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High juvenile mortality in amphibians during overwintering related to fungal pathogen exposure

Samantha L. Rumschlag*, Michelle D. Boone

Department of Biology, Miami University, Oxford, OH 45056, USA

ABSTRACT: The result of pathogen exposures may depend upon trade-offs in energetic demands for immune responses against host growth and survival. Environmental conditions may influence these trade-offs by affecting host size, or trade-offs may change across seasons, altering impacts of pathogens. We exposed northern leopard frog *Lithobates pipiens* tadpoles to different larval environments (low leaf litter, high density of conspecifics, atrazine, caged fish, or controls) that influenced size at metamorphosis. Subsequently, we exposed metamorphs to *Batrachochytrium dendrobatidis* (*Bd*), a fungal pathogen, just after metamorphosis and/or prior to overwintering 12 wk later. *Bd* exposure dramatically reduced survival during overwintering, with the strongest effects when hosts were exposed at both time points. Larval environments resulted in differences in host size. Those exposed to caged fish were 2.5 times larger than the smallest (those exposed to high density of conspecifics), but larval environment did not influence *Bd* effects on growth and survival. The largest frogs exposed to caged fish had greater survival through overwintering, but in the absence of *Bd*. We built stage-structured models to evaluate if overwinter mortality from *Bd* is capable of having effects on host populations. Our models suggest that *Bd* exposure after metamorphosis or before overwintering can reduce population growth rates. Our study demonstrates that hosts suffer little effects of *Bd* exposures following metamorphosis and that small body size did not hamper growth and survival. Instead, we provide evidence that winter mortality from *Bd* exposure is capable of reducing population sizes, providing a plausible mechanism for amphibian declines in temperate regions.

KEY WORDS: Host–pathogen interactions · Disease ecology · Wildlife diseases · Seasonality · *Batrachochytrium dendrobatidis*

INTRODUCTION

Emerging infectious diseases represent a major threat to public health, global economies, and wildlife populations (Binder 1999, Daszak et al. 2000, Morens et al. 2004). Thus, to protect populations at risk to disease outbreaks, understanding factors that determine the likelihood of host population declines is key. Environmental factors can simultaneously influence hosts and pathogens; the net effect of these forces determines the likelihood of host population declines. For instance, fluctuations in environmental conditions (including temperature and resource availability) can result in predictable patterns of disease-driven mortality of hosts (Altizer et al. 2006). For a pathogen, environmental factors can influence survival and reproductive rates, which are key components of pathogen virulence and persistence in the host population (Voyles et al. 2012). Environmental factors can determine a host's ability to limit infection and/or damage caused by a pathogen (Pulkkinen & Ebert 2004). For example, when conditions are physiologically stressful, little energy may be devoted to pathogen defenses relative to basal metabolic activities such as host growth and survival (Blaustein et al. 2012). Common environmental factors, which induce these trade-offs, may contribute to detrimental effects of pathogens on hosts that are otherwise not

*Corresponding author: rumschsl@gmail.com

impacted by certain pathogens. Environmental factors might particularly influence the ability of the juvenile host life stages to respond to pathogens because pathogen defenses, including immune responses, have not been fully developed in juveniles when compared to adults (Koop et al. 2013).

Batrachochytrium dendrobatidis (hereafter *Bd*) is a fungal pathogen that causes the disease chytridiomycosis, which some suggest is the greatest infectious disease threat to vertebrates in modern time (Murray et al. 2011). The impacts of this pathogen may be amplified by environmental conditions that influence host quality and pathogen virulence. *Bd* can infect hundreds of amphibian species and is present on all continents where amphibians exist (Olson et al. 2013). Exposure to *Bd* can result in decreased host growth (Caseltine et al. 2016) and death (Searle et al. 2011, Wise et al. 2014), and is linked with worldwide population declines of amphibians (Berger et al. 1998, Muths et al. 2003, Lips et al. 2006). Effort has been focused on understanding the disease ecology of chytridiomycosis in regions where mortality events have been sudden and widespread, such as Central and South America and Australia. Comparatively little is known about the influence of *Bd* on amphibian populations in temperate climates in North America, such as the Midwest and eastern USA (Talley et al. 2015). While there are no clear examples of *Bd*-related declines in these regions, there also has been less attention given to the disease ecology of *Bd* here even though pathogens could still have important impacts on populations and communities. In particular, there is a gap in our knowledge about the effects of *Bd* on individuals across life stages and seasons, which limits our understanding of host–pathogen interactions in this system.

Host body size, which is determined in part by larval environment in the current system, could be an important predictor of pathogen exposure outcomes during the terrestrial juvenile period through winter. In temperate climates, larger hosts may be better suited to sustain overwintering and pathogenic exposures because immune responses and overwinter survival are energetically costly processes. Body size can predict fitness and host condition in many vertebrates (Dobson 1992, Bachman & Widemo 1999, Shine et al. 2001). For instance, larger body size in amphibians at metamorphosis is associated with increased overwinter survival and with earlier time to first reproduction and increased fecundity (Semlitsch & Wilbur 1988, Berven 1990, Schmidt et al. 2012, Earl & Whiteman 2015). Larger individuals are likely better adapted to sustain the negative effects of pathogen exposures through a superior ability to mount energetically costly immune responses. Further, the probability of being infected with *Bd* can decrease with increasing body size (Murray et al. 2013), which suggests that larger individuals might better defend against pathogens.

The objective of the present research was to determine how the larval environment impacts the effects of *Bd* exposure just after metamorphosis and prior to overwintering on northern leopard frog *Lithobates pipiens* juveniles in the first year of life. Northern leopard frogs may be a reservoir species (Woodhams et al. 2008, Gahl et al. 2012), allowing for this species to spread the pathogen to more sensitive species while suffering little to no ill effects themselves. In contrast, northern leopard frogs could be sensitive to *Bd* during enigmatic periods of the life cycles, such as during overwintering, a time in which amphibians could suffer increased risks of mortality to *Bd*. However, impacts during overwintering have never been considered, leaving a gap in our understanding of how temperate species are impacted by pathogens. Northern leopard frogs are commonly infected in natural populations (Ouellet et al. 2005, Woodhams et al. 2008, Rodriguez et al. 2009, Voordouw et al. 2010) and have been shown to suffer decreases in growth in short-term experiments (Caseltine et al. 2016).

To determine how larval environment influences the effects of *Bd* exposure on long-term host growth and survival, we altered the quality of the larval environment by manipulating the amount of leaf litter, the density of conspecifics, the presence of 40 µg l^{-1} of the herbicide atrazine, and the presence of caged fish. These conditions were designed to influence amphibian body size, which affects overwinter survival (Berven 1990, Earl & Whiteman 2015) and could mediate the impacts of *Bd* on growth and survival through overwintering. As leaf litter is a nutrient source for the primary food resource of tadpoles — algae (Peacor & Pfister 2006) — we expected low leaf litter and high density of conspecifics to result in smaller size at metamorphosis because of increased competition and decreased per capita resource availability (Semlitsch 1987, Peacor & Pfister 2006, Purrenhage & Boone 2009). We expected atrazine to result in small size because atrazine is toxic to algae at 40 µg l^{-1} (Schafer et al. 1993, 1994) and can influence the growth of amphibians through effects on thyroid hormones (Rohr & McCoy 2010). We expected exposure to caged fish to result in small size because of reduced foraging and/or induction of a

hormonal stress response (Benard 2004). Alternatively, presence of fish could result in larger frogs because of decreases in tadpole energy expenditure and increased nutrient turnover, driving increases in algae (Werner & Peacore 2003, Benard 2004). We hypothesized that (1) suboptimal conditions in the larval environment, which impact juvenile sizes of amphibian hosts, increase the likelihood of negative effects of *Bd* exposure on juveniles and (2) *Bd* exposure and timing of exposure alter overwinter survival of juvenile amphibians.

MATERIALS AND METHODS

Animal collection and care

Eight partial northern leopard frog *Lithobates pipiens* egg masses were collected on 31 March 2013 from Talawanda High School Pond (39° 29′ 16″ N, 84° 43′ 42″ W) in Oxford, OH, USA. Egg masses were held in the laboratory at room temperature (22–23°C) until 2 April 2013, when they were moved to a climate-controlled room where they were held at 18.3°C on a 14 h:10 h light:dark cycle until they reached free-swimming stage (Gosner stage 25; Gosner 1960). Tadpoles were fed ground TetraMin tropical fish flakes (Tetra Holding) ad libitum until they were transferred on 16 April 2013 (experimental Day 0) to outdoor mesocosms at Miami University's Ecology Research Center (Oxford, OH, USA). To test how larval environment, which generates different sizes classes of hosts, influenced the effects of *Bd* exposure, we manipulated the larval environment (control, leaf litter, density of conspecifics, atrazine exposure, caged fish) in experimental mesocosms (183 cm in diameter, 70 cm deep). Each randomly assigned treatment was replicated 5 times for a total of 25 mesocosms. Treatments were randomly assigned to mesocosms. Each mesocosm contained 1000 l of water, was inoculated with zooplankton and algae from a local pond, and was covered with a screen lid. Mesocosms in the low leaf litter treatment contained 300 g leaf litter from a mixed deciduous forest, while mesocosms associated with all other treatments contained 1 kg leaf litter. These amounts of leaf litter represent low and high amounts found in nature (Wallace et al. 1999, Finzi et al. 2001, Mitchell et al. 2001, Colon-Gaud et al. 2008). In addition, these amounts result in differences in amphibian size at metamorphosis because they drive differences in available algal resources (Rubbo et al. 2008, Semlitsch & Boone 2009, Boone & Sullivan 2012). Ponds with a high density of conspecifics contained 90 northern leopard frog tadpoles, while ponds associated with all other treatments contained 30 northern leopard frog tadpoles. Amphibian larval densities vary widely in natural populations; the selected densities used in the present study fall within the observed range (e.g. 14 to 4238 tadpoles per 1000 l; Morin 1983, Petranka 1989). The atrazine treatments consisted of a single exposure to 40 µg l^{-1} of the herbicide atrazine (42.2% atrazine; Drexel Chemical Company); ponds assigned to all other treatments were not exposed to atrazine. This treatment simulated a single pulse of atrazine into a pond as a result of seasonal pre-emergent application. To reach treatment exposure of atrazine, we dissolved 0.948 g atrazine in 1000 ml of water and added 100 ml of the solution evenly over the surface of the pond on 23 April 2013 (experimental Day 7). This atrazine treatment falls within the range of expected environmental concentrations and thus represents a realistic exposure (Fairchild et al. 1998). To confirm the initial atrazine concentration, we collected a composite water sample from all 5 mesocosms exposed to atrazine 24 h after application and sent it to the Mississippi State Chemical Laboratory (Mississippi State, MS, USA). Analyses resulted in a measured concentration of 53 µg l^{-1}. For caged-fish treatments, on 15 April 2013 we collected 30 bluegill sunfish (15.7 ± 1.26 cm [mean ± SD]) from Acton Lake (College Corner, OH, USA) via electroshocking. On 23 April 2013 (experimental Day 7), we added 2 fish per mesocosm to floating cages made from plastic baskets (64.77 × 45.42 × 25.7 cm) with plastic floats that provided cover for the fish; ponds assigned to all other treatments contained floating cages and cover floats without fish as a sham control. The density of fish used in the present study falls within the range of densities observed in natural populations (Bettoli et al. 1993). Fish within experimental cages were fed 5 to 10 live northern leopard frog tadpoles weekly. Additional fish were held in 2 holding mesocosms (1000 l) with water and opaque foam floats that provided cover. In holding mesocosms, fish were fed earthworms ad libitum. Fish from holding mesocosms were rotated into and out of floating cages in experimental ponds weekly. Northern leopard frogs from 2 mesocosm ponds in the caged fish treatment developed infections of parasitic copepods (possibly *Lernea cyrinacea*), so individuals metamorphosing from these ponds were excluded from the experiment. Tadpoles were reared in mesocosms through forelimb emergence (Gosner stage 42), at which point they were transferred to the laboratory. Meta-

morphosis was recorded once the tail was fully absorbed (Gosner stage 46; experimental Days 59 to 77). A subsample of northern leopard frogs within each treatment was used in the terrestrial phase of the study.

The terrestrial phase of the experiment began after metamorphosis and ended before overwintering, lasting for 12 wk (until experimental Day 164). During this portion of the experiment, we housed northern leopard frogs individually within terraria that consisted of 2 l beakers containing layers of pea gravel (~2.5 cm) and topsoil (~4 cm) and a small water dish with a fiberglass screen attached to the tops of beakers. Treatments in the terrestrial phase of the experiment were assigned randomly to beakers. Northern leopard frogs were held at 23°C on a 14 h:10 h light:dark cycle in controlled environment chambers. Metamorphs were fed increasing amounts of crickets dusted in calcium powder that varied between two 0.635 cm and four 1.27 cm crickets 3 times per week. After 12 wk of these conditions in the terrestrial phase, we initiated terrestrial overwintering conditions according to James (2003), beginning on 11 September 2013 (experimental Day 148). While northern leopard frogs can overwinter aquatically, they have also been observed to overwinter terrestrially (Logier 1952, Pinder et al. 1992, Tattersall & Ultsch 2008), and experimental studies of terrestrially overwintering northern leopard frogs have observed high rates of survival (Parris 2001, Distel & Boone 2010). In the laboratory, we gradually decreased the amount of crickets provided to the northern leopard frogs for 3 feeding days until no crickets were provided. Feeding was discontinued before temperatures were dropped to allow for gut clearance and to avoid the possibility of intestinal infection during overwintering. Beginning on 14 September 2013 (experimental Day 151), we gradually acclimated the northern leopard frogs to 17°C by drawing down the temperature by 1°C d^{-1} until experimental Day 156; then we held frogs at 17°C until experimental Day 163. On experimental Day 163 at 17°C, northern leopard frogs were exposed to *Bd* before overwintering (see below). To initiate hibernation and burrowing behavior after exposure to *Bd*, northern leopard frogs were transferred to 2 l beakers with 10 cm topsoil, a layer of leaf litter, and a fiberglass screen attached to the tops of beakers, and moved to an environmental chamber set to 7°C on experimental Day 164. Amphibians generally tolerate this drop in temperature well (James 2003); we saw no mortality with this drop in temperature. Beakers were haphazardly arranged on shelves for the remainder of the experiment. Northern leopard frogs were held until experimental Day 166 at 7°C, then temperature was reduced to 6°C for 3 d before it was reduced to 5°C on a 10 h:14 h light:dark cycle for the remainder of the experiment (25 March 2014, experimental Day 343). Each week, we sprayed containers with dechlorinated water to maintain soil moisture during overwintering. While our intent was to mimic overwintering in the terrestrial environment, we did not replicate non-linearities of natural decreasing winter temperatures.

Experimental design and *Bd* exposures

At metamorphosis, we assigned northern leopard frogs from different larval environments (low leaf litter, high density of conspecifics, 40 µg l^{-1} atrazine, caged fish, or control; see above) to *Bd* exposure 'just after' metamorphosis (*Bd* present, *Bd* absent) and to *Bd* exposure 'before overwintering' (*Bd* present, *Bd* absent) (Fig. 1). To summarize, we had 5 larval environment × 2 *Bd* exposures just after metamorphosis × 2 *Bd* exposures before overwintering, which resulted in a total of 20 treatments. As a result, our experimental design included hosts that were not exposed to *Bd*, hosts that were only exposed to *Bd* just after metamorphosis, hosts that were only exposed before

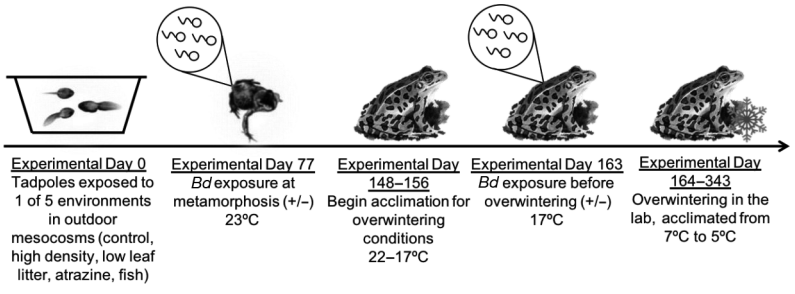

Fig. 1. Experimental timeline of events. Northern leopard frog *Lithobates pipiens* tadpoles were reared in mesocosms in which they were exposed to different larval environments in groups (control, high density of conspecifics, low leaf litter, 40 µg l^{-1} atrazine, caged bluegill). Exposure to *Bd* (*Bd* present or *Bd* absent) occurred just after metamorphosis. Frogs were reared for 12 wk in the terrestrial environment before they began acclimation to overwintering conditions. They were then exposed to *Bd* just before overwintering (*Bd* present or absent), and then frogs were overwintered in the laboratory until the end of the experiment. Image of tadpoles and metamorph by Jane Kim of Ink Dwell. Image of northern leopard frog by Gina Mikel. Images used with permission

overwintering, and hosts that were exposed both just after metamorphosis and before overwintering. The experimental unit was the individual frog. Treatments were replicated 7 to 12 times for a total of 192 experimental units across all 20 treatments. *Bd* treatments were assigned randomly to individual frogs within individual treatments.

After metamorphosis on 2 July 2013 (experimental Day 77) and before overwintering on 27 September 2013 (experimental Day 164), we exposed individual frogs to *Bd* (*Bd* present or *Bd* absent) for 12 h. Because timing of metamorphosis varied with larval treatment, frogs exposed to *Bd* just after metamorphosis were exposed at up to 18 d since tail absorption, and frogs exposed to *Bd* before overwintering were exposed between 87 and 105 d after metamorphosis. Preliminary analyses revealed that these differences in age at time of exposure did not influence the impacts of *Bd* exposures measured in the present study. Temperate amphibians are likely exposed to *Bd* around metamorphosis and before overwintering in the first year of life because they frequently use waterbodies as habitat. As frogs metamorphose from a waterbody, indirect *Bd* exposure can occur via contact with water, soil, and vegetation (Johnson & Speare 2003, Rachowicz et al. 2005, Kolby et al. 2015). Contact with *Bd* in the environment is likely again in the late fall as frogs often return to waterbodies to overwinter in the surrounding upland habitat (Pilliod et al. 2002). In addition, densities of amphibians are high in waterbodies during spring metamorphosis and late fall migrations, which could result in increased contact rates and direct *Bd* exposure between infected and non-infected hosts (Rowley & Alford 2007). Spring and fall represent times during which midwestern species are commonly infected with *Bd* (Talbott et al. 2018).

To expose frogs to *Bd*, we placed individuals in ventilated plastic Petri dishes (100 mm diameter) with 7 ml dechlorinated water and 1 ml of the assigned treatment solution (see below). After 12 h, individuals were returned to their assigned terrarium. We cultured *Bd* (isolate JEL 213 isolated from *Rana muscosa* in the Sierra Nevada Mountains, USA, obtained from J. Longcore, University of Maine, Orono, ME, USA) on 1% tryptone agar plates using standard protocols (Longcore et al. 1999); in a study with American toads evaluating the effect of *Bd* isolate, this isolate had similar effects compared to a local Ohio isolate (Burrow et al. 2017). *Bd* zoospores were harvested using 5 ml dechlorinated water. For *Bd*-absent treatments, we added dechlorinated water to 1% tryptone agar plates without *Bd* cultures. After 30 min, we collected the water from the plates into 2 solutions: one containing *Bd* zoospores and one that was free of *Bd* zoospores. We calculated the concentrations of *Bd* zoospores using a hemocytometer. The *Bd*-present treatment solution that frogs were exposed to after metamorphosis contained 5.2×10^6 zoospores ml^{-1}. The *Bd*-present treatment solution that frogs were exposed to before overwintering contained 2.1×10^6 zoospores ml^{-1}. These concentrations fall within the range of concentrations used in other studies (Rumschlag et al. 2014, Wise et al. 2014, Caseltine et al. 2016). At the end of the experiment, frogs were euthanized using a 1% solution of MS-222 (tricaine methanesulfonate) buffered with sodium bicarbonate.

Statistical analyses

In the larval portion of the present study, we determined mass at metamorphosis, time to metamorphosis, and survival to metamorphosis. Mesocosm was the experimental unit. We tested for the effect of the larval environment on the proportion of northern leopard frogs surviving to metamorphosis within each mesocosm using an ANOVA. We logit transformed survival to metamorphosis. We tested for the effect of larval treatment on mesocosm means of mass at metamorphosis and time to metamorphosis of individuals used in the terrestrial portion of the experiment with a multivariate ANOVA. Pond means of mass at metamorphosis and time to metamorphosis were log-transformed to meet assumptions of normality.

In the terrestrial portion of the present study, we measured survival daily, and individual frogs were weighed weekly to measure growth. After completion of the overwintering portion of the study, we measured survival at the conclusion of the experiment. For the terrestrial and overwintering portions of the study, the individual was the experimental unit because frogs were housed individually. We tested for the effects of larval environment, exposure to *Bd* just after metamorphosis, and the interaction of these treatments on the survival of individuals in the terrestrial environment prior to overwintering using a logistic regression. We used a repeated-measures ANOVA to determine the effects of larval environment, exposure to *Bd* just after metamorphosis, and the interaction of these treatments on mass of leopard frogs throughout the course of the terrestrial phase of the experiment (from 1 wk post-*Bd* exposure just after metamorphosis through just before overwinter-

ing) using log-transformed mass of individuals as the repeated measure. In addition, to analyze the effects of larval environment, *Bd* exposure just after metamorphosis, and the interaction of these treatments on terrestrial growth of northern leopard frogs, we used an ANOVA on change in terrestrial mass (final terrestrial mass before overwintering − mass at 1 wk post-*Bd* exposure just after metamorphosis). We examined the influence of larval environment, *Bd* exposure just after metamorphosis, *Bd* exposure before overwintering, and the interaction between *Bd* exposure just after metamorphosis and before overwintering on overwinter survival of individuals using a logistic regression. Northern leopard frogs that died prior to exposure of *Bd* before overwintering were excluded from analyses of overwinter survival. Non-significant interaction terms, except the 2-way interaction between *Bd* exposures just after metamorphosis and before overwintering, were removed from this model to conserve degrees of freedom. In preliminary analyses, we included the 2- and 3-way interactions of larval environment, *Bd* exposure just after metamorphosis, and *Bd* exposure before overwintering, and in this preliminary model none of the treatments or their interactions were significant. Because of low survival through the overwintering phase, we were unable to test for the effect of treatments on amphibian size through overwintering. Pairwise differences for larval survival, mass at metamorphosis and time to metamorphosis were evaluated for ANOVAs using Scheffe's multiple comparison tests. To test for the effect of host body size and *Bd* exposures just after metamorphosis and prior to overwintering, accounting for the non-independence of larval treatment, we used a generalized linear mixed model with a binomial error distribution (lme4 package, glmer function in R). Overwinter survival was the response and the predictors were log-transformed mass before overwintering, *Bd* exposure just after metamorphosis, *Bd* exposure before overwintering, the 2-way interactions between mass and *Bd* exposures, and larval treatment was the random intercept term. To visualize the effects of host body on overwinter survival, we dropped the non-significant interaction terms in the model and used a conditional plot (visreg package in R) that shows the effect of host body size on overwinter survival controlling for *Bd* exposures and larval treatment. Analyses were completed using SAS 9.2 (SAS Institute) and R 3.4.1. ANOVAs were constructed using generalized linear models (PROC GLM in SAS) with Gaussian distributions, and results were evaluated using Type III error with $\alpha = 0.05$. Logistic regressions (PROC LOGISTIC in SAS 9.4) were built with a binary distribution and a logit link function, and results were evaluated using Type III analysis of effects with $\alpha = 0.05$.

Stage-structured population model

To evaluate if overwinter mortality observed in the experiment is capable of affecting population growth, we built stage-based Lefkovitch (Caswell 2000) annual projection matrices using 3 stages representing female northern leopard frog populations with a birth pulse (Biek et al. 2002) under 3 conditions: no exposure to *Bd*, exposure of recently metamorphosed frogs to *Bd*, and exposure of metamorphs to *Bd* before overwintering. Our models consisted of 3 life-history stages: pre-juvenile (embryo, larva, and overwintering metamorph), juvenile, and reproductive adult. The projection matrix representing a population that has not been exposed to *Bd* is:

$$\begin{bmatrix} 0 & \begin{bmatrix}\text{probability of juvenile}\\ \text{becoming adult} \times\\ \text{probability of laying} \times\\ \text{clutch size}\end{bmatrix} & \begin{bmatrix}\text{adult survival} \times\\ \text{probability of}\\ \text{laying} \times\\ \text{clutch size}\end{bmatrix} \\ \begin{bmatrix}\text{embryo survival} \times\\ \text{larval survival} \times\\ \text{metamorph survival}\end{bmatrix} & \begin{bmatrix}\text{probability of}\\ \text{remaining a}\\ \text{juvenile}\end{bmatrix} & 0 \\ 0 & \begin{bmatrix}\text{probability of juvenile}\\ \text{becoming adult}\end{bmatrix} & [\text{adult survival}] \end{bmatrix}$$

To model the effects of *Bd* exposure, we reduced the overwintering metamorph survival rate based on the experimental data from the present study. To represent the effects of *Bd* exposure just after metamorphosis, we reduced overwintering metamorph survival by 42%; likewise, to represent the effects of *Bd* exposure prior to overwintering, we reduced overwintering metamorph survival by 77%.

Mean vital rates used in elements of the matrices were drawn from scientific literature (Table 1). When possible, mean vital rates were specific to northern leopard frogs. When vital rates for northern leopard frogs were not available, we used vital rates of congeneric species. Using vital rates of congeners is a common practice in demographic modeling when the vital rates of a focal species for a specific stage are unknown (Biek et al. 2002, Kesler & Haig 2007, Wanless et al. 2009, Lawson et al. 2010). The mean embryo and larval survival rates are based on field survey data for wood frogs *Rana sylvatica* that showed that survival from eggs to metamorphosis ranges from 0 to 5% (Berven 1990). The product of the mean embryo survival and the mean larval survival is the midpoint of this range, 2.5%. The mean

overwintering metamorph survival and standard deviation used in the model is based on survival rates of overwintering northern leopard frog metamorphs in terrestrial enclosures where groups of 10 recently metamorphosed northern leopard frogs were overwintered in 2 × 2 m terrestrial enclosures with pits filled with leaf litter to allow for frogs to burrow below the frost line (Distel & Boone 2010). The mean juvenile survival rate is based on the estimates for mean juvenile survival of northern red-legged frogs *Rana aurora* (Biek et al. 2002).

The formula used for the mean probability of a juvenile remaining a juvenile is $p_1 = [(1 - p_i^{d_i-1}) \times p_i]/(1 - p_i^{d_i})$, where p_i is the annual probability of survival for a juvenile in year i and d_i is the number of years spent as a juvenile (Crouse et al. 1987). We estimated female northern leopard frogs reaching sexual maturity in 2 to 3 yr (Force 1933, Vogt 1981). The mean probability of a juvenile remaining a juvenile is estimated from the midpoint using the above formula and the mean juvenile survival rate and 2 or 3 yr to sexual maturity. The formula used for the mean probability of a juvenile becoming an adult is $p_2 = [p_i^{d_i} \times (1 - p_i)]/(1 - p_i^{d_i})$ (Crouse et al. 1987). The mean probability of a juvenile becoming an adult is estimated from the midpoint using the formula above and the juvenile survival rate with 2 or 3 yr to sexual maturity.

The mean adult survival rate and standard deviation used in the models are based on measurements of annual female wood frog survival in a natural population (Berven 2009). A probability of laying a clutch of 1.00 and a standard deviation of 0 was assumed based on a similar assumption by Biek et al. (2002). The mean clutch size is based on the midpoint between the range of reported values of clutch sizes for northern leopard frogs, 645 to 7648 (Hupf 1977, Corn & Livo 1989, Watermolen 1995). Our model represents only female northern leopard frogs, so within the matrices, clutch size is divided by 2 assuming a 1:1 sex ratio.

Standard deviations for mean vital rates were taken from Biek et al. (2002) with the exception of metamorph survival and adult survival as described previously (Table 1); standard deviation for clutch size for northern leopard frogs was based on the standard deviation for boreal toads *Anaxyrus boreas*, a species with a similar mean clutch size, and standard deviation for all other vital rates were based on a closely related species, the northern red-legged frog.

To determine the influence of reduced overwintering metamorph survival, we calculated λ (the finite rate of increase of population growth) at a stable age distribution for 2000 replicate matrices, which were generated by randomly selecting clutch sizes from a log-normal distribution and all other vital rates from β-distributions that were constructed with 2000 observations using means and standard deviations in Table 1 based on Biek et al. (2002).

Additionally, to assess the influence of vital rates on λ on our 3 annual projection matrices, we used sensitivity analysis to quantify how relatively small changes in each vital rate would affect λ when the other vital rates are held constant, and elasticity analysis to quantify how proportional changes in each vital rate would affect λ when all other vital rates are held constant (De Kroon et al. 2000). Sensitivity and elasticity analyses are based on mean vital rates (Table 1). We compared sensitivity and elasticity analyses across our 3 projection matrices to determine if *Bd* exposure of juveniles influences which stage of a population is more vulnerable to small fluctuations in its vital rates. Modeling exercises were completed in R version 3.2.1 with code adapted from Stevens (2010).

Table 1. Vital rates and transition probabilities that make up the stage-structured Lefkovitch projection matrices representing females of a northern leopard frog *Lithobates pipiens* population. Vital rates and matrix elements are for females only and are annual with the exception of embryo, larval, and metamorph survival, which together represent transitions within the first year of life

Vital rate	Mean	SD
Embryo survival	0.700 (Berven 1990)	0.049 (Biek et al. 2002)
Larval survival	0.036 (Berven 1990)	0.012 (Biek et al. 2002)
Metamorph survival	0.410 (Distel & Boone 2010)	0.050 (Distel & Boone 2010)
Juvenile survival[a]	0.335 (Biek et al. 2002)	0.093 (Biek et al. 2002)
Juvenile to juvenile[b]	0.280 (Crouse et al. 1987)	0.036 (Biek et al. 2002)
Juvenile to adult[c]	0.055 (Crouse et al. 1987)	0.056 (Biek et al. 2002)
Adult survival	0.178 (Berven 2009)	0.026 (Berven 2009)
Probability of laying	1 (Biek et al. 2002)	–
Clutch size	4146.50 (Hupf 1977, Corn & Livo 1989, Watermolen 1995)	856.00 (Biek et al. 2002)
Age at sexual maturity (yr)	2 to 3 (Force 1933, Vogt 1981)	–

[a]Juvenile survival is the sum of the probability of a juvenile remaining a juvenile and the probability of a juvenile becoming an adult
[b]Probability of a juvenile remaining a juvenile
[c]Probability of a juvenile becoming an adult

Table 2. Summary of analyses on survival to metamorphosis (ANOVA) and metamorphic response (MANOVA) of northern leopard frogs *Lithobates pipiens* in response to exposure to varying larval environments (control, high density of conspecifics, low leaf litter, 40 µg l^{-1} atrazine, caged bluegill)

Response	Source of variation	df	*F*	p
Survival to metamorphosis	Larval environments	4	5.05	0.0056
	Error	20		
Metamorphic response	Larval environments	8, 38	15.41	<0.0001
Time to metamorphosis	Larval environments	4	16.99	<0.0001
	Error	20		
Mass at metamorphosis	Larval environments	4	32.40	<0.0001
	Error	20		

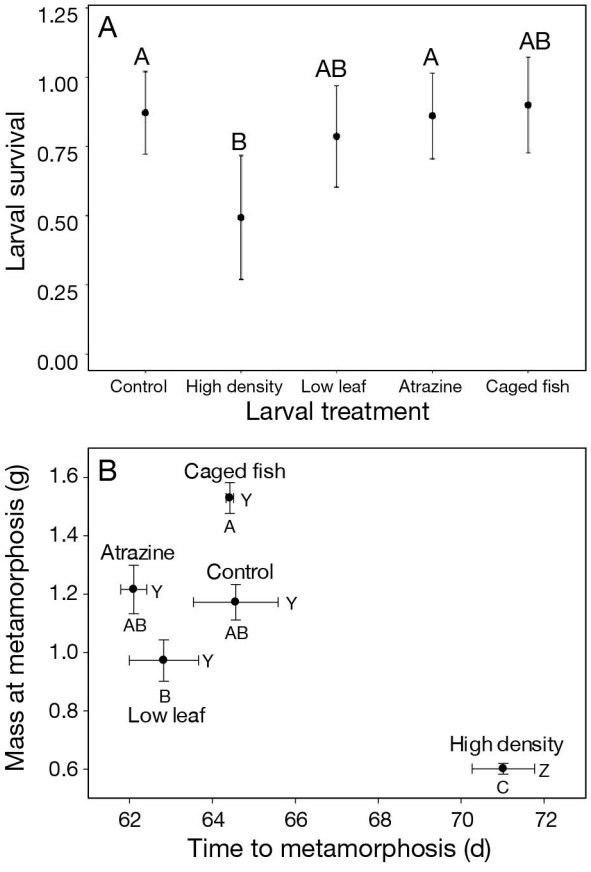

Fig. 2. Effects of larval environment on metamorphic endpoints. Shared letters indicate no significant difference according to Scheffe's multiple comparisons test. (A) Survival of northern leopard frog *Lithobates pipiens* larvae exposed to varying larval environments (control, high density of conspecifics, low leaf litter, 40 µg l^{-1} atrazine, caged bluegill). Plotted values are means ± 1 binomial SE. (B) Time to metamorphosis and mass at metamorphosis for northern leopard frog larvae exposed to varying larval environments. Different letters indicate differences among treatments for time to metamorphosis (YZ) or differences among treatments for mass at metamorphosis (ABC). Plotted values are means ± 1 SE.

RESULTS

Larval treatments impacted survival to metamorphosis, time to metamorphosis, and size at metamorphosis, which set the stage for testing how host condition would influence the impact of *Bd* on the terrestrial life stage (Table 2, Fig. 2). High density and low leaf litter reduced survival to metamorphosis compared to exposure to 40 µg l^{-1} atrazine, caged fish, or the control (Fig. 2A). Small to large size classes were generated by larval environmental conditions. Tadpoles exposed to caged fish, atrazine, or control conditions reached the largest mass at metamorphosis, while exposure to the low leaf litter or high density of conspecifics led to smaller sizes (Fig. 2B). A high density of conspecifics increased the time to metamorphosis relative to the control, caged fish, atrazine, and low leaf litter treatments (Fig. 2B).

Hypothesis 1: suboptimal larval environment increases the likelihood of negative effects of *Bd* exposure

Larval environment did not influence terrestrial survival (Table 3) but did impact terrestrial growth (Table 4). Initial size differences at metamorphosis, corresponding with larval environment, were maintained through the terrestrial period (Fig. 3). However, individuals exposed to different larval conditions were not differentially impacted by *Bd* exposure in the terrestrial or overwintering periods. There were no significant effects of the interaction of *Bd* exposures and larval environment on survival (Table 3) or growth (Table 4).

Hypothesis 2: *Bd* exposure and timing of exposure alters overwinter survival of juvenile amphibians

Timing of *Bd* exposure had a strong effect on the likelihood of surviving the winter (Table 3). Northern leopard frogs never exposed to *Bd* had a 73% probability of surviving the winter, while those exposed to *Bd* just after metamorphosis had a 42% reduction in survival. Individuals exposed immediately prior to overwintering, however, had a 77% reduced probability of surviving the winter com-

Table 3. Summary of logistic regressions of northern leopard frog *Lithobates pipiens* (1) terrestrial survival in response to varying larval environments (control, high density of conspecifics, low leaf litter, 40 µg l^{-1} atrazine, caged bluegill), *Bd* exposures just after metamorphosis (*Bd* present, *Bd* absent), and the interaction of these treatments; (2) overwinter survival in response to varying larval environments, *Bd* exposures just after metamorphosis, and *Bd* exposures just before overwintering (*Bd* present, *Bd* absent); and (3) overwinter survival in response to mass before overwintering, *Bd* exposures just after metamorphosis, *Bd* exposures before overwintering, and the 2-way interactions between *Bd* exposures and mass with larval environment as a random effect

Response	Source of variation	df	Wald χ^2	p
Terrestrial survival	Larval environments	4	1.2743	0.8657
	Bd just after metamorphosis	1	0.0000	0.9999
	Larval environments × *Bd* just after metamorphosis	4	0.8802	0.9274
Overwinter survival	Larval environments	4	10.2253	0.0368
	Bd just after metamorphosis	1	7.2942	0.0069
	Bd before overwintering	1	27.9044	<0.0001
	Bd just after metamorphosis × *Bd* before overwintering	1	0.0138	0.9064
Overwinter survival	Mass before overwintering	1	4.7041	0.0301
	Bd just after metamorphosis	1	4.9615	0.0259
	Bd before overwintering	1	29.3240	<0.0001
	Mass before overwintering × *Bd* just after metamorphosis	1	1.6392	0.2004
	Mass before overwintering × *Bd* before overwintering	1	2.2228	0.1360

Table 4. Summary of repeated-measures ANOVA on juvenile mass through time and ANOVA of change in mass of northern leopard frogs *Lithobates pipiens* over the terrestrial portion of the experiment in response to exposure to varying larval environments (control, high density of conspecifics, low leaf litter, 40 µg l^{-1} atrazine, caged bluegill), to *Bd* just after metamorphosis (*Bd* present, *Bd* absent), and the interactions of these treatments. Additionally, summary of ANOVA on change in mass of northern leopard frogs over the overwintering portion of the experiment in response to exposure to varying larval environments, to *Bd* just after metamorphosis, and the interactions of these treatments

Response	Source of variation	df	*F*	p
Terrestrial growth				
Between subjects	Larval environments	4	58.76	<0.0001
	Bd just after metamorphosis	1	0.03	0.8699
	Larval environments × *Bd* just after metamorphosis	4	1.00	0.4062
	Error	190		
Within subjects	Time	11	6004.76	<0.0001
	Time × Larval environments	44	24.50	<0.0001
	Time × *Bd* just after metamorphosis	11	1.09	0.3660
	Time × Larval environments × *Bd* just after metamorphosis	44	0.58	0.9882
	Error	2090		
Change in mass	Larval environments	4	1.51	0.2007
	Bd just after metamorphosis	1	1.52	0.2197
	Larval environments × *Bd* just after metamorphosis	4	0.60	0.6649
	Error	191		

pared to controls (Fig. 4A). Likewise, larval environments also influenced overwinter survival, with individuals raised with caged fish, which were the largest at metamorphosis, having the highest survival probability (Table 3, Fig. 4B). While host size was a positive predictor of overwinter survival accounting for larval environment, frogs with a smaller body size were not less likely to survive the winter when they were exposed to *Bd* at either time point (e.g. no significant *Bd* by body mass interactions; Table 3, Fig. 4C).

Population model

Exposure of metamorphs to *Bd* (modeled as decreases in overwintering metamorph survival) had differential effects on the finite rate of increase of population growth, λ. In the model that represented a population that has not been exposed to *Bd*, mean λ was 1.14, which predicts a growing population. When recently metamorphosed frogs were exposed to *Bd*, which decreased their overwinter survival and probability of transitioning to the juvenile life stage

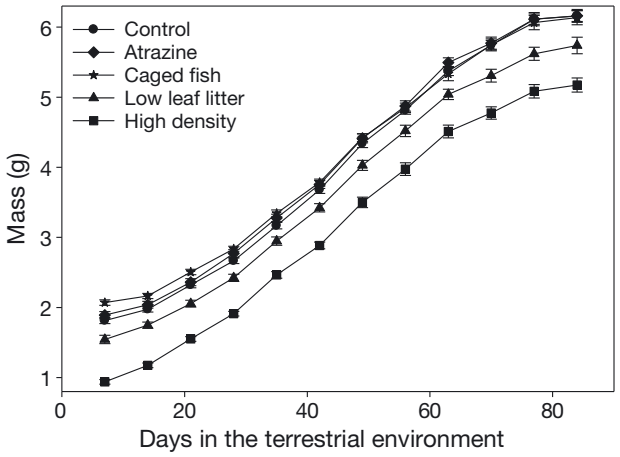

Fig. 3. Mass of northern leopard frog *Lithobates pipiens* metamorphs over time in the terrestrial phase according to varying larval environments (control, high density of conspecifics, low leaf litter, 40 µg l^{-1} atrazine, caged bluegill). Plotted values are means ± 1 SE

by 42%, mean λ decreased by 19% (Fig. 5), which predicts a declining population. When metamorphic frogs were exposed to *Bd* just before overwintering, which decreased their overwinter survival and probability of transitioning to the juvenile stage by 77%, mean λ decreased by 41% relative to the model with no *Bd* exposure (Fig. 5).

Sensitivity analyses across the 3 annual projection matrices — representing populations of northern leopard frogs with no exposure to *Bd*, exposure of recently metamorphosed frogs to *Bd*, and exposure of metamorphs to *Bd* before overwintering — showed that λ was most sensitive to changes in survival from the pre-juvenile (embryo, larva, and overwintering metamorph) to juvenile stage relative to other matrix elements (Table 5). Small changes in transition probability of pre-juveniles to juveniles caused the biggest changes in λ. Similarly, elasticity analyses within each of these 3 projection matrices showed that λ was most elastic to changes in survival from the pre-juvenile to juvenile stage relative to other matrix elements (Table 5). Small proportional changes in this transition relative to all other matrix elements had the greatest impact on λ.

