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Out of the Frying Pan, Into the Fire? Yosemite Toad (*Anaxyrus canorus*) Susceptibility to *Batrachochytrium dendrobatidis* After Development Under Drying Conditions

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***Abstract.*—The Yosemite Toad (*Anaxyrus canorus*) has experienced declines in distribution and abundance in recent decades. The declines in this species have been anecdotally attributed to infectious disease and increased frequency and intensity of drought, but neither of these factors have been formally tested. We investigated the combined effects of reduced water availability and susceptibility to the disease chytridiomycosis across *A. canorus* life stages. Specifically, we reared *A. canorus* tadpoles under drying conditions and exposed metamorphosed toadlets to *Batrachochytrium dendrobatidis* (*Bd*), the pathogenic fungus that causes the lethal disease chytridiomycosis. We examined (1) the time to and size at metamorphosis of *A. canorus* tadpoles reared under drying conditions, (2) the susceptibility of post-metamorphic *A. canorus* to *Bd*, and (3) the synergistic effects of drying conditions and disease on post-metamorphic toadlet survival. We found that recently metamorphosed *A. canorus* toadlets are highly susceptible to lethal *Bd* infection. Although we did not detect an effect of reduced water availability on disease risk, we suggest follow-up experiments in both the laboratory and the field to better understand the direct and indirect roles that drought and disease play in *A. canorus* population declines.**

Key Words.—amphibian declines; disease ecology; drought; hydroperiod; Sierra Nevada

Introduction

Amphibian species and populations are declining worldwide at an alarming rate (Stuart et al. 2004). While there are many factors contributing to declines (e.g., habitat loss and overexploitation), infectious diseases pose major threats to amphibians (Daszak et al. 2003; Stuart et al. 2004). One of the most notable amphibian diseases is chytridiomycosis, which is caused by the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*; Berger et al. 1998; Longcore et al. 1999; Pessier et al. 1999). Chytridiomycosis has been implicated in global declines of over 200 amphibian species and is considered one of the greatest disease threats to biodiversity (Skerratt et al. 2007; Wake and Vredenburg 2008).

Amphibian mortality from disease may be compounded by a variety of environmental factors, including those associated with a changing climate (Alford et al. 2007; Rohr and Raffel 2010; Hof et al. 2011; Raffel et al. 2013; Cohen et al. 2018). Climate change models predict increasing temperatures and increased frequency and intensity of drought in some regions (Pachