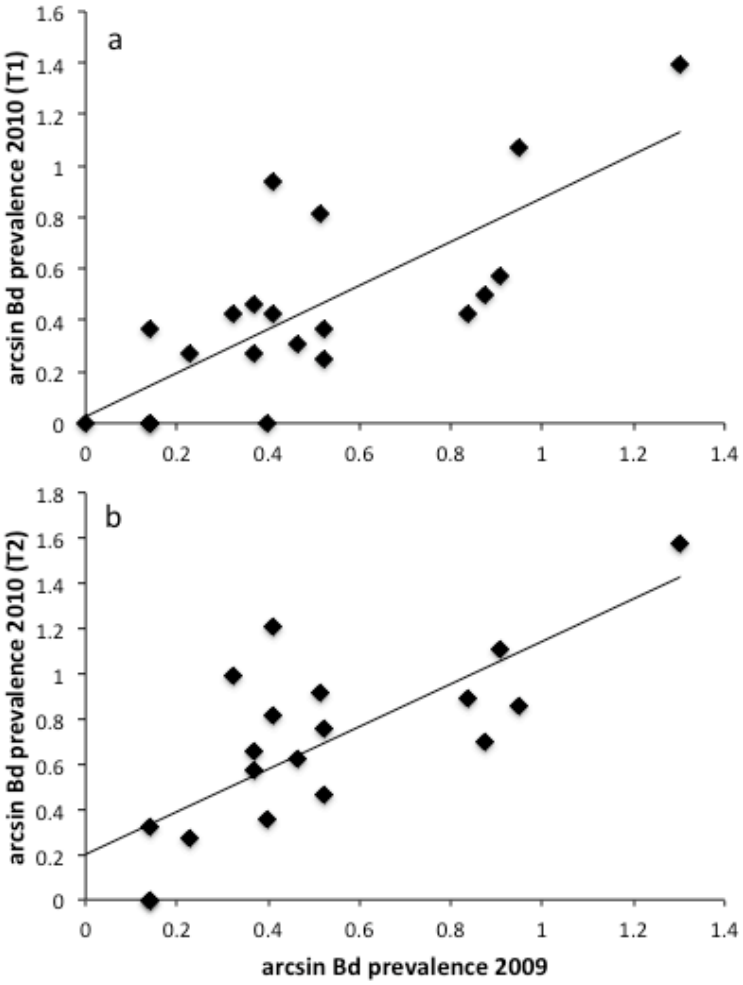

Figure 6. Prevalence of *Batrachochytrium dendrobatidis* (Bd) in populations of boreal chorus frogs in 2009 is correlated with Bd prevalence in the same populations in the following year. (a) Correlation between Bd prevalence in 2009 and T1 in 2010 ($ANOVA_{df=19}$, $r^2 = 0.57$, $p = 0.0001$). (b) Correlation between Bd prevalence in 2009 and T2 in 2010 ($ANOVA_{df=18}$, $r^2 = 0.55$, $p = 0.0003$)

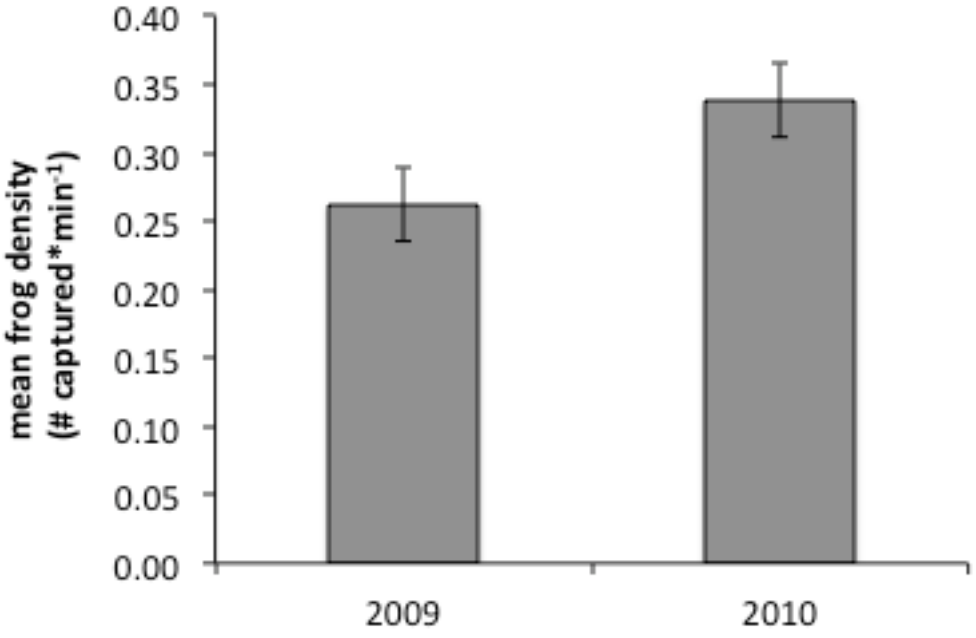

Figure 7. Relative frog densities, as measured by capture rate in each pond, increased significantly from 2009 to 2010 (paired t-test$_{df = 19}$, p = 0.0003), despite high prevalence of *Batrachochytrium dendrobatidis*

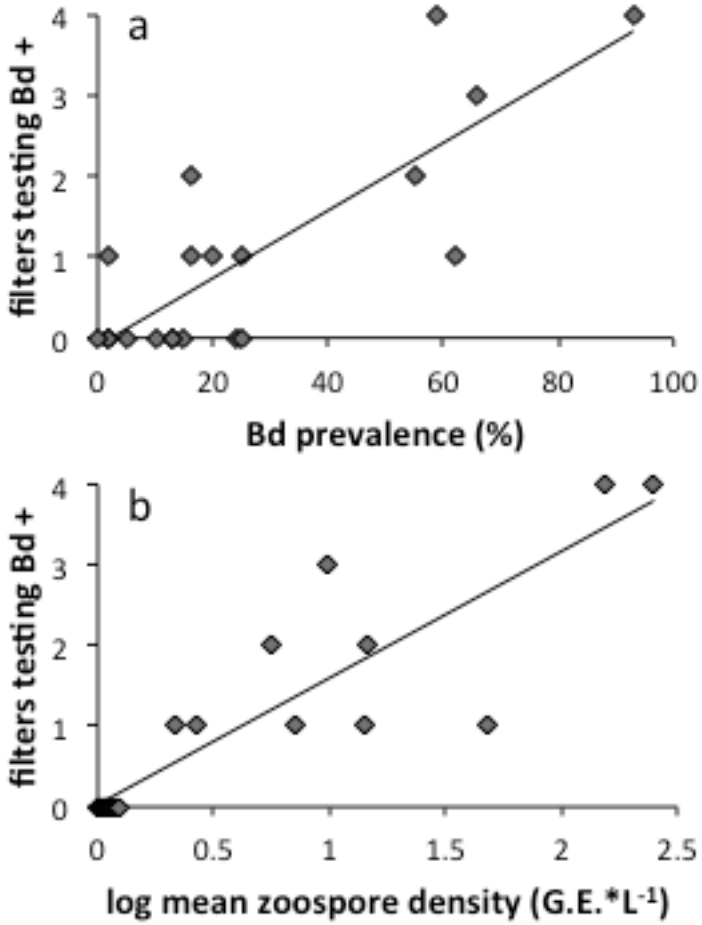

Figure 8. The number of filters detecting *Batrachochytrium dendrobatidis* (Bd) within a pond was positively correlated with Bd prevalence in breeding adult chorus frogs (Spearman's *Rho* = 0.70, p = 0.0006) as well as the mean concentration of aquatic zoospores detected within a pond (Spearman's *Rho* = 0.95, p < 0.0001). G.E. = genome equivalents

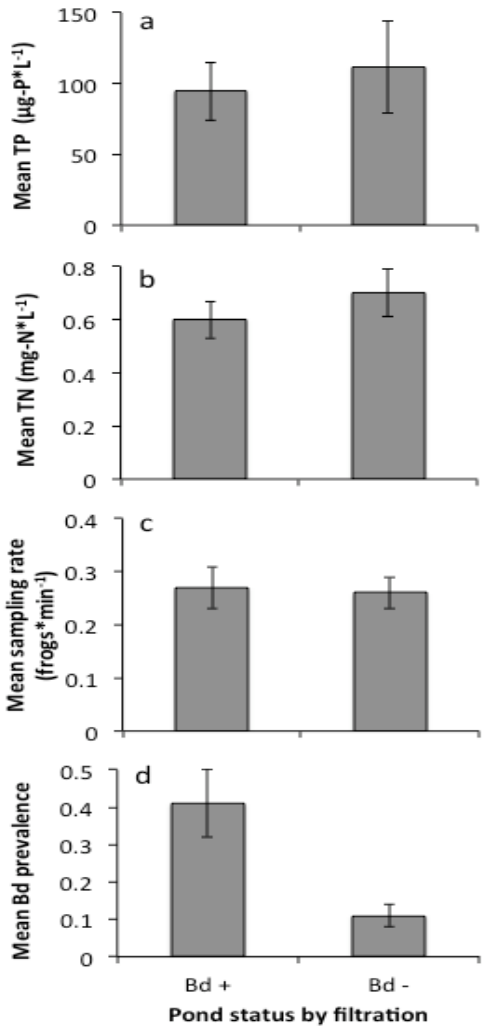

Figure 9. Comparison of mean (a) total phosphorus (TP), (b) total nitrogen (TN), (c) frog sampling rate, and (d) disease prevalence from ponds testing positive (n=10) versus negative (n=10) for the presence of *Batrachochytrium dendrobatidis* (Bd) by water filtration. Ponds testing Bd positive by filtration had significantly higher Bd prevalence in host animals ($t\text{-test}_{df = 18}$, $p = 0.006$). Total P, TN, and frog density were not significantly different in ponds with water testing Bd positive versus Bd negative ($t\text{-test}_{df\text{-}18}$, $p = 0.8$, 0.4, and 0.8, respectively)

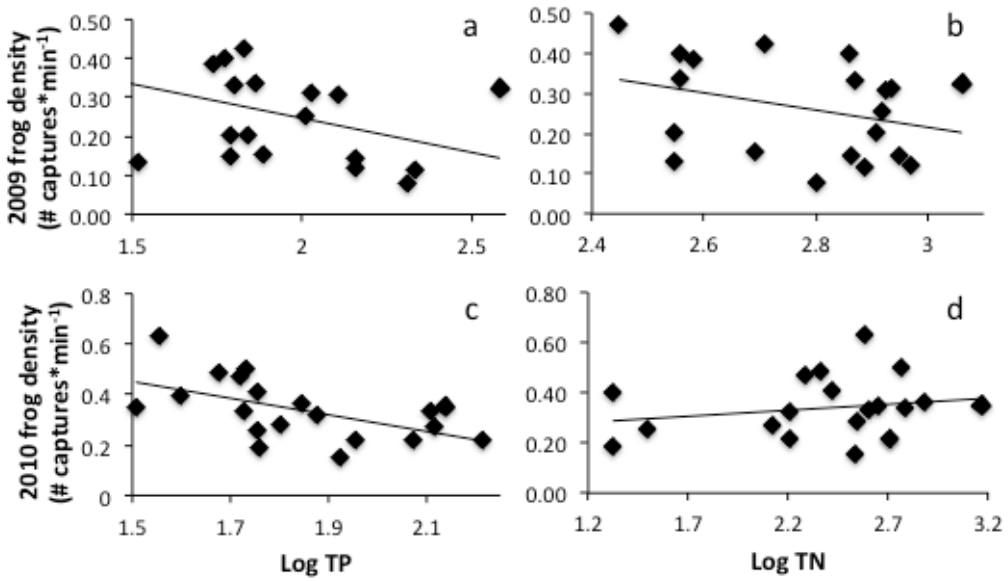

Figure 10. Rates of chorus frog capture within each pond were negatively correlated with the aquatic concentrations of total phosphorus in 2009 (a; $ANOVA_{df=19}$, $p = 0.04$) and 2010 (c; $ANOVA_{df=19}$, $p = 0.01$). There were no consistent or statistically significant trends between host density and TN in either year (b & d)

Chapter 4

HOST LIFE HISTORY AND ENVIRONMENT FACILITATE PERSISTENCE WITH A VIRULENT PATHOGEN

ABSTRACT

The amphibian disease, chytridiomycosis, is an unambiguous example of the negative effects infectious diseases can have on wildlife populations. This disease, caused by the fungal pathogen *Batrachochytrium dendrobatidis* (Bd), is linked to amphibian declines across the globe. Yet there is considerable variation in population-level responses to Bd introductions, ranging from host extinction to long-term persistence with Bd. Detailed descriptions of Bd dynamics in populations of susceptible hosts are fundamental to identifying the factors that enable these populations to persist with this pathogen.

In Arizona, populations of boreal chorus frogs (*Pseudacris maculata*) may persist with Bd for several years at high host and pathogen densities. The mechanisms underlying this ability to persist with Bd remain unclear. One hypothesis is that chorus frogs may be tolerant of Bd infections, enabling individuals to survive and reproduce despite high levels of Bd infection. Alternatively, high temperatures reached in ephemeral breeding pools commonly used by chorus frogs may "rescue" host populations by reducing or removing Bd infections from developing larvae, resulting in increased survival of young-of-the-year to maturity and enabling population persistence.

Here I analyze a simple, matrix-based model of a chorus frog population to understand the relative importance of juvenile and adult survivorship for projected population growth (λ). Then, the prevalence and intensity of Bd infections during chorus frog breeding and development are described in detail and combined with laboratory experiments to test the roles of host tolerance and environmental rescue in chorus frog population persistence.

A time-based matrix population model found that when females are highly fecund, projected population growth is most sensitive to rates of survival in young-of-the-year froglets as opposed to adults. The model also shows that chorus frog populations can persist with 100% annual mortality in adults, but not young-of-the-year. This suggests that mechanisms that enhance survival of recently metamorphosed froglets will be important for chorus frog population persistence. Controlled laboratory experiments found that adult frogs with natural Bd infections suffered 85% mortality with no mortality in uninfected controls, providing minimal support for host tolerance of Bd infections playing a role in population persistence. Field studies found Bd prevalence in summer-emerging froglets was significantly lower than Bd prevalence in adults sampled from the same ponds in spring (paired $t\text{-test}_{df=14}$, $p = 0.0001$), with 11 of 15 spring-positive ponds testing negative when sampling froglets in the summer. Experimental heat-treatment at naturally relevant temperatures (29 °C) removed Bd infections from developing chorus frog larvae, indicating that warm water temperatures remove Bd infections during tadpole development. Also, intensive weekly surveys from two ponds found that Bd infections were completely absent from the larval cohort

in a warmer, ephemeral pond, while Bd persisted in larvae and newly metamorphosed froglets in a deeper, cooler, perennial pond.

These results support the hypothesis that warm water temperatures reduce Bd prevalence in developing larvae and explain the reduction and absence of Bd from summer emerging froglets, which may ultimately enhance the likelihood of chorus frog population persistence. In combination, these results provide evidence that host life history and environment may interact to enable populations of susceptible hosts to persist despite high levels of disease-induced mortality.

INTRODUCTION

Infectious disease emergence may be increasing in frequency (Daszak et al. 2000, Harvell et al. 1999, Aguirre & Tabor 2008, Jones et al. 2008, Smith et al. 2009). This trend has been noted in several emerging wildlife diseases, resulting in large-scale population declines and even species extinctions (Daszak et al. 1999, Dobson & Foufopoulos 2001, Harvell et al. 1999, Johnson & Paull 2011). Infectious diseases present new challenges to historical conservation strategies because they readily cross park and preserve borders (Cheng et al. 2011, Lips et al. 2006). In cases where we cannot reliably prevent pathogen introduction, identifying the factors that influence host-pathogen dynamics and enable populations of susceptible hosts to persist will enrich our understanding of the processes that control these interactions while contributing to management decisions (Bielby et al. 2008). This study examines the mechanisms that enable

host populations of boreal chorus frogs (*Pseudacris maculata)* to persist with the emerging infectious disease chytridiomycosis.

Chytridiomycosis is an example of the challenges infectious diseases present to species conservation. This disease is caused by the pathogenic fungus *Batrachochytrium dendrobatidis* (Bd; Berger et al. 1998). Bd is linked to the decline and extinction of numerous amphibian species, many from "pristine" habitats and preserves (Cheng et al. 2011, Lips et al. 2006, Rachowicz et al. 2006). There is, however, variation in the responses of amphibian hosts to Bd, with some host populations declining markedly while others persist (Briggs et al. 2010, Collins & Crump 2009, Daszak et al. 2005, Hale et al. 2005, Lips et al. 2003, Lips et al. 2006, Murphy et al. 2009, Pearl et al. 2009, Pilliod et al. 2010, Rachowicz et al. 2006, Piovia-Scott et al. 2010, Retallick et al. 2004, Ryan et al. 2008, Voordouw et al. 2010). Evidence supports several non-mutually exclusive hypotheses to explain this variation, including host resistance/tolerance (Harris et al. 2009, Savage & Zamudio 2011, Voordouw et al. 2010), pathogen attenuation (Retallick & Miera 2007), normal variation in density-dependent host pathogen dynamics (Briggs et al. 2010), and environmental conditions (Forrest & Schlaepfer 2011, Murphy et al. 2009, Puschendorf et al. 2011).

Bd occurs in several populations of boreal chorus frogs, *Pseudacris maculata*, in Arizona (Hyman & Collins 2012) with adults suffering up to 80% mortality as a result of Bd infection (Retallick & Miera 2007). Yet, populations of chorus frogs may persist with Bd for several years at high host and pathogen densities (Collins & Miera 2005, O. Hyman, unpubl. data). This study provides a

detailed description of the seasonal dynamics of Bd during chorus frog breeding and development and combines these data with laboratory experiments and a mathematical model to test two hypotheses for the mechanisms underlying the ability of chorus frog populations to persist with Bd; host tolerance and environmental rescue.

Explanation of Hypotheses

Host tolerance. The simplest explanation for chorus frog population persistence is that despite harboring high intensity Bd infections (Hyman & Collins 2012), chorus frogs do not contract the disease chytridiomycosis. If chorus frogs can tolerate Bd infections without increased mortality or fitness related costs, the population could persist with Bd. To test this hypothesis, we compared rates of mortality in naturally Bd-infected and uninfected frogs collected from a wild population and held under laboratory conditions ideal for the pathogen. If chorus frogs do not contract chytridiomycosis, then individuals with natural Bd infections should incur similar rates of mortality as those without Bd infections.

Environmental rescue. If chorus frogs are not Bd tolerant, then environmental factors, such as temperature, may play a role in their ability to persist with Bd. For example, in culture, Bd will not grow at ≥ 28 °C and is killed at ≥ 30 °C (Piotrowski et al. 2004). High temperatures ranging from 26-37 °C also remove Bd infections from animals in captivity (Geiger et al. 2011, Retallick & Miera 2007, Woodhams et al. 2003) and potentially in the wild (Forrest & Schlaepfer 2011, Puschendorf et al. 2011).

Chorus frogs use mostly ephemeral bodies of water for breeding. These ponds warm and dry as frogs develop. The warm temperatures reached in these ponds could potentially eliminate Bd infections from larvae and newly metamorphosed froglets (Fig. 1). For example, Geiger et al. (2011) found that seven of eight midwife toad larvae lost Bd infections when held at 26 °C for five days. This environmentally induced reduction in Bd prevalence may "rescue" chorus frog populations from Bd-related decline by clearing the juvenile cohort of infections each year, thereby increasing survivorship to adulthood. If larvae and newly metamorphosing froglets are losing Bd infections as ponds warm, then (1) heat-treated tadpoles should lose Bd infections, (2) Bd prevalence should be reduced in larvae and newly metamorphosed froglets, and (3) warmer, ephemeral ponds should have lower Bd prevalence in these life stages in comparison to cooler, perennial ponds (Fig. 1). These predictions were tested using field studies and laboratory experiments.

In addition a matrix-based model of a chorus frog population was constructed to examine how relative rates of adult and juvenile survival influence projected chorus frog population growth under varying levels of stage-specific survival. The purpose of this model was to explore the relative importance of juvenile versus adult survival for chorus frog population growth in the context of the tolerance and environmental rescue hypotheses.

METHODS

Mathematical Model

Chorus frog life cycle. Chorus frogs are annual breeders. Adults breed for several weeks following snowmelt in late February to early April. Individual females can lay between 500-1500 eggs (Moriarity & Lannoo 2005) with an estimated hatching success of 37-87% (Kramer 1978). Once hatched, tadpoles develop over ~2.5-month period with estimates of survival to metamorphosis ranging from 10-100% in natural habitats (Smith 1983). Following breeding and metamorphosis, adults and young-of-the-year froglets (metas) forage in surrounding woodlands and overwinter under leaves, rocks, and logs beneath the snow (Moriarity & Lannoo 2005). Metas typically mature within one year with an estimated 19% survival from metamorphosis to maturity (Smith 1987). Longevity of this species is not known, but closely related species (*P. nigrita*) live for one-three years (Caldwell 1987). Smith (1987) estimated 14% annual adult survival following breeding in Michigan populations.

Model construction. Based on the available knowledge of the chorus frog life cycle we constructed a simple population model to explore the relationships between fecundity, stage-specific survival, and projected population growth rate. The model presented herein is the simplest time-based matrix population model that separates the adult stages from the juvenile (meta) stages and accounts for differences in the survival and fecundity of each of these stages. Ponds in Arizona, including those used in this study, are typically ≥1 km apart, well

beyond the 200 m maximum dispersal distance of chorus frogs (Kramer 1973). Thus, immigration and emigration are not included in the model as these factors are likely to make negligible contributions to individual populations in a typical breeding cycle.

In the model, individual female adults (≥ 1 yr old) and metamorphosed juveniles (metas; < 1 yr old) are counted in the summer of each year (t+n) following breeding and metamorphosis. A schematic representation of the model is shown in Fig. 2. The adult (A_n) → meta (M_n) transition describes the production and survival of eggs, larvae, and tadpoles to metamorphosis during the ~2.5 mo of development from egg to meta. This model assumes that these are constant rates encapsulated by the parameter γ. The M_n → A_n transition describes meta survival to maturity, σ. The A_n → A_n transition describes adult survival (following breeding) to breeding in the following year, Σ. Both Σ and σ are assumed to be constant proportions that encapsulate natural and Bd related mortality defined as:

$$\Sigma = \Psi(1-I*D) \quad (1)$$

$$\sigma = \Omega(1-i*d) \quad (2)$$

where Ψ and Ω represent the fraction of adults and metas, respectively, that typically survive to breed the following year in the absence of Bd, I and i represent the fraction of adults and metas that become infected each year, and D and d represent the fraction of infected animals that die from Bd infections.

Following the results from of my field surveys and laboratory experiments (discussed below), the model assumes that annual adult mortality occurs following breeding and meta mortality follows metamorphosis in each time step.

The dynamics of this system can be formulated as a system of two first-order, linear difference equations:

$$A_{t+1} = \Sigma A_t + \sigma M_t \quad (3)$$

$$M_{t+1} = \gamma A_{t+1} \quad (4)$$

$$= \gamma \Sigma A_t + \gamma \sigma M_t$$

where t is measured in years, Σ and σ are annual survivorship of adults and metas as decribed above, and γ is the number of female metas produced per an adult female each year. These equations can take the general form:

$$N_{t+1} = MN_t \quad (5)$$

where

$$M_n = \begin{bmatrix} \Sigma & \sigma \\ \gamma\Sigma & \gamma\sigma \end{bmatrix} \text{ and } N_t = \begin{bmatrix} A \\ M \end{bmatrix}_t \quad (6)$$

This 2 x 2 matrix (6) can be converted to the general quadratic characteristic equation where λ represents the eigenvalues of the matrix (M):

$$\lambda^2 - \beta\lambda + \delta = 0 \qquad (7)$$

where

$$\beta = \Sigma + \gamma\sigma$$

$$\delta = \Sigma\gamma\sigma - \Sigma\gamma\sigma = 0$$

The dominant eigenvalue λ of this matrix is equal to e^r, where r is the intrinsic rate of increase of the population in the equation

$$N_t = N_0 e^{rt}$$

Thus, if $\lambda = e^r = 1$, then $r = 0$, and the population size remains stable, if $\lambda > 1$ the population grows, and $\lambda < 1$ the population shrinks to zero. Solving for λ in (7) we obtain the following non-trivial solution:

$$\lambda = \Sigma + \gamma\sigma \qquad (8)$$

Thus, the chorus frog population will grow or remain stable when:

$$\gamma\sigma \geq 1 - \Sigma \qquad (9)$$

By substituting (1) and (2) into (9) we find the population grows when:

$$\gamma[\Omega(1-i*d)] > 1 - \Psi(1-I*D) \quad (10)$$

Equations (9) and (10) were used to examine the lower limits of annual adult and juvenile survivorship that the population can tolerate and still maintain positive or neutral projected population growth ($\lambda \geq 1$). In addition, equation (10) was used to examine how λ responds to incremental, proportional (1%) changes to individual survival-related parameters while all others were held constant (Caswell 1989). Parameter values from the literature and results from this study were used in this analysis (Table 1). The lowest estimates of survival and fecundity were chosen from the literature to ensure estimates of frog population growth were conservative. The highest rates of infection in adults (100% following breeding; see 2011 field results below) and emerging metas (85%; see 2011 field results below) were used as default values to err on the conservative side of lower population growth. The model assumes that the fraction of adult mortality from Bd infections (D) found in the laboratory-based tolerance/resistance experiment (85%) is indicative of survival in the field. The model also assumed 85% Bd-induced mortality in metas (d) as found by Searle et al. (2011). Female fecundity (γ) was estimated using the following equation:

$$\gamma = \delta\theta\eta*0.5 \quad (11)$$

where δ is the number of eggs produce per female, θ is egg hatching success, η is larval survival to metamorphosis, and 0.5 represents the fraction of females in each cohort assuming a 1:1 sex ratio.

Tolerance Experiment

On March 5, 2011, 40 adult boreal chorus frogs were collected from a single pond, Salmon Lake, located in Coconino National Forest, Coconino county, Arizona (Fig. 3; see Table S1 for GPS coordinates). Bd was detected in chorus frogs in this pond at 16% and 88% prevalence during spring breeding in 2009 and 2010 (O. Hyman, unpubl. data), respectively, indicating that this population has been exposed to Bd for at least two generations. I collected frogs from this pond and placed them individually into pre-labeled, 177-ml polypropylene containers with lids (Biologix no. 51-17720) along with an autoclaved rock and 50 ml of 20% Holfreter's solution. Each frog was handled wearing vinyl gloves that were changed between animals to prevent cross contamination. Animals were transported to the laboratory and kept on a 12-hr light cycle (0700-1900) at 23 °C. Animals were randomly arranged on a single shelf to control for any spatial effects. All animals were fed three crickets biweekly and had water changed daily and containers changed weekly until sphagnum moss was added on May 4, 2011 (see below). Two additional laboratory-reared adult chorus frogs that previously tested Bd negative by quantitative PCR (qPCR) were placed in identical containers and interspersed among treatment animals to act as negative controls against contamination during water changes, ensuring that any detection of Bd infections in field caught

animals was a result of previous Bd exposure in the field, not infection in the lab. All animals were swabbed for Bd on the date of death, and all live animals were swabbed on April 1, October 4, 2011 and March 5, 2012 following Retallick and Miera (2007) to confirm and reconfirm disease status. Containers were checked daily for dead animals. Presence of skin sloughs (indicative of Bd infection) and whether the animal was in or out of the water in each container was also recorded until May 4, 2011. On this date, sphagnum moss was added to each container to simulate the transition from aquatic to terrestrial habitats that chorus frogs make following the end of breeding. Dead animals were swabbed and preserved in 70% ethanol. All swabs were extracted in Prepman Ultra^{TM} following Retallick and Miera (2007) and examined for the presence of Bd DNA by qPCR (Boyle et al 2004) using internal positive controls and bovine serum albumin to test for and reduce PCR inhibition following Hyman & Collins (2012).

Differences in mortality among groups were tested using Kaplan-Meier survival analysis. Skin sloughing and water avoidance were non-normally distributed (Shapiro-Wilk W test, $p < 0.01$) and therefore these data were compared for infected and uninfected animals using non-parametric Wilcoxon signed-rank tests, with all animals dying previous to May 4, 2011 dropped from analyses to eliminate biases created by animals that did not survive for the entire time period in water. A Wilcoxon signed-rank test was also used to compare the initial loads (on April 1, 2011) of frogs that survived with Bd infections to those that died.

Environmental Rescue

Laboratory heating experiment. This experiment's goal was to test if naturally occurring water temperatures are sufficiently warm to remove Bd infections from chorus frog tadpoles during development. Fifty-two chorus frog tadpoles were collected on April 17, 2011 from 27 Mile Lake in Coconino National Forest, Coconino county, Arizona (Fig. 3; see Table S1 for GPS coordinates). Tadpoles were held in one large tank filled with aged tap water kept at 17 °C on a 13:11 light cycle (0630-1930) and fed Wardly® Premium Algae Discs™ (Hartz Mountain Corp. Item #416) weekly until June 21, 2011, when they were staged (Gosner 1960) and placed individually into, 177-ml polypropylene containers (Biologix no. 51-17720) filled with 75 ml of 20% Holtfreter's solution. Forty-eight tadpoles were individually inoculated with ~1,000 zoospore*ml^{-1} of a Bd strain isolated from 27 Mile Lake (Retallick & Miera 2007). Zoospores were counted three times on a haemocytometer and 1 ml of pure culture was added to each container using a Rainin 1000-µL micropipette to ensure that each container received similar zoospore doses. High doses were used to ensure animals became infected. In addition, a single, fiber-tipped DNA swab (Fisher no. 14-959-79) was submerged in the bottom of the containers of four randomly selected hot and cold treated animals (eight total swabs) on the day of inoculation. These swabs acted as "remnant DNA controls" to help ensure that any Bd DNA detected at the end of the experiment was from true Bd infections, as opposed to remnant DNA (Dejean et al. 2011) on the surface of objects

transferred during water/container changes from either initial Bd inoculations or tadpole produced zoospores. The remaining four tadpoles were sham inoculated with Bd-free culture medium to act as negative controls against cross contamination. Following inoculations, all containers were placed on a single shelf in an environmental chamber kept at 20 °C on a 13:11 light cycle (0630-1930) for one week to allow Bd infections to develop.

Tadpoles and controls were then individually transferred into autoclaved, 177-ml containers with 170 ml of 20% Holtfreter's solution, sorted by Gosner stage, and randomly assigned (within each Gosner stage) to one of two treatments, hot or cold. Cold treatments were kept at 20 °C from 0630-1930 and 17 °C from 1930-0630 to replicate diel cycles typical of these ponds. Hot treatments followed the temperature cycles of cool treatments, with the exception that during the hottest part of the day (1200 – 1600) they were warmed to 29 °C to replicate natural "warm" water temperatures. Twelve individual Bd-exposed tadpole containers were placed into each of four separate, time-heated, water baths (48 total animals). In addition, one negative control tadpole was added to each water bath (four total controls). Water baths were then placed in an environmental chamber kept at 17 °C on a 13:11 light cycle (0630-1930). The placement of hot and cold water-baths within the chamber was alternated following a Latin-square design to neutralize spatial effects on disease outcome. Thermistors in individual containers recorded water temperatures at 0800, 1500, and 1900 hours within ± 0.5 °C of the desired range for each treatment for the first three and last three days of the experiment, confirming that water temperatures remained consistent

throughout the course of the experiment. All tadpoles and controls were transferred to new, autoclaved containers and fed 2 mg of ground Wardly® Premium Algae Discs™ weekly following sterile protocols to prevent contamination.

On July 4, 2011 (2 wk post-inoculation), following the second water change, eight exposed and one sham-exposed tadpole from each treatment (18 total) were euthanized in MS-222 and tested for the presence of Bd by extracting their mouthparts (Retallick et al. 2006) to ensure Bd inoculations and sham inoculations were successful. One DNA remnant control swab was also removed from each treatment and extracted as described above. The remaining tadpoles were fed and changed weekly until reaching Gosner stage 42, at which time they were individually transferred to a tilted plastic shoebox filled with enough 20% Holtfreter's solution to submerge half of the container. DNA remnant control swabs were transferred to individual shoeboxes on the same date as the animal with which they shared a 177-ml container. Once tadpoles reached Gosner stage 46, they were euthanized in MS-222, placed into individual vials with 70% ethanol, and stored at -20 °C. Any animals that died prior to reaching stage 46 were dipped in MS-222 and stored as above. DNA remnant control swabs were stored on the day their matching frog died following the same process. On August 4, 2011 one foot and one hand was removed from each preserved froglet and extracted following the mouthpart extraction protocol described in Retallick et al. (2006). DNA remnant controls were extracted as described in Hyman and Collins (2012). Presence of Bd DNA was determined by qPCR as described above. This

method was chosen because it is the most sensitive technique available (Hyatt et al. 2007).

Chi-squared tests were used to compare the number of froglets testing Bd positive at the end of the experiment and the number of animals to reach stage 46 between hot and cold treatments. Wilcoxon signed-rank tests were used to compare mean Gosner stage between hot and cold treatments at the start of the experiment and the number of days animals in each treatment took to reach Gosner stage 42. Wilcoxon signed-rank tests were used to compare Bd loads of tadpoles euthanized 2 wk post initial Bd exposure between treatments.

Field observations 2010. If warmer water temperatures remove Bd infections from tadpoles and newly metamorphosed froglets, Bd prevalence should be reduced in young-of-the-year froglets. This prediction was tested by comparing Bd prevalence in breeding adults to that of newly metamorphosed froglets from the same ponds. In 2010, ~30 adult chorus frogs were swabbed for Bd from each of 15 ponds during breeding. Details of sampling are described in Hyman & Collins (2012). All ponds were located in ponderosa pine dominated habitat in Coconino National Forest (Fig. 10; see Table S1 for GPS coordinates) Each of these ponds was revisited one to two months later in mid-late June to sample ~30 newly emerging metamorphs for Bd infections following Garner et al. (2009). Bd prevalence was compared between adults and young-of-the-year within ponds using a paired t-test.

Field observations 2011. If high water temperatures are removing Bd infections from larvae and newly metamorphosing froglets, then cooler/deeper

ponds should have higher Bd prevalence in tadpoles and newly metamorphosed froglets than warmer/shallower ponds (Fig. 1). This prediction was tested by comparing water temperatures and seasonal Bd dynamics in two ponds: a relatively deep, perennial pond (27 Mile Lake) and a shallow, ephemeral pond (Twin Pond). Both ponds were located in Coconino National Forest (Fig 3; Table S1) and surrounded by a similar habitat of mostly ponderosa pine and oak dominated forest (Brown, 1994). The shallow, ephemeral pond (Twin Pond) was located at an elevation of 2,178 m and was completely dry by the last sampling date. Bd has been detected annually in breeding chorus frogs from this pond since 2009 with infection prevalence in adults ranging from 12-15% during breeding (O. Hyman unpubl. data). The deeper, perennial pond (27 Mile Lake) was located at 2,119 m and had a maximum depth of 1.69 m at the last sampling date. Bd was first detected in chorus frogs in this pond in 2001 (Retallick & Miera 2007) and was detected at 71, 66, and 77% prevalence in adult frogs during breeding in 2008, 2009, and 2010 respectively (O. Hyman unpubl. data). Chorus frog populations have persisted at high host densities at both of these sites, despite high Bd prevalence for several generations (O. Hyman, unpubl. data).

Each pond was visited weekly from the start to the end of adult breeding, three times during larval development, and three times during the emergence of young-of-the-year froglets. During each of these visits (with the exception of times larvae were sampled) up to 33 adult frogs/froglets were swabbed for Bd as described in Hyman & Collins (2012). All adult frogs and froglets were collected from within the perimeter of the pond and between 1900 and 2400 hrs to control

for diel effects. Newly metamorphosed froglets were not present at the shallow, ephemeral pond on the final visit, as they had already dispersed to the surrounding forest. When water was present, ~600 ml of pond water was filtered at each visit to test for Bd in the water column using the methods described in Hyman & Collins (2012). One hundred fifty larvae were sampled on each of the three visits during larval development. Five larvae were captured in hand-nets from each of 30 haphazardly selected locations within a pond (150 total larvae). These locations were stratified across pond microhabitats to include shallow, deep, open, shaded, rocky, and vegetated areas. Different nets were used at each within-pond location, to limit potential disease transfer. Once captured, larvae were euthanized in individual vials containing MS-222, transferred to new individual vials containing 70% ethanol, and placed on ice until they were stored in the lab at -20 °C. Bd infection status of individual larvae was determined by extracting DNA from excised mouthparts following Retallick et al. (2006), including positive and negative controls. Samples were analyzed in duplicate by qPCR as described above.

In addition to disease data, the presence of each life stage (adults, larvae, juveniles) within ponds was assessed during each visit. Adult presence was determined by calling. Initial presence of larvae was determined after the first egg mass was detected by dip-netting 50 times in the areas of highest egg densities. Later in the season, larval presence was easily determined by visual surveys. Presence/absence of young-of-the-year froglets was determined by time-limited, 1-hr visual surveys around pond edges. These surveys were used to determine the

relative amount of overlap between each life stage, which could be important for disease transmission across life stages, especially adults to larvae.

Pond water temperature was measured daily at 2-hr intervals using iButton™ temperature loggers (1-Wire Thermochron™) sealed in Plasti-dip™ and placed on pond bottoms at a depth of 1 m. Temperature loggers were moved on each visit to maintain them at this depth as ponds evaporated and shrank. The shallow, ephemeral pond was < 1 m deep on the second date of larval sampling (May 5, 2011), so the temperature logger was left at the deepest point (0.89 m). Both temperature loggers were removed from ponds on June 24, 2011 when the ephemeral pond was completely dry. We compared the total number of days water temperatures exceeded 26 °C (removes Bd infections from *A lytes* larvae), 28 °C (no Bd growth *in vitro*), and 30 °C (Bd death *in vitro*) in each pond. Mean daily maximum water temperatures during tadpole development (April 1– June 1, 2011) were compared between ponds using t-tests.

RESULTS

Frog Population Model

In biological terms, equation (9) states that the frog population is projected to grow when the per capita number of offspring that survive to maturity each year ($\gamma\sigma$) exceeds the fraction of adults that die each year ($1-\Sigma$). Thus, even in situations where there is no adult survivorship ($\Sigma = 0$), as long as each breeding adult produces ≥ 1 female frog that survives to maturity in the subsequent generation ($\gamma\sigma \geq 1$), the population will grow. Though this is likely to be an

overstatement of the robustness of chorus frog populations to adult mortality, it theoretically supports the hypothesis that chorus frog populations can persist even with 100% annual adult mortality, assuming that a sufficient number of juveniles survive to reproduction in the following year (i.e. $\gamma\sigma \geq 1$). The reverse, however, is not true. The population cannot grow or persist if there is no meta survivorship ($\sigma = 0$).

Annual meta survivorship, also had a strong influence on projected population growth (λ) relative to adult survivorship (Fig. 4; Table 2). Incrementally increasing individual parameters by a fixed proportion (1%) while holding all others constant revealed that λ is most strongly influenced by parameters related to meta survivorship and least strongly by those related adult survivorship and fecundity (Fig. 4; Table 2). This response, however, is modulated by fecundity. Higher levels of fecundity (γ) result in increased responses of λ to meta survival parameters (Fig. 5). Thus, at higher fecundities, which are typical for chorus frogs (Moriarity & Lannoo 2005), projected population growth is most sensitive to meta survival. At lower fecundities, however, meta survival becomes less important relative to adult survival for population growth.

Tolerance Experiments

Of the 40 animals collected, 27 tested Bd positive and 13 tested Bd negative on the first sampling date following collection (April 1, 2011). All Bd-negative animals and the negative controls remained Bd negative at the middle and last pathogen sampling dates on October 4, 2011 and March 5, 2012,

respectively, indicating no cross contamination or development of infections during the course of the experiment. All Bd-positive animals tested Bd positive on the date of death. Three of the four Bd-positive animals that did not die during the course of the experiment tested Bd positive on the last day of sampling on March 5, 2012. One Bd-positive animal lost its Bd infection by October 4, 2011.

Bd-positive animals suffered significantly greater mortality ($X^2_1 = 22.1$, $p < 0.0001$) with 85% (23 of 27) of the Bd-positive animals dying and no death in Bd-negative animals (Fig. 6). Median time to death was 53 days, with the earliest death occurring 17 days after collection and the last death occurring after 218 days (Fig. 6). The four surviving Bd-positive frogs have lived at least 366 days (> 1 yr) with infections. Bd-infected frogs sloughed skin and avoided water significantly more often than uninfected individuals (Mann Whitney$_{df=1}$, $p < 0.001$ and < 0.0001, respectively). Bd-positive animals had a median load of ~12,000 zoospore genomic equivalents (GE; limits: 16-221,600 zoospore GE) at the time of death. Surviving infected frogs (n=4) had a median load of 266 zoospore GE (limits: 23 – 4,600 zoospore GE) on April 1, 2011, 678 zoospore GE (limits: 0 – 2,352 zoospore GE) on October 4, 2011 and 284 zoospore GE (limits: 0-3,520 zoospore GE) on March 5, 2012. Animals that survived to the end of the experiment with infections had significantly lower Bd loads at the start of the experiment (April 1, 2011) than those that died (Wilcoxon$_{df=1}$, $p = 0.006$; Fig. 7).

Laboratory Heating Experiment

Significantly fewer heat-treated animals tested Bd positive compared to cold-treated animals ($X^2_1 = 12.5$, $p = 0.0004$). In fact, 0 of 16 heat-treated animals

tested Bd positive, in contrast to 9 of 16 (56%) cold-treated animals (Fig. 8). All hot- and cold-treated animals, except one heat-treated tadpole, tested Bd positive two weeks post exposure, but cold-treated tadpoles had significantly higher Bd loads than heat-treated tadpoles after only one week in the treatments ($Wilcoxon_{df=1}$, $p = 0.003$. Fig. 9). Gosner stage (mean ±SE) was not significantly different between heat- (33.2 ± 0.5) and cold- (34.2 ± 0.5) treated animals at the start of the experiment ($Wilcoxon_{df=1}$, $p = 0.50$). Heat-treated tadpoles reached stage 42 significantly faster than cold-treated animals ($Wilcoxon_{df=1}$, $p = 0.001$), with a mean difference of approximately seven days. There was no significant difference in mortality between hot- and cold- treatments ($X^2_1 = 1.19$, $p = 0.28$), with three cold- and one heat-treated animal dying before reaching stage 46. All negative control animals and DNA-remnant controls tested Bd negative.

Field Observations 2010

A minimum of 24 adults and 28 newly metamorphosed froglets were sampled from each pond (Fig. 10; Table S7). Bd was detected in chorus frog adults from 13 of 15 ponds sampled, with Bd prevalence ranging from 0-100% (mean 43 ± 8%; Fig. 10; Table S7). Bd prevalence was significantly lower in young-of-the-year froglets (paired $t\text{-test}_{df=14}$, $p = 0.0001$) with Bd detected in only four of 15 ponds and prevalence ranging from 0-10% (mean 2 ± 0.8%; Fig. 10; Table S7).

Field Observations 2011

Water temperature. Mean daily maximum water temperatures during larval development were not significantly different in the ephemeral versus

perennial pond (t-test$_{df=116}$, p = 0.84), with a mean (± SE) of 22.5 ± 0.7 °C and 22.7 ± 0.6 °C, respectively. However in the ephemeral pond maximum daily water temperatures at 1-m depth exceeded 26 °C on 19 days, 28 °C (no Bd growth *in vitro*) on seven days, and 30 °C (Bd death *in vitro*) on two days during larval development, while the deeper perennial pond exceeded these temperatures on 13, four, and zero days, respectively (Fig. 11d; Table S8).

Bd dynamics. During breeding, Bd prevalence generally increased in both ponds, reaching 100% for the final three weeks of calling. In the cooler perennial pond, Bd prevalence was initially high (~55%), dropped to ~20% prevalence, then increased to a steady 100% prevalence for the final three weeks of adult sampling. In the warmer ephemeral pond, Bd prevalence during breeding was initially low (~0-10%) then followed a similar pattern as the perennial pond, with prevalence quickly climbing to 100% during the final three weeks of breeding (Fig. 11a).

Following breeding, Bd prevalence slowly increased in larvae as they developed in the perennial pond. However, in the ephemeral pond Bd was not detected in any larvae throughout development (Fig. 11a). Bd prevalence in newly metamorphosed froglets followed a similar pattern, with slowly increasing prevalence from June 1 to July 18 at the perennial pond, and no Bd detected in newly metamorphosed froglets from the ephemeral pond on either of the sampling dates on June 1 or 24th (Fig. 11a).

Bd loads mirrored patterns of prevalence, peaking at the end of breeding with a mean of ~19,400 (max: 116,000) and 11,700 (max: 37,600) zoospore GE

in the perennial and ephemeral pond, respectively (Fig. 11b). In the perennial pond, mean Bd load steadily rose from 0 to 1,554 to 2,506 zoospore GE in larvae, while mean load in young-of-the-year froglets initially increased from 13 to 512 zoospore GE then decreased to 162 zoospore GE, despite the rise in Bd prevalence (Fig. 11b). Loads were zero for all larvae and froglets collected from the ephemeral pond (Fig. 11b).

Bd densities in the water column followed a similar pattern, peaking near the end of breeding, with densities as high as 244 zoospore GE l^{-1} and 121 zoospore GE l^{-1} in the perennial and ephemeral pond, respectively (Fig. 11c). Water filters consistently detected Bd DNA in the perennial pond at each sampling point throughout larval development, including times when larvae did not test Bd positive (Fig. 11c). Conversely, water filters never detected Bd DNA after the end of breeding in the ephemeral pond (Fig. 11c).

Adult and larval chorus frog life stages showed similar degrees of overlap in the perennial and ephemeral pond, with a minimum of three and four weeks of overlap, respectively, demonstrating that larvae were present in both ponds at times of high Bd prevalence, loads, and aquatic zoospore densities (Fig. 11a). In the perennial pond, larvae and newly metamorphosed froglets overlapped for at least two weeks, while there was only one day of detected overlap between these stages in the ephemeral pond (Fig. 11a). This single day of "detected" overlap in the ephemeral pond is almost certainly an underestimate, because the ephemeral pond had dried completely before the next sampling period, precluding the detection of larvae.

DISCUSSION

My model presents evidence that projected chorus frog population growth is most sensitive to changes in parameters associated with juvenile survival. Other matrix-based models of frog populations have found similar results. For example, Govindarajulu et al. (2005) found that bullfrog population growth rate is most affected by early post-metamorphic survival rates and recommended culling of young-of-the-year bullfrogs as the most effective way to reduce population growth. The model also predicts that chorus frog populations could theoretically persist with 100% annual adult mortality, but not 100% annual mortality of metas. These findings suggest that factors enhancing the survival of young-of-the-year chorus frogs will increase the likelihood of chorus frog population persistence, even in the face of high levels of annual adult mortality.

Adult boreal chorus frogs brought into the laboratory with natural Bd infections incurred high rates of mortality (85%). These results parallel previous reports of up to 80% mortality in artificially infected boreal chorus frogs collected from an adjacent site (Retallick & Miera 2007). It is clear that under conditions ideal for the pathogen, most adult chorus frogs in this population are susceptible to chytridiomycosis. This is in contrast to a similar study, which found no Bd-related mortality in closely related *Pseudacris regilla* collected from populations that have persisted with Bd for six years in California (Reeder et al. 2012). The high level of Bd-related mortality in infected adults does not support the hypothesis that innate or adaptive means of Bd resistance or tolerance are a ubiquitous trait in chorus frogs that is responsible for their persistence with Bd.

Assuming that Bd causes similar rates of mortality in the field, it is surprising that such a high proportion of susceptible individuals would survive in a host population several generations post-Bd introduction. This suggests that either host resistance/tolerance is not a heritable trait or that some extrinsic factor, unrelated to host resistance/tolerance is enabling these populations to persist with high numbers of susceptible host phenotypes.

My results suggest a combination of chorus frog life history traits and environmental conditions enable populations of chytridiomycosis-susceptible frogs to persist with Bd. In my laboratory study, median time to death was 53 days, with the earliest death occurring 17 days after collection and the last death occurring after 218 days. Retallick & Miera (2007) found similar trends, with all frogs dying between 19 and 70 days post-exposure. Assuming frogs suffer mortality at a similar rate in the wild, even the most susceptible individual has at least 2.4 weeks post-initial Bd exposure to survive and reproduce. Most frogs enter ponds Bd free at the time of breeding. If susceptible frogs breed before suffering mortality from chytridiomycosis this would negate selection for more resistant or tolerant individuals, enabling susceptible phenotypes to be retained in the population. This only works, however, if subsequent offspring are either not exposed to Bd, display different levels of resistance or tolerance than parents, or are exposed to Bd under conditions that are not favorable to the pathogen, hence allowing susceptible offspring to reach maturity.

My results indicate that environmental conditions in the field reduce Bd prevalence in offspring. Bd prevalence was significantly reduced in young-of-the-

year froglets emerging from ponds in 2010, with Bd detected in only four of fifteen ponds sampled during this life stage. The absence of detectable Bd infections in larvae and froglets in the ephemeral pond sampled in 2011 indicates that Bd can be entirely removed from the larval cohort following breeding. Similar dynamics may underlie the absence of Bd infection in froglets observed in eleven other ponds in 2010 (Fig. 9).

Heat treatment experiments and field studies support the hypothesis that high water temperatures are responsible for the absence of Bd infections in larvae and froglets collected from these ponds. Heat-treated larvae lost detectable Bd infections following metamorphosis. Although the high-temperature regime used in this experiment may have exposed tadpoles to 29 °C at a higher frequency than these temperatures were recorded in the field, the absolute temperatures were within the range experienced in nature. This demonstrates that temperatures within the range of those occurring in nature can remove Bd infections during larval development. This inference is further supported by the absence of Bd infection from a heat-treated tadpole only two weeks post-inoculation.

Bd infections were not detected in larvae or recently metamorphosed froglets collected from the shallower, ephemeral pond. This was in contrast to the deeper, perennial pond, in which Bd was detected from both of these life stages. Although mean temperatures of these ponds were not significantly different during larval development, water temperatures in the ephemeral pond exceeded critical temperatures that kill Bd or limit its growth more often than in the deeper perennial pond, where temperatures never exceeded 30 °C. I cannot conclude that

shallower sections (< 1 m) of the perennial pond did not exceed 30 °C, because the temperature logger was kept at a shallower depth (≤ 0.89 vs. ≤ 1 m) in the ephemeral pond during the times that it exceeded these critical temperatures. However, because the temperature logger was in the deepest part of the ephemeral pond, I can conclude that when water temperatures in this pond reached critical temperatures, there were no deeper areas of the pond that might provide thermal refuge for Bd, whereas these thermal refugia would have to be present in deeper sections of the perennial pond. This may explain why Bd persisted in the perennial pond and not the ephemeral pond despite relatively similar water temperatures.

The results of the heat treatment experiment and the absence of Bd infections in larvae and froglets collected from the ephemeral pond supports a role for temperature in reducing Bd infections in chorus frogs, but does not definitively demonstrate that the absence of Bd in the larval cohort is only due to high water temperatures. Other factors that may differ between the two ponds such as host densities, pond habitat structure, tadpole behavior, reduced abundance of reservoir hosts, or simple stochasticity in Bd transmission from adults to larvae could also explain this trend. Future studies should test these possibilities.

Previous studies of 20 Arizona chorus frog populations found that Bd was present in breeding adults at 17 sites in both 2009 and 2010 (O. Hyman unpubl data). The persistence of Bd at these sites means that Bd is either surviving in the environment, alternative hosts such as tiger salamanders (*Ambystoma tigrinum)*,

or chorus frogs that survive through the winter with Bd infections. The three frogs that maintained chronic Bd infections for an entire year in the laboratory demonstrate that infected chorus frogs have the potential to survive long enough to maintain Bd in the population across breeding seasons, which would explain Bd's re-occurrence each year. These chronically infected frogs had significantly lower initial levels of Bd infection than animals that died (Fig. 7) suggesting that the outcome of Bd infections may be dose dependent (Brunner et al. 2005, Carey et al. 2006) and that initial dose may subsequently affect the ability of Bd to persist across breeding seasons.

The inter-annual persistence of Bd in these populations also means that any chorus frogs surviving from the previous year are likely to be exposed (or re-exposed) to Bd when they return to ponds each breeding season. Thus, the persistence of chorus frog populations with high frequencies of susceptible animals would also require that either a sufficient number of adults survive to the following breeding season to reproduce or their offspring reach sexual maturity within a year. Smith (1987) found that *P. triseriata* in Michigan reached sexual maturity within the first year following metamorphosis. I hypothesize that early maturation in combination with the loss of infection during larval development may enable populations of susceptible amphibian hosts to persist with Bd without experiencing selection for more Bd resistant or tolerant phenotypes or marked population declines. This hypothesis is supported by my field and laboratory studies; however, more work is needed to confirm that higher temperatures increase meta survival. Future studies should employ mark-recapture techniques

to determine whether meta survival to maturity is enhanced by the loss of Bd infections.

In some cases Bd was not lost from the larval cohort. For example, Bd was detected in froglets from four ponds in 2010 and persisted in larval and juvenile froglets throughout development in the perennial pond (27 Mile Lake) in 2011. Assuming environmental removal of Bd infections from larval and juvenile life stages plays an important role in chorus frog population persistence, frog populations at these sites should decline. Yet animals at the perennial site (27 Mile Lake) have persisted with Bd at high host and pathogen densities for at least nine years (Collins & Miera 2005, Hyman and Collins 2012). My model demonstrates that as long as the number of offspring surviving to maturity is sufficient to replace those adults that were lost, chorus frog populations can persist. Thus, even in situations where Bd causes high levels of mortality in young-of-the-year froglets, the population can grow. Alternatively, the persistence of Bd in the larval cohort may alter the selective pressure of Bd at these sites, potentially resulting in stronger selection for host resistance or tolerance or lowered pathogen virulence. In this scenario, we would predict a higher proportion of Bd-infected frogs collected from these perennial ponds to survive with Bd infections than those collected from ponds where Bd is completely lost from the larval cohort. Future studies should employ common garden experiments to determine whether chorus frogs display between-pond variation in their tolerance of local Bd strains (e.g. Tobler & Schmidt 2010).

CONCLUSIONS

Environmental context shapes the selective pressures that a pathogen exerts on its hosts and the evolutionary process in general (Thompson 1999). Geographic variation in evolutionary processes can result in very different outcomes of species interactions, including hosts and their pathogens (Thompson 1999). For example, Retallick & Miera (2007) demonstrated significant differences in the virulence of Bd strains isolated from chorus frog populations separated by only a few dozen kilometers. This study demonstrates that despite taking place in relatively similar habitats, the host-pathogen dynamics of chorus frogs and Bd can be very different at the pond-level. These subtle environmental differences may alter the selective pressures that Bd exerts on local chorus frog populations potentially resulting in a geographic mosaic of host susceptibility and pathogen virulence at scales as small as individual ponds. This idea is supported by previous results of Retallick & Miera (2007), and has important implications for the movement of Bd strains, even in areas where Bd is endemic. For example, if there is pond-level variation in frog susceptibility to chytridiomycosis, individuals that do not develop chytridiomycosis at one site may not be resistant to or tolerant of Bd strains at another location where the selective regime is different (Retallick & Miera 2007).

I present evidence in support of the hypothesis that in some cases rapid host maturation can combine with environmental conditions unfavorable for the pathogen to enable host populations with high proportions of susceptible individuals to persist with this pathogen at high host and pathogen densities.

However, in other cases environmental factors do not remove Bd from the system, potentially resulting in a different selective regime that will alter the shape of this host-pathogen relationship. Future studies should examine whether these contexts influence the evolution of chorus frogs and Bd and the ability of chorus frog populations to persist with this pathogen.

Table 1. List and description of parameter values used in the model

Parameter	Value	Description	Source
δ	500	number of eggs produced per female	Moriarity & Lannoo 2005
θ	0.38	egg hatching success	Kramer 1978
η	0.10	larval survival to metamorphosis	Smith 1983
γ	9.50	number of female metamorphs produced by an adult female	$\delta*\theta*\eta*0.5$
Ω	0.19	fraction of metamorphs that survive to the next breeding season in Bd free conditions	Smith 1987
Ψ	0.14	fraction of adults that survive to the next breeding season in Bd free conditions	Smith 1987
i	0.85	fraction of metamorphs that emerge with Bd infections each year	This study
I	1.00	fraction of adults that become infected during breeding each year	Hyman & Collins 2012 This study
d	0.85	fraction of infected metas that die from Bd infections	Searle et al. 2011
D	0.85	fraction of adults that die from Bd infections	Retallick & Miera 2007 This study
Σ	0.021	fraction of adults that survive to the next breeding season in the presence of Bd	$\Psi(1-I*D)$
σ	0.053	fraction of metamorphs that survive to the next breeding season in the presence of Bd	$\Omega(1-i*d)$

Table 2. Relative response of projected population growth (λ) to 1% increases in each individual parameter while all other parameters were held constant. λ shows a greater response, as assessed by slope, to changes in parameters associated with meta than adult survival

Parameter	Description	Relative Response (Slope of line)
γ	number of female metamorphs produced by an adult female	0.05
Ω	fraction of metamorphs that survive to the next breeding season in Bd free conditions	2.63
Ψ	fraction of adults that survive to the next breeding season in Bd free conditions	0.15
i	Fraction of metamorphs that emerge with Bd infections each year	-1.53
I	Fraction of adults that become infected during breeding each year	-0.13
d	Fraction of infected metas that die from Bd infections	-1.53
D	Fraction of adults that die from Bd infections	-0.13

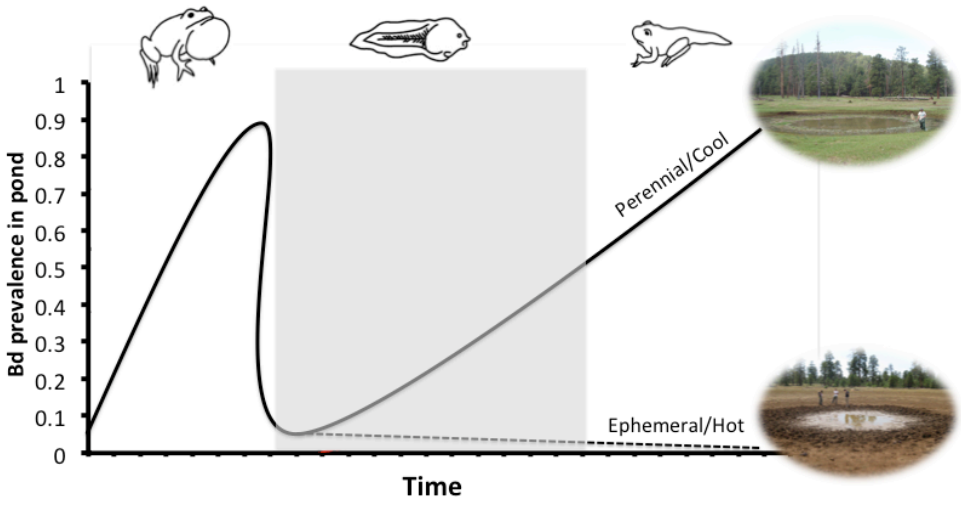

Figure 1. Predicted change in *Batrachochytrium dendrobatidis* (Bd) prevalence over the course of boreal chorus frog (*Pseudacris maculata)* breeding and development in a perennial (solid line) versus ephemeral (dashed line) pond. Grey block represents life stage transitions from eggs to larvae to young-of-the-year froglets. Bd is maintained in larvae and froglets in the cooler perennial pond, but lost from the warmer ephemeral pond due to high water temperatures that Bd cannot tolerate

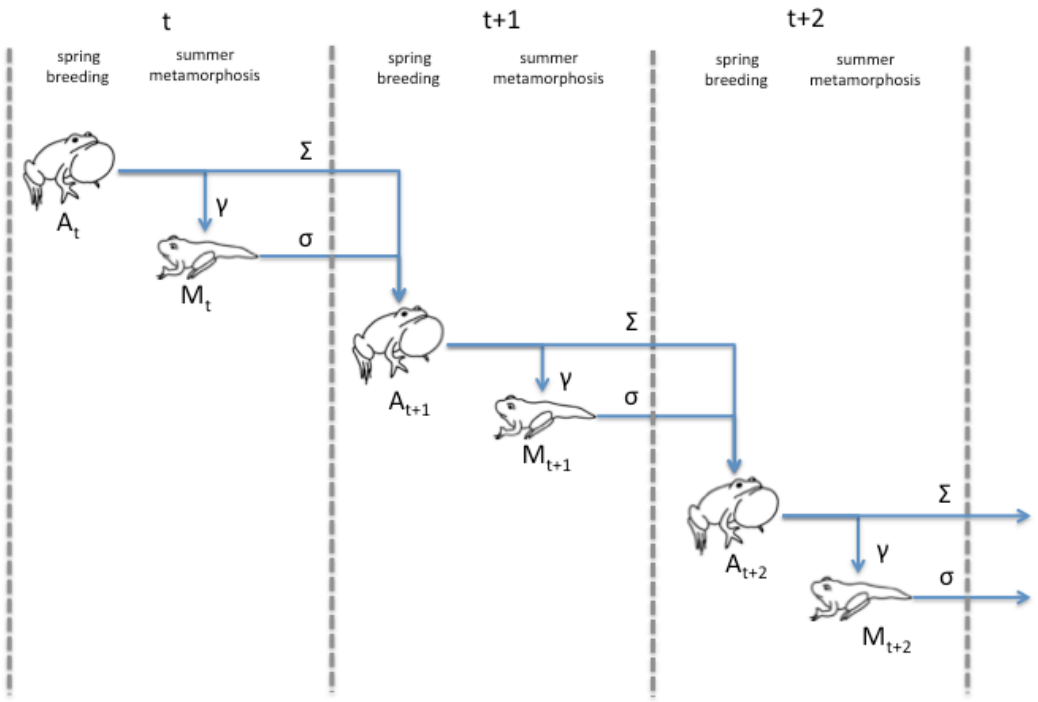

Figure 2. Diagram of *Pseudacris maculata* lifecycle. γ represents the number of metamorphs (M) produced by an adult (A) female each year. Σ and σ are survival of adults and metamorphs, respectively, to the next breeding season

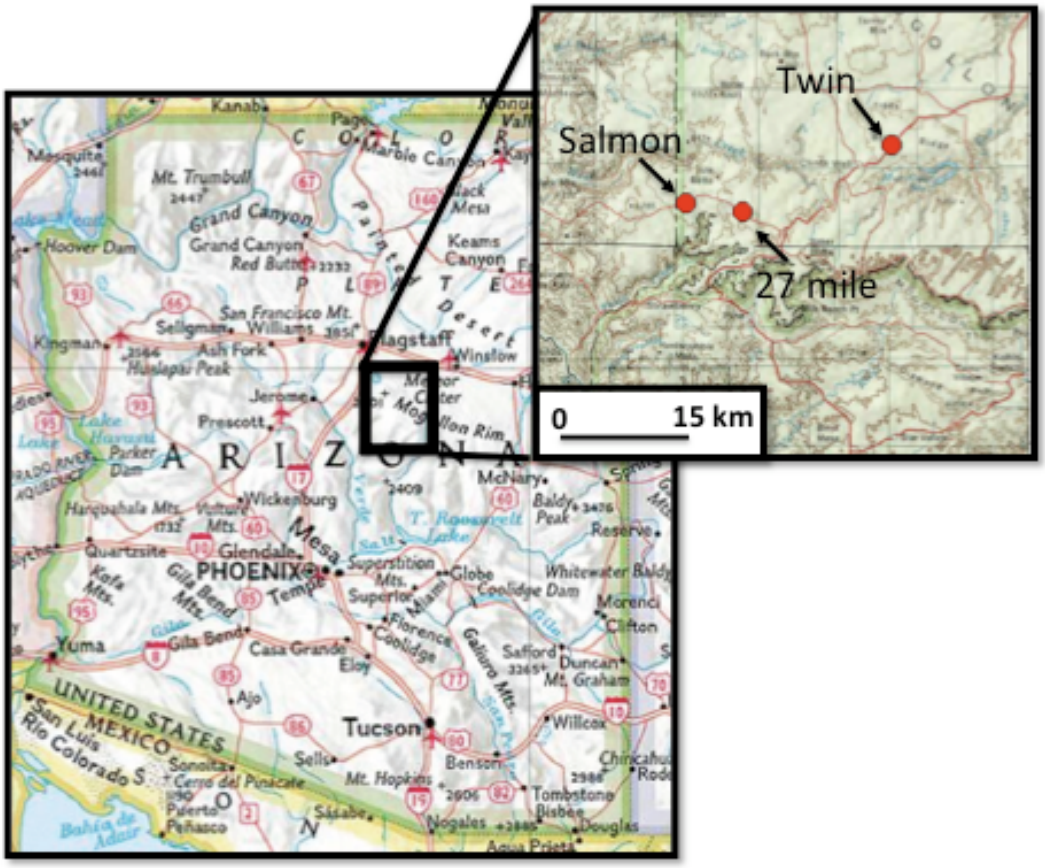

Figure 3. Location of 3 ponds used in this study. Adult frogs used in tolerance experiments were collected from Salmon Lake. Tadpoles used in heat treatment experiments were collected from 27 Mile Lake. Twin pond was the ephemeral pond and 27 Mile Lake was the perennial pond in 2011 field surveys. GPS coordinates available in Table S1