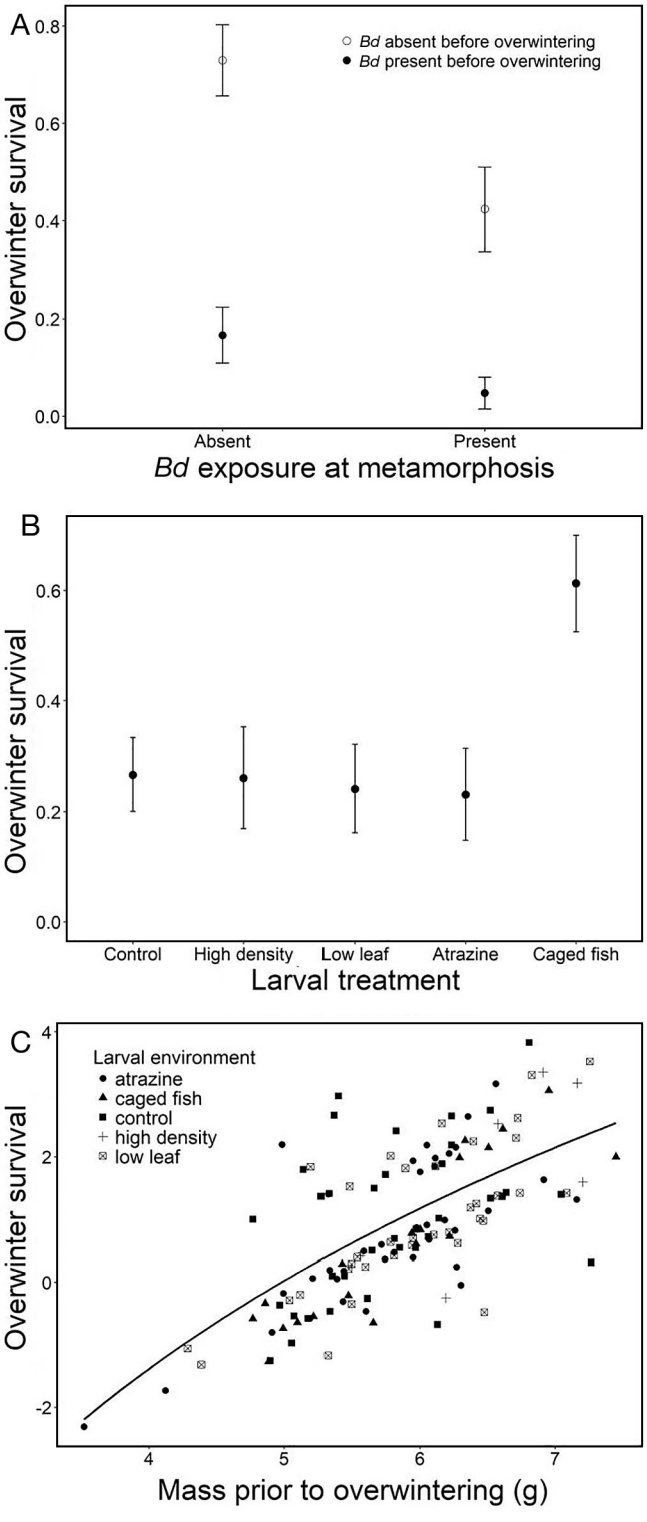

Fig. 4. (A) Overwinter survival of northern leopard frog *Lithobates pipiens* metamorphs exposed to *Bd* at different time points, just after metamorphosis (*Bd* present, *Bd* absent) and before overwintering (*Bd* present, *Bd* absent). Plotted values are means ± 1 binomial SE. (B) Overwinter survival of metamorphs exposed to varying larval environments (control, high density of conspecifics, low leaf litter, 40 µg l^{-1} atrazine, caged bluegill). Plotted values in A and B are means ± 1 binomial SE. (C) Conditional plot showing the effect of host body mass before overwintering on overwinter survival, controlling for the effects of *Bd* exposures after metamorphosis and before overwintering with larval environment as a random effect. The reference categories in the model shown are the control treatment for the larval environment and *Bd*-absent treatments for *Bd* exposures

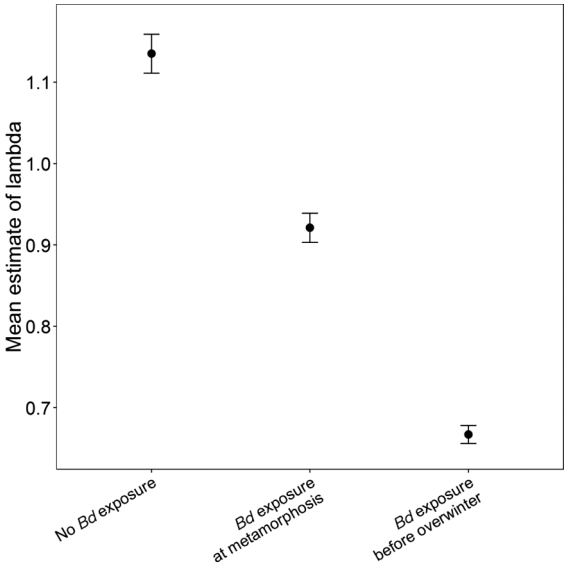

Fig. 5. Mean λ (the finite rate of increase of population growth) values with 95% confidence intervals representing the impact of *Bd* exposures on overwinter northern leopard frog *Lithobates pipiens* metamorph survival to the juvenile stage

DISCUSSION

Determining how the environment influences virulence of pathogens in early development of hosts remains a largely unanswered question in disease ecology (Lively et al. 2014). Variation in seasonal weather patterns and resource availability in early development represent 2 universal forces that drive population dynamics (e.g. Fretwell 1972); yet, in temperate climates we lack an understanding of how host–pathogen interactions in wildlife populations respond to these changes over a given year. We set out to examine how larval environment influenced host vulnerability to pathogen exposures. Despite large differences in metamorph size (an indirect measure of fitness), we did not find that individuals were differentially affected by pathogen exposure. However, overwinter survival was disproportionally impacted by pathogen treatment. Our study demonstrated how pathogen exposure can appear to have 'no effect' for a period of time (12 wk) and then result in dramatically high mortality during winter. Our models of population growth rates suggest the hypothesis that mortality during winter could result in population-level declines. Although *Bd* has been found to cause local extinction of hosts (e.g. Skerratt et al. 2007), local extirpation during winter has not been directly observed and could offer an explanation for enigmatic declines of temperate species. A similar pattern of increased pathogen-related mortality has been observed in bats with white-nose syndrome (Langwig et al. 2015).

Table 5. Sensitivity and elasticity values for projection matrices representing no *Bd* exposure, *Bd* exposure of recently metamorphosed northern leopard frogs *Lithobates pipiens*, and *Bd* exposure of metamorphs before overwintering. The first number listed is the sensitivity value, and the second is the elasticity value

	Pre-juvenile	Juvenile	Adult
No *Bd* exposure			
Pre-juvenile	–	0.004, 0.356	0.000, 0.056
Juvenile	52.479, 0.412	0.523, 0.111	–
Adult	–	1.333, 0.056	0.064, 0.009
***Bd* exposure just after metamorphosis**			
Pre-juvenile	–	0.003, 0.325	0.000, 0.066
Juvenile	68.795, 0.390	0.531, 0.140	–
Adult	–	1.263, 0.066	0.079, 0.013
***Bd* exposure of metamorphs before overwintering**			
Pre-juvenile	–	0.002, 0.263	0.000, 0.082
Juvenile	109.261, 0.344	0.549, 0.205	–
Adult	–	1.114, 0.082	0.107, 0.025

1: suboptimal larval environments do not increase negative effects of *Bd* exposure

Exposure to stressors during early life stages can have lasting impacts on health, increasing susceptibility to infectious pathogens during later life stages (Rohr et al. 2013). We predicted that exposure to suboptimal larval environments could increase the effects of pathogen exposure on host growth (e.g. Caseltine et al. 2016) and survival (e.g. Burrow et al. 2017), but we found no support for this effect in this species. Manipulating the larval environment led to hosts of different size classes, and these size classes were sustained over the course of the experiment. However, even though previous studies have shown that small size is linked to reduced ability to respond to pathogens (Dobson 1992, Bachman and Widemo 1999, Shine et al. 2001, Burrow et al. 2017), hosts of smaller sizes did not suffer greater effects of *Bd* exposure in terms of growth and survival compared to hosts of larger sizes. Even using a range of environmental factors that could influence the impacts of a pathogen regardless of size (e.g. the

herbicide atrazine; Rohr et al. 2013), the conditions of the larval environment did not differentially alter the effect of *Bd* exposure. Our results suggest that the ability of hosts to respond effectively to pathogen exposures as measured by their growth and survival is not strongly impacted by larval environment in this system. These results are consistent with other studies that show that the pesticides atrazine, carbaryl, and glyphosate do not increase the impacts of *Bd* exposure on frog survival or infection with *Bd* (Davidson et al. 2007, Gahl et al. 2011, Buck et al. 2012, Paetow et al. 2012).