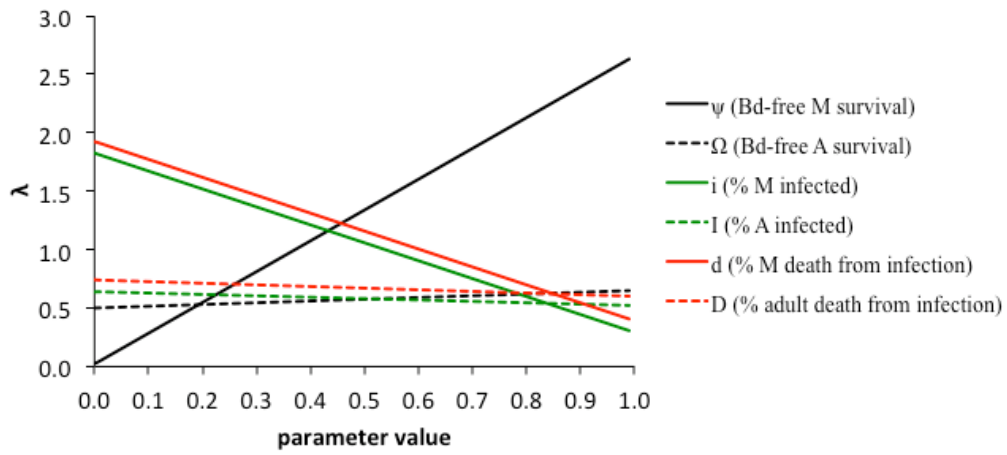

Figure 4. Relative response of projected population growth (λ) to 1% increases in individual survival-related parameters while all other parameters were held constant. Dotted lines represent parameters related to adult (A) survival. Solid lines represent parameters related to meta (M) survival. Colors code for equivalent parameters for each life stage. λ consistently shows a stronger response (depicted by steeper slopes) to changes in parameters associated with meta vs. adult survival. D and d are purposefully offset by 0.1 for ease of viewing

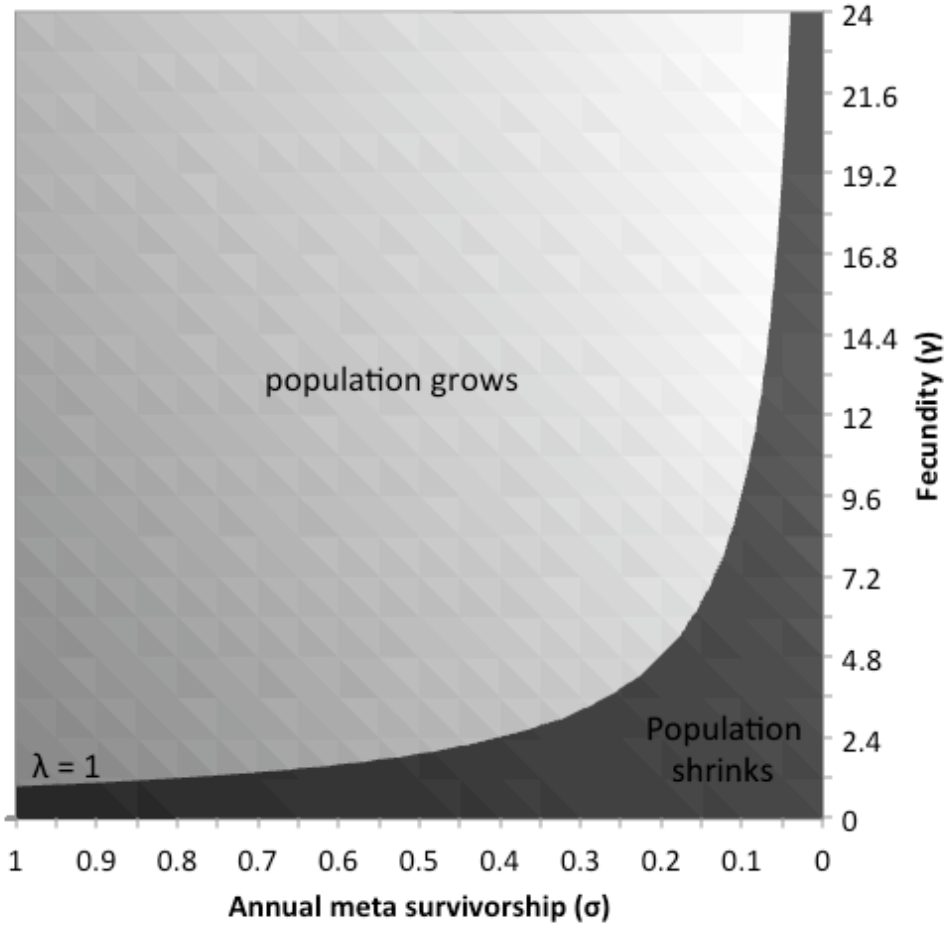

Figure 5. Diagram depicting the relationship of projected population growth (λ) to annual meta survivorship (σ) and fecundity (γ) when all other parameters are held constant (see Table 1 for values). At higher fecundities, populations maintain positive growth for a wide range of meta survivorship. When fecundity is low, meta survivorship becomes increasingly important to maintain positive population growth. At the lowest fecundities, however, even 100% meta survival cannot maintain positive population growth

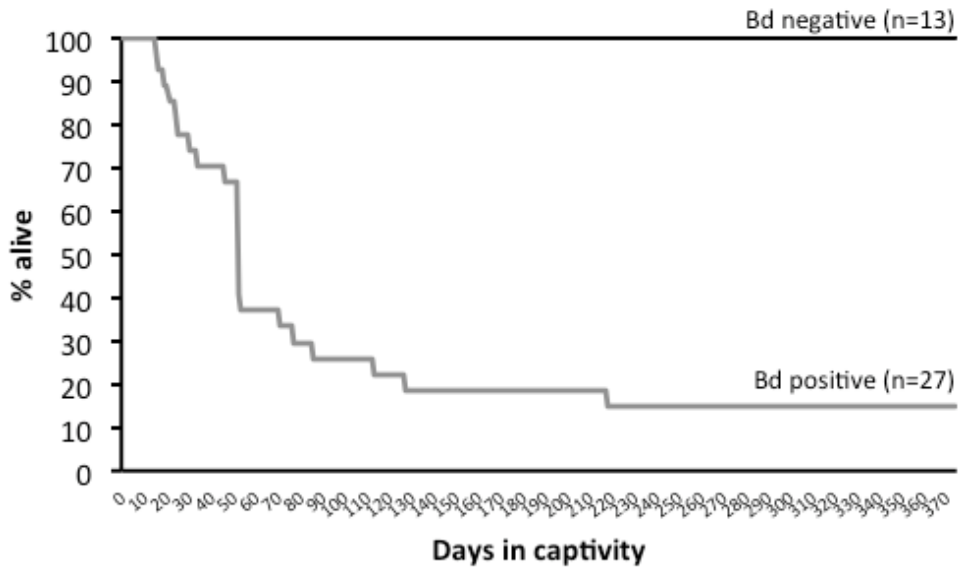

Figure 6. Boreal chorus frogs (*Pseudacris maculata*) with naturally obtained *Batrachochytrium dendrobatidis* (Bd) infections suffered significantly greater mortality (85%) than Bd-free controls (0%; $X^2_1 = 22.1$, $p < 0.0001$)

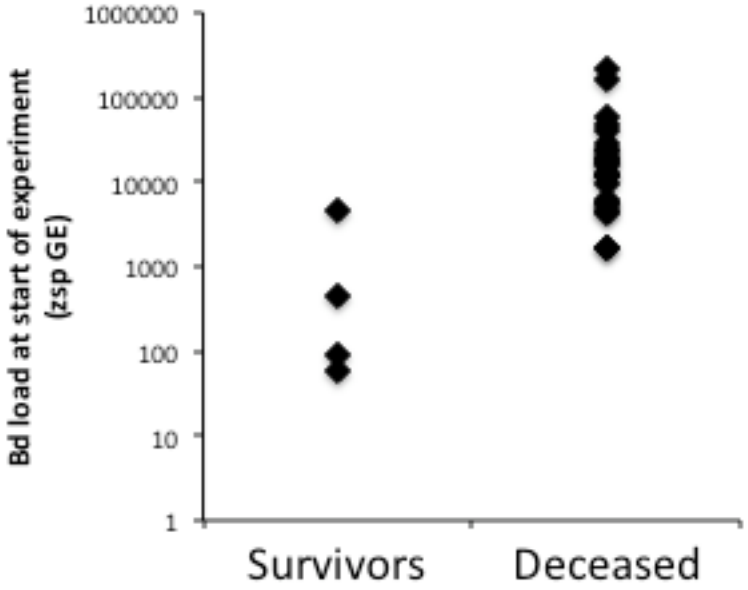

Figure 7. The four boreal chorus frogs (*Pseudacris maculata*) that survived with *Batrachochytrium dendrobatidis* (Bd) had significantly lower Bd loads at the start of the experiment than the 24 frogs that died ($Wilcoxon_{df=1}$, $p = 0.006$)

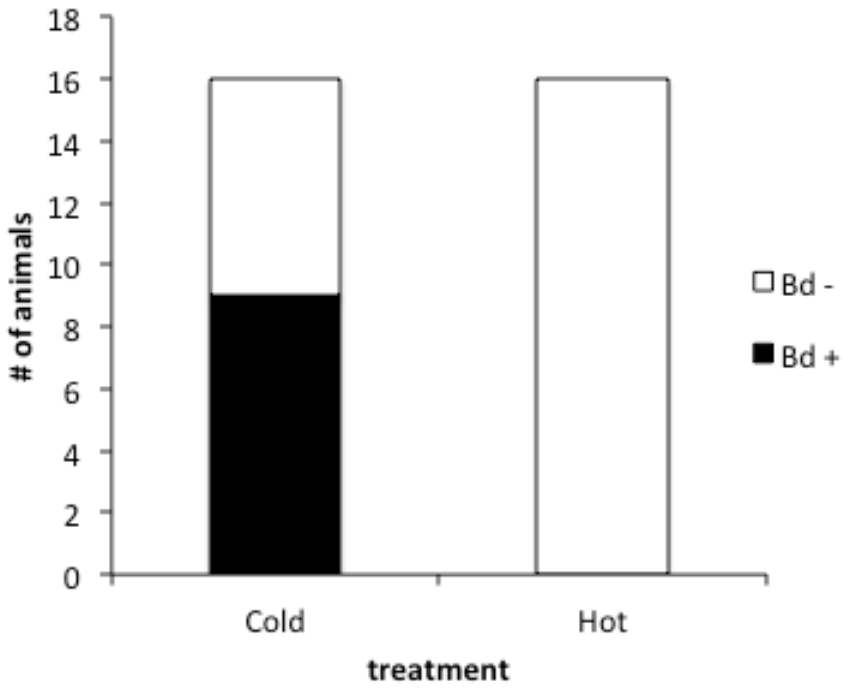

Figure 8. Results of qPCR analyses of hand and foot samples taken from newly metamorphosed boreal chorus frogs (*Pseudacris maculata*) exposed to *Batrachochytrium dendrobatidis* (Bd) as larvae and allowed to develop in naturally relevant warm temperatures (hot) versus cooler (cold) temperatures. Animals developing in cooler temperatures were more likely to test Bd positive following metamorphosis ($X^2_1 = 12.5$, $p = 0.0004$)

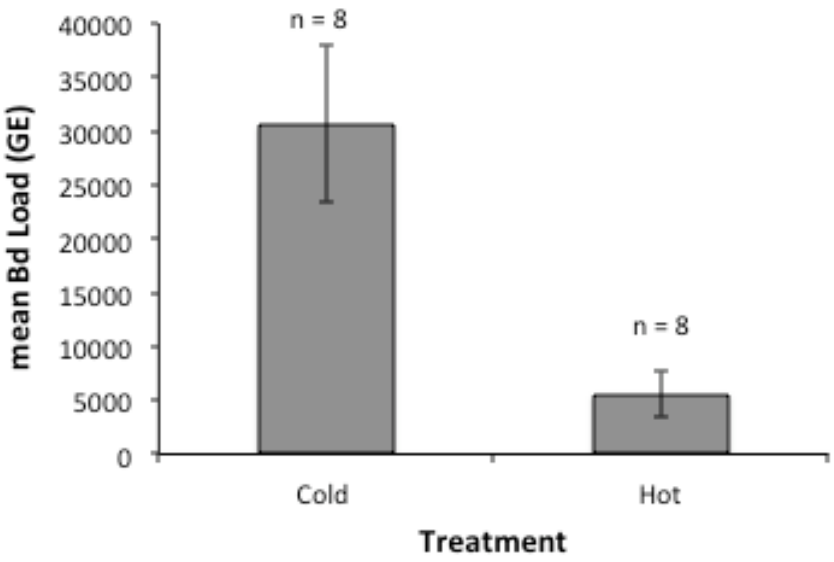

Figure 9. Comparison of mean *Batrachochytrium dendrobatidis* (Bd) loads (±1 SE) on the mouthparts of boreal chorus frog (*Pseudacris maculata*) tadpoles two weeks after initial Bd exposure and one week after being kept at 20 °C (cold) or 29 °C for four hours daily (hot). Loads were significantly lower in heat-treated animals ($Wilcoxon_{df=1}$, $p = 0.003$). One heat-treated animal tested Bd-negative

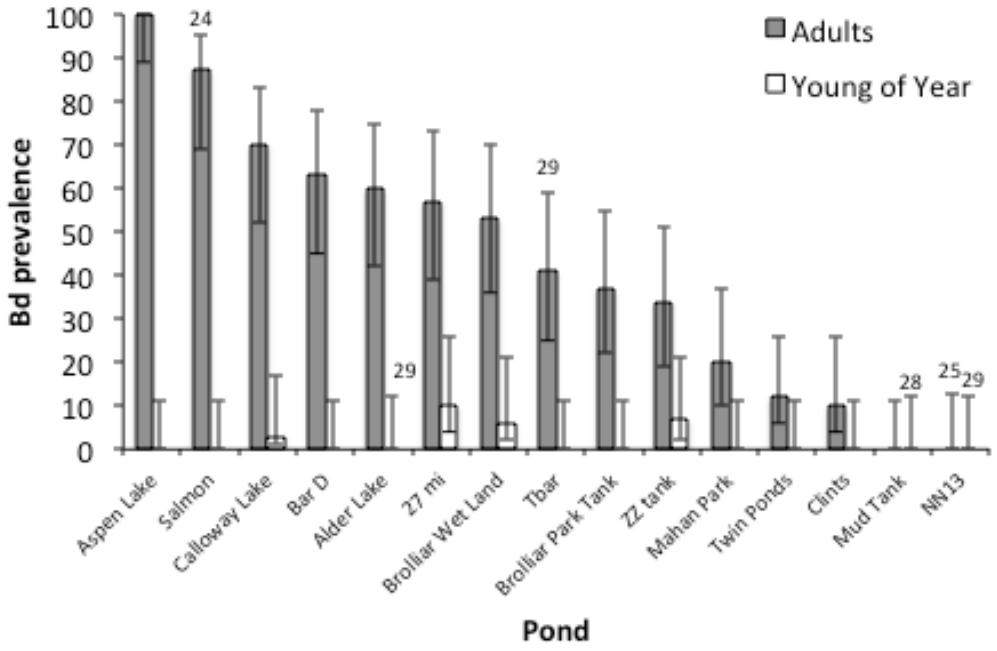

Figure 10. *Batrachochytrium dendrobatidis* (Bd) prevalence (± 95% confidence intervals) in adult versus young-of-the-year boreal chorus frogs (*Pseudacris maculata*) collected from each of 15 ponds on Arizona's Mogollon Rim in 2010. Bd prevalence was significantly lower in newly emerging froglets (paired t-test$_{df = 14}$, p = 0.0001), with only four ponds having froglets test Bd positive. Numbers above bars represent samples size if < 30

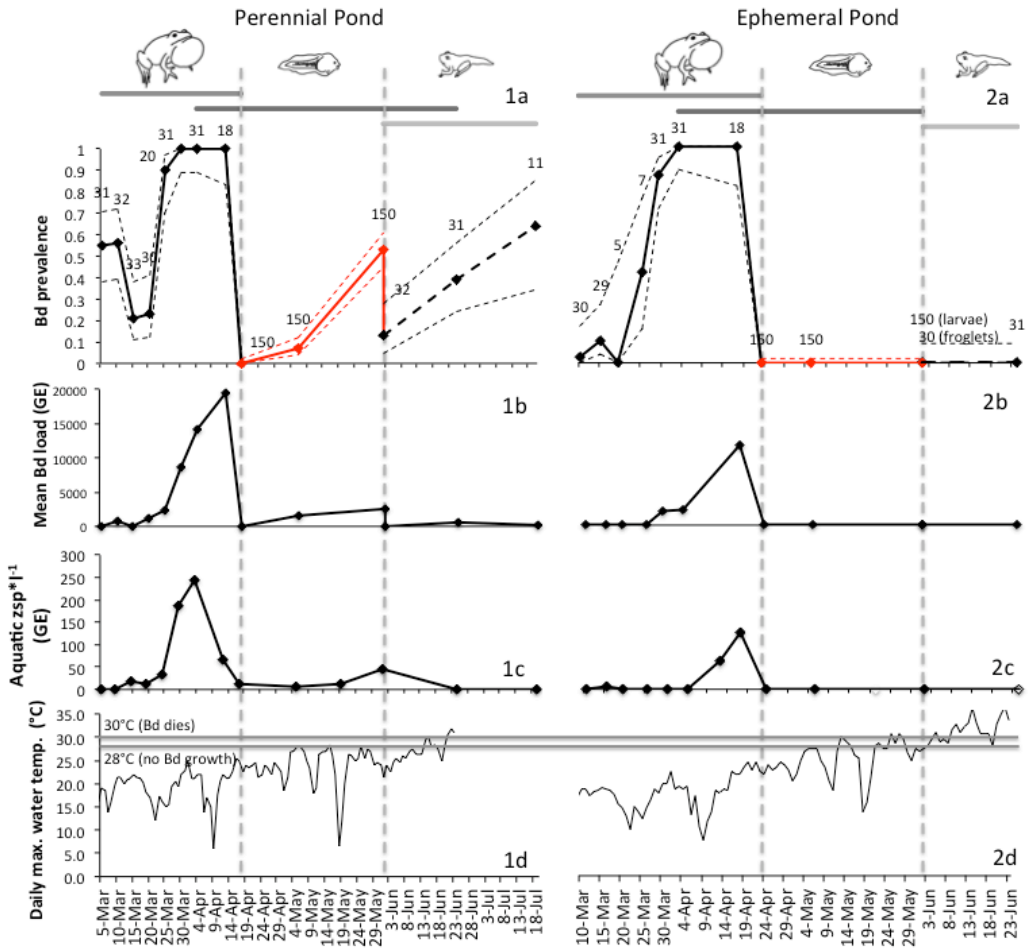

Figure 11. Comparison of (a) *Batrachochytrium dendrobatidis* (Bd) prevalence, (b) mean Bd loads (c) aquatic densities of Bd zoospores (zsp), and (d) water temperatures at 1 m depth in a perennial pond (1) versus an ephemeral pond (2) over an approximately four month period coinciding with the breeding and development of the boreal chorus frog (*Pseudacris maculata)*. Vertical dashed grey lines represent the transition to sampling of new life stages for Bd. Solid markers on lines represent sampling points. In (a) solid horizontal grey lines represent time periods of detection and overlap of each life stage within each pond. Numbers above points represent sample size, dotted lines represent 95%

confidence intervals for Bd prevalence, solid black lines represent Bd prevalence in adult frogs, red lines represent prevalence in larvae, and dashed black lines represent prevalence in young of the year. Horizontal grey lines in (d) represent temperatures relevant to Bd growth and survival. Water temperatures in shallow ponds exceeded 30°C (causes Bd death) during larval development and Bd was not detected in larvae, young-of-the-year, or water in this pond after breeding. GE = genomic equivalents

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APPENDIX A

SUPPPLEMENTARY TABLES

Table S1. Name, location, and general description of ponds used in this study

Pond Name	Lat (N) NAD27	Long (W) NAD27	Description
27 Mile	34 30.049'	111 27.877'	perennial, natural depression
Alder Lake	34 22.685'	110 57.886'	ephemeral, natural depression
Aspen Lake	34 17.832'	110 51.339'	ephemeral, natural depression
Baker Lake	34 27.263'	111 23.750'	marsh, natural depression
Bar D	34 40.788'	111 24.084'	semi-perennial, man-made cattle tank
Brolliar Park	34 50.788'	111 28.368'	semi-perennial, man-made cattle tank
Brolliar Wetlnd	34 51.322'	111 28.275'	ephemeral, man-made depression
Calloway Lake	34 30.832'	111 27.550'	semi-perennial, man-made cattle tank
Clints	34 35.331'	111 17.334'	semi-perennial, man-made cattle tank
Mahan Park	34 37.533'	111 22.062'	semi-perennial, man-made cattle tank
McClure Lake	34 35.416'	111 16.701'	perennial, man-made cattle tank
Mud	34 29.597'	111 34.923'	semi-perennial, man-made cattle tank
No Name 13	34 30.643'	111 19.020'	semi-perennial, man-made cattle tank
No Name 7	34 20.759'	110 58.494'	ephemeral, natural depression
Salmon Lake	34 30.624'	111 32.410'	semi-perennial, man-made cattle tank
T bar 2 (3rd)	34 43.841'	111 31.510'	perennial, man-made cattle tank
Tinny	34 50.337'	111 26.068'	ephemeral, man-made cattle tank
Twin Ponds	34 34.427'	111 16.150'	ephemeral, natural depression
Van Deren	34 50.137'	111 26.700'	perennial, natural spring
ZZ Tank	34 49.642'	111 26.306'	semi-perennial, man-made cattle tank

Table S2**.** *Batrachochytrium dendrobatidis* (Bd) prevalence and load data from swabs collected from boreal chorus frogs from 20 ponds in Arizona in 2009. GE = genome equivalents. N = sample size

Pond	N	% Bd prevalence (Bayesian 95% central CI)	mean Bd Load (GE)
27 Mile	61	66 (52-76)	653
Alder Lake	60	55 (42-67)	853
Aspen Lake	60	93 (84-97)	1123
Baker Lake	60	2 (<1-9)	18
Bar D	54	24 (14-37)	660
Brolliar Park Tank	60	13 (7-24)	1752
Brolliar Wet Land	44	16 (8-29)	475
Calloway Lake	60	10 (5-20)	108
Clints	60	2 (<1-9)	8
Mahan Park	60	25 (16-37)	2749
McClure Lake	60	5 (2-14)	169
Mud	47	2 (<1-11)	4
No Name 13	30	0 (0-11)	0
No Name 7	63	62 (50-73)	629
Salmon Lake	61	16 (9-28)	337
T bar 2	56	59 (48-72)	124
Tinny	60	25 (16-37)	661
Twin Ponds	60	15 (8-26)	496
Van Deren	60	13 (7-24)	330
ZZ tank	60	20 (12-32)	211
Total	1136	27 (25-30)	--
mean	56.80	27	568
SD	7.89	26	675
SEM	1.76	6	151

Table S3. *Batrachochytrium dendrobatidis* (Bd) prevalence, Δ Bd prevalence, and load data from swabs collected from boreal chorus frogs from 20 ponds in Arizona in 2010. Mean load is calculated within each pond using only Bd infected animals. T1 = swabs collected at beginning of breeding season. T2 = swabs collected 1-2 weeks later as breeding winds down. GE = genome equivalents. CI = Bayesian Confidence Interval

Pond	Days between T_1 and T_2	N_{T1}	N_{T2}	% Bd prevalence$_{T1}$ (95% CI)	% Bd prevalence$_{T2}$ (95% CI)	Δ Bd prevalence (% change day^{-1})	Mean load$_{T1}$ (zsp G.E.±1 S.E.M.)	Mean load$_{T2}$ (zsp G.E.±1 S.E.M.)	Δ Bd load (Load$_{T2}$ – Load$_{T1}$)
27 mi	7	30	30	77 (59 - 88)	57 (39 - 72)	-2.9	3020 (±1728)	5531 (±2691)	2511
Alder Lake	7	30	30	17 (7 - 34)	60 (42 - 75)	6.1	1409 (±925)	3785 (±3214)	2376
Aspen Lake	7	30	30	96 (83 - 99)	100 (89 - 100)	0.4	12574 (±5336)	42834 (±9299)	30260
Baker Lake	10	20	11	0 (0 - 16)	0 (0 - 26)	0.0	0 (na)	0 (na)	0
Bar D	11	30	30	53 (36 - 70)	63 (45 - 78)	0.9	1196 (±919)	1508 (±822)	312
Brolliar Park Tank	7	30	30	7 (2 - 21)	37 (22 - 55)	4.3	20 (±18)	3360 (±2426)	3340
Brolliar Wet Land	7	30	30	17 (7 - 34)	53 (36 - 70)	5.1	97 (±88)	10101 (±7206)	10004
Calloway Lake	8	30	30	17 (7 - 34)	70 (52 - 83)	6.6	260 (±234)	6254 (±3673)	5994
Clints	7	30	30	13 (5 - 30)	10 (4 - 26)	-0.4	37 (±31)	14 (±8)	-23
Mahan Park	7	17	30	6 (1 - 27)	20 (10 - 37)	2.0	107 (na)	4059 (±3830)	3952
McClure Lake	10	30	27	7 (2 - 21)	7 (2 - 24)	0.0	209 (±207)	222 (±191)	13
Mud	7	30	30	0 (0 - 11)	0 (0 - 11)	0.0	0 (na)	0 (na)	0
No Name 13	.	25	0	0 (0 - 13)	na		0 (na)	na	na
No Name 7	7	21	30	29 (14 - 50)	80 (63 - 90)	7.3	2533 (±2034)	618 (±194)	-1915
Salmon Lake	7	31	24	65 (47 - 79)	88 (69 - 95)	3.2	1632 (±1284)	3577 (±1744)	1945
T bar 2	7	31	29	23 (11 - 40)	41 (25 - 59)	2.6	295 (±189)	995 (±706)	700
Tinny	13	30	30	13 (5 - 30)	47 (30 - 64)	2.6	601 (±345)	1328 (±944)	727
Twin Ponds	7	30	40	0 (0 - 11)	12 (6 - 26)	1.7	0 (na)	1893 (±1885)	1893
Van Deren	13	30	30	20 (10 - 37)	29 (17 - 48)	0.7	2179 (±2051)	2021 (±1733)	-158
ZZ tank	10	30	29	9 (4 - 26)	34 (20 - 53)	2.5	222 (±75)	6666 (±6339)	6444
Total	-	565	550	25 (21 - 28)	43 (39 - 47)	--	--	--	--
mean	-	28.25	27.50	24	40	2.3	1320	4738	3599
SD	-	4.08	8.22	28	31	2.7	2812	9375	7063
SE	-	0.91	1.84	6	7	0.6	629	2096	1620

Table S4. Explanatory variables collected from individual ponds in 2009 and 2010. TP = total phosphorus, TN = total nitrogen