2: *Bd* exposure alters overwinter survival of juvenile amphibians

The impacts of pathogens on hosts vary throughout a given year (Altizer et al. 2006). Because of decreases in resources and changes in weather, winter is a time that coincides with increased impacts of pathogen across classes of hosts including humans (Dowell 2001). Our results show decreased survival of pathogen-exposed hosts during overwintering. This finding is noteworthy because it is the first to demonstrate that a temperate species, which has previously been considered a pathogen reservoir (Woodhams et al. 2008, Gahl et al. 2012) and which has appeared resistant to infection and the effects of *Bd* exposure (Gahl et al. 2012, Paetow et al. 2012, Ortiz-Santaliestra et al. 2013), can suffer significant mortality during winter.

Currently, we lack an understanding of the impacts of *Bd* on the population dynamics for a majority of host species. To address this gap for northern leopard frogs, we evaluated if increased mortality during winter from *Bd* exposure could result in population-level impacts using a theoretical stage-structured population model, a widespread tool in conservation ecology. These models provide a quantitative link between experimentally observed reductions in vital rates and population dynamics of northern leopard frogs. They allow us to evaluate if increased mortality during winter is capable of having population-level consequences, which is important because equivalent changes in vital rates across life stages can have differential impacts on overall population growth (Caswell 2000). Our models provide support that decreased population growth rates could result from mortality during winter, a time when observations of host mortality would be difficult.

In this system, low survival during winter as a result of *Bd* exposure might go unnoticed in natural host populations because amphibians are difficult to track during the non-breeding season and reduced recruitment could be linked to a number of other plausible factors including predation or variation in climate. Although *Bd* mass mortality events have not been documented in the eastern and midwestern USA, populations of amphibian hosts including northern leopard frogs are commonly infected with *Bd* (Daszak et al. 2005, Ouellet et al. 2005, Longcore et al. 2007, Woodhams et al. 2008, Rodriguez et al. 2009, Voordouw et al. 2010), and many midwestern amphibians are experiencing unexplained population declines (Lannoo 2005). For instance, studies have documented northern leopard frog population declines throughout some of the northern portion of its range (Hecnar & M'Closkey 1996, Rorabaugh 2005, Voordouw et al. 2010). Because of the absence of widespread mortality events in temperate zones, less attention has been given to understanding the disease ecology of *Bd* in these regions compared to those in which mortality events have been sudden and widespread, such as Central and South America and Australia. To strengthen our hypothesis concerning the link between winter mortality of *Bd*-exposed hosts and temperate population declines, we call for additional empirical testing and population monitoring to determine the effects of winter on seasonal changes in infection prevalence and load, host social behavior, contact rates among hosts, host birth and death rates, and changes in host immune function and pathogen physiology.

Proposed mechanisms of winter mortality

The present study lacked data on infection prevalence and load, so careful consideration must be given in proposing the mechanisms of observed *Bd* mortality. We propose the following possible hypotheses, which are not mutually exclusive, to describe the observed patterns. (1) *Bd* exposure does not result in infection but triggers a physiologically costly immune response via host resistance. The combined physiological costs associated with immune responses and hibernation result in mortality. (2) Host tolerance to *Bd* decreases through winter. The host's ability to limit *Bd* infection and growth via an immune response is reduced because of the physiological costs of hibernation during winter, resulting in mortality. (3) The cooler temperatures that coincide with the onset of winter favor the pathogen's growth, infection, and persistence, increasing the virulence of the pathogen and driving observed pat-

terns in mortality. These hypotheses remain to be tested.

In the first 2 hypotheses, mortality might be a result of the combined physiological costs of immune responses and hibernation of amphibians during winter. In temperate regions, low temperatures during winter necessitate hibernation in amphibians, which initiates a series of energetically costly physiological processes that are associated with decreased immune responses (Maniero & Carey 1997, Carey et al. 1999, Rollins-Smith & Woodhams 2011) that could increase the impact of *Bd* on amphibians. Northern leopard frogs can defend against *Bd* infections by sloughing epidermal layers where *Bd* infection occurs (Paetow et al. 2012) and by producing antimicrobial skin peptides that inhibit *Bd* (Pask et al. 2012). These responses may be reduced or inhibited during overwintering because of reductions in temperature.

Our results also suggest that timing of *Bd* exposure and number of exposures dramatically alter mortality risk during winter. For temperate hosts, the time just before overwintering might be a critical vulnerable window for exposure to pathogens because of changes in host immune defenses, resulting in increased mortality when hosts were exposed just before overwintering compared to when they were exposed just after metamorphosis. Exposure to *Bd* multiple times, which resulted in the lowest rates of survival, may compound the physiological costs of either resisting or tolerating infection, resulting in increased mortality during winter when frogs were exposed twice compared to once.

Our third hypothesis suggests that the cooler temperatures that coincide with the onset of winter may favor *Bd* growth, infection, and environmental persistence. Across climatic zones, *Bd* infections in amphibians commonly increase during months associated with decreases in temperatures relative to the annual mean (Ouellet et al. 2005, Woodhams & Alford 2005, Bosch et al. 2007), which may be caused by the thermal performance of *Bd* and its ability to persist at low temperatures (4–14°C). In culture, across a range of temperatures from 13 to 28°C, initial release of zoospores, zoospore longevity, and maximum zoospore production is greatest at lower temperatures (13–15°C; Stevenson et al. 2013). The balance between host immunity and pathogen virulence may shift with decreases in temperature caused, at least in part, by the ability of *Bd* to thrive at low temperatures (Rollins-Smith et al. 2011), which may coincide with the onset of winter temperatures in the Midwest.

CONCLUSION

In conclusion, the present study shows that various larval environments that result in different host sizes do not alter the impacts of pathogen exposure on host growth and survival during the first year of life in this system. Instead, our results indicate that hosts exposed to pathogens could suffer mortality during winter, which our population model predicts could have formative effects on population growth and persistence by reducing recruitment of breeding adults. The population model in the present study is best used to generate the hypothesis that *Bd*-related mortality during winter is capable of influencing population growth rates, rather than generating estimates of specific population-level declines, which are likely dependent on local factors. Because there is little emphasis on the study of overwintering on amphibian–pathogen interactions, we may be underestimating the impact that *Bd* has on temperate species. For instance, winter-associated reduced resources and temperatures may alter host–pathogen interactions in ways that do not occur in tropical climates. There may be differences in disease dynamics inherent to climatic region that could shape amphibian populations in the eastern and midwestern USA, regions generally not believed to be influenced by *Bd*-related declines. Our interpretations of the mechanisms driving these patterns in mortality are limited because we lack data on infection prevalence and load throughout the seasons. Which mechanisms drive the observed effects is an important topic that could be elucidated in future studies. Our results point to the need to evaluate the influence of overwintering on natural host–pathogen interactions in temperate climates, a topic that is understudied in the field of infectious disease. For hosts across taxa that overwinter in covert locations, including amphibians, arthropods, reptiles, and small mammals, the impacts of pathogen exposures on host responses likely change with dramatic reductions in temperature and food availability but are difficult to discern from other causes of reduced spring recruitment such as predation and climate effects (Altizer et al. 2006). Examining the influence of pathogens across the life cycle of a host that encompasses a broad range of environmental conditions experienced in the wild is critical if we are to understand how seasonality influences natural populations in this climatic region.

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