Tank	TP (µg-P L^{-1}) 2009	TP (µg-P L^{-1}) 2010	TN (µg-N L^{-1}) 2009	TN (µg-N L^{-1}) 2010	Capture rate (animals min^{-1}) 2009	Capture rate (animals min^{-1}) 2010
27 Mile	60.9	62.34	730	350	0.15	0.28
Alder	18.1	46.42	280	230	0.47	0.49
Aspen	58.4	53.06	720	580	0.40	0.50
Baker	75.9	162.30	490	510	0.15	0.22
Bar D	142.2	68.82	930	760	0.12	0.37
Brolliar Park	53.9	51.28	380	190	0.39	0.47
Brolliar Wet	68.6	127.52	810	610	0.20	0.34
Calloway	381.1	136.25	1150	1460	0.32	0.35
Clints	100.8	55.72	830	30	0.25	0.26
Mahan	143.0	129.44	890	130	0.15	0.27
McClure	62.4	52.36	740	400	0.33	0.34
Mud	203.3	82.44	630	340	0.08	0.15
No Name 13	31.9	56.16	350	20	0.13	0.19
No Name 7	60.7	74.42	350	160	0.20	0.32
Salmon	126.1	117.08	840	510	0.31	0.22
T bar 2	212.7	89.06	770	160	0.11	0.22
Tinny	72.3	38.72	360	20	0.34	0.40
Twin	104.7	56.08	860	260	0.31	0.41
Van Deren	22.5	31.14	360	440	0.40	0.35
ZZ	66.4	34.94	510	380	0.43	0.63
mean	103.29	76.28	650	380	0.26	0.34
SEM	18.9	8.5	60	70	0.03	0.03
min	18.10	31.14	280	20	0.08	0.15
max	381.07	162.30	1150	1460	0.47	0.63

Table S5. Fit of linear and quadratic responses of frog capture rates ($n \geq 30$ per pond) to concentrations of total phosphorus (TP) and total nitrogen (TN) from 20 ponds on Arizona's Mogollon Rim in 2009 and 2010. Quadratic responses were not fit to TN data due to their small range. Capture rate decreased significantly with increased TP indicating lower frog densities in high P ponds. No consistent trends were found for TN. Bold signifies statistically significant relationships ($\alpha = 0.05$).

Response variable	Explanatory variable	Relationship	N	R^2 (linear, quadratic)	p (linear, quadratic)
capture rate (2009)	**TP (2009)**	**-**	**20**	**0.17,** 0.17	**0.04,** 0.08
capture rate (2009)	TN (2009)	-	20	0.03, na	0.23, na
capture rate (2010)	**TP (2010)**	**-**	**20**	**0.27, 0.29**	**0.01, 0.02**
capture rate (2010)	TN (2010)	+	20	-0.04, na	0.58, na

Table S6. Fit of linear and quadratic responses of 2010 *Batrachochytrium dendrobatidis* (Bd) prevalence at the start of breeding (T_1), one week later (T_2), and loads (T_1 and T_2) to total phosphorus (TP), total nitrogen (TN), and host densities (capture rate) of adult chorus frogs ($n \geq 30$ per pond) collected during breeding from 20 ponds on Arizona's Mogollon Rim in 2009 and 2010. Bold signifies statistically significant relationships

Response variable	Explanatory variable	Relationship	N	Adjusted R^2 (linear, quadratic)[a]	p (linear, quadratic)
Arcsin Bd Prevalence$_{T1}$	TP (2009)	-	20	-0.05, -0.11	0.92, 0.92
Arcsin Bd Prevalence$_{T1}$	TN (2009)	+	20	0.001, NA	0.32, NA
Arcsin Bd Prevalence$_{T1}$	Capture rate (2009)	+	20	-0.02, NA	0.49, NA
Arcsin Bd Prevalence$_{T1}$	TP (2010)	-	20	-0.05, -0.05	0.79, 0.63
Arcsin Bd Prevalence$_{T1}$	TN (2010)	+	20	-0.008, NA	0.37, NA
Arcsin Bd Prevalence$_{T1}$	Capture rate (2010)	+	20	0.05, NA	0.18, NA
Arcsin Bd Prevalence$_{T2}$	TP (2009)	-	19	-0.05, -0.10	0.67, 0.84
Arcsin Bd Prevalence$_{T2}$	TN (2009)	+	19	-0.05, NA	0.74, NA
Arcsin Bd Prevalence$_{T2}$	Capture rate (2009)	+	19	0.05, NA	0.18, NA
Arcsin Bd Prevalence$_{T2}$	TP (2010)	-	19	-0.05, -0.07	0.80, 0.72
Arcsin Bd Prevalence$_{T2}$	TN (2010)	+	19	0.03, NA	0.23, NA
Arcsin Bd Prevalence$_{T2}$	Capture rate (2010)	+	19	0.08, NA	0.12, NA
log mean Bd load$_{T1}$	TP (2009)	-	20	-0.27[a]	0.26, na
log mean Bd load$_{T1}$	TN (2009)	-	20	-0.09[a]	0.71, na
log mean Bd load$_{T1}$	Capture rate (2009)	+	20	0.32[a]	0.16, na
log mean Bd load$_{T1}$	TP (2010)	-	20	-0.23[a]	0.34, na
log mean Bd load$_{T1}$	TN (2010)	+	20	0.23[a]	0.34, na
log mean Bd load$_{T1}$	Capture rate (2010)	+	20	0.32[a]	0.16, na
log mean Bd load$_{T2}$	TP (2009)	-	19	-0.22[a]	0.35, na
log mean Bd load$_{T2}$	TN (2009)	+	19	0.17[a]	0.47, na
log mean Bd load$_{T2}$	Capture rate (2009)	+	19	0.43[a]	0.06, na
log mean Bd load$_{T2}$	TP (2010)	-	19	-0.07[a]	0.76, na
log mean Bd load$_{T2}$	TN (2010)	+	19	0.37[a]	0.12, na
log mean Bd load$_{T2}$	**Capture rate (2010)**	**+**	**19**	**0.55**[a]	**0.02, na**

**One pond (NN13) was not sampled at T2 and therefore dropped from analyses that involved data from this time point*

[a]*Values represent non-parametric Spearman's Rho correlation coefficients, which were used in place of simple linear correlations to account for the non-normal distribution of mean Bd loads in 2010.*

Table S7. Sample size, date of collection, and prevalence of *Batrachochytrium dendrobatidis* (Bd) sampled from 2 life stages of boreal chorus frogs (*Pseudacris maculata*) from 15 ponds on Arizona's Mogollon Rim.

	Adults			Young-of-year		
Pond	Date sampled	n	Bd prevalence (± 95% CI)	Date sampled	n	Bd prevalence (± 95% CI)
27 Mile	6-Apr-10	30	57 (39-73)	15-Jun-10	30	10 (4-26)
Alder Lake	5-May-10	30	60 (42-75)	29-Jun-10	29	0 (0-12)
Aspen Lake	5-May-10	30	100 (89-100)	18-Jun-10	30	0 (0-11)
Bar D	24-Apr-10	30	63 (45-78)	28-Jun-10	31	0 (0-11)
Brolliar Park Tank	4-May-10	30	37 (22-55)	5-Jul-10	30	0 (0-11)
Brolliar Wet Land	4-May-10	30	53 (36-70)	5-Jul-10	33	6 (2-21)
Calloway Lake	8-Apr-10	30	70 (52-83)	15-Jun-10	30	3 (1-17)
Clints	14-Apr-10	30	10 (4-26)	28-Jun-10	30	0 (0-11)
Mahan Park	9-Apr-10	30	20 (10-37)	16-Jun-10	30	0 (0-11)
Mud Tank	23-Mar-10	30	0 (0-11)	17-Jun-10	28	0 (0-12)
NN13	15-Apr-10	25	0 (0-13)	15-Jun-10	29	0 (0-12)
Salmon	24-Mar-10	24	88 (69-95)	8-Jun-10	30	0 (0-11)
Tbar 2	25-Mar-10	29	41 (25-59)	8-Jun-10	30	0 (0-11)
Twin Ponds	10-Apr-10	40	12 (6-26)	16-Jun-10	30	0 (0-11)
ZZ tank	26-Apr-10	30	34 (19-51)	27-Jun-10	30	7 (2-21)

Table S8. Water temperatures and prevalence of *Batrachochytrium dendrobatidis* (Bd) in boreal chorus frogs (*Pseudacris maculata)* collected during breeding and development from a perennial (27 Mile) and ephemeral (Twin) pond in Arizona.

Pond	date	life stage	n	Bd prevalence (95% CI)	mean load (GE)	Cumulative days above 28 C	Cumulative days above 30 C
27 Mile	4-Mar-11	adult	31	55 (38-71)	44.3	0	0
27 Mile	9-Mar-11	adult	32	56 (39-72)	692.8	0	0
27 Mile	14-Mar-11	adult	33	21 (11-38)	26.1	0	0
27 Mile	19-Mar-11	adult	30	23 (12-41)	1224.0	0	0
27 Mile	24-Mar-11	adult	20	90 (70-97)	2318.0	0	0
27 Mile	29-Mar-11	adult	31	100 (89-100)	8636.0	0	0
27 Mile	3-Apr-11	adult	31	100 (89-100)	14135.0	0	0
27 Mile	12-Apr-11	adult	18	100 (83-100)	19427.0	0	0
27 Mile	17-Apr-11	larvae	150	0 (0-2)	0.0	0	0
27 Mile	5-May-11	larvae	150	7 (4-12)	1554.0	1	0
27 Mile	1-Jun-11	larvae	150	53 (45-61)	2506.0	4	0
27 Mile	1-Jun-11	juvenile	32	13 (5-28)	13.0	4	0
27 Mile	24-Jun-11	juvenile	31	39 (24-56)	512.0	12	4
27 Mile	19-Jul-11	juvenile	11	64 (34-85)	162.0	na	na
Twin	10-Mar-11	adult	30	3 (1-17)	6.9	0	0
Twin	15-Mar-11	adult	29	10 (4-27)	30.1	0	0
Twin	19-Mar-11	adult	5	0 (0-46)	0.0	0	0
Twin	25-Mar-11	adult	7	42 (16-76)	134.7	0	0
Twin	29-Mar-11	adult	31	87 (71-95)	1960.0	0	0
Twin	3-Apr-11	adult	31	100 (89-100)	2256.0	0	0
Twin	17-Apr-11	adult	18	100 (82-100)	11740.0	0	0
Twin	23-Apr-11	larvae	150	0 (0-2)	0.0	0	0
Twin	5-May-11	larvae	150	0 (0-2)	0.0	0	0
Twin	1-Jun-11	larvae	150	0 (0-2)	0.0	7	2
Twin	1-Jun-11	juvenile	30	0 (0-9)	0.0	7	2
Twin	24-Jun-11	juvenile	31	0 (0-9)	0.0	26	17

APPENDIX B

SUPPLEMENTARY FIGURES

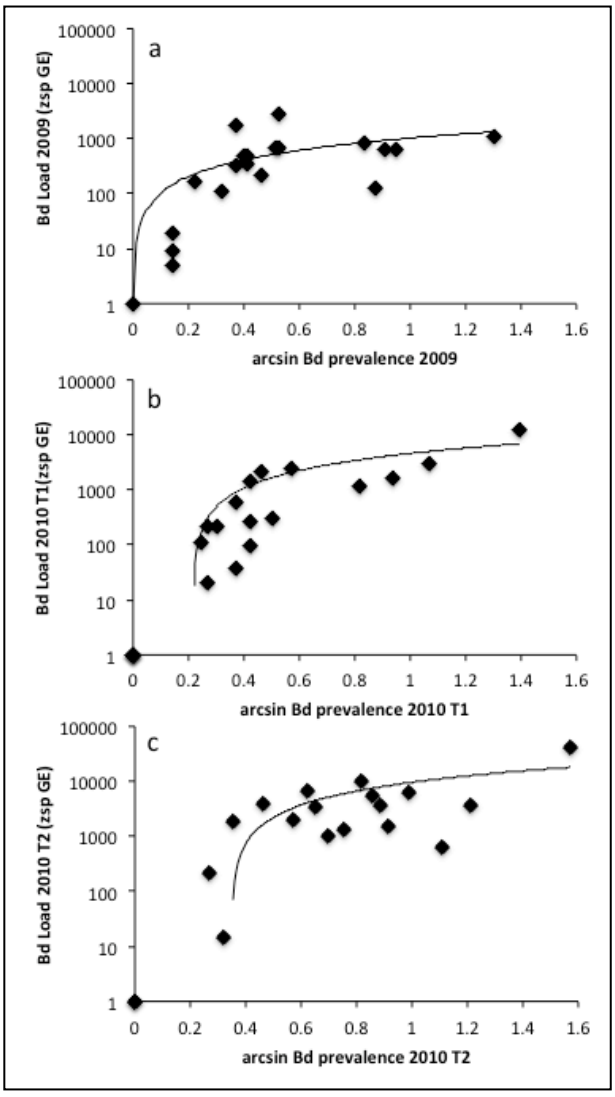

Figure S1. Average *Batrachochytrium dendrobatidis* (Bd) load was significantly positively correlated with Bd prevalence within ponds in (a) 2009 ($ANOVA_{df=19}$, $r^2 = 0.71$, $p < 0.0001$), as well as (b) T1 ($ANOVA_{df=19}$, $r^2 = 0.87$, $p < 0.0001$) and (c) T2 ($ANOVA_{df=18}$, $r^2 = 0.74$, $p < 0.0001$) in 2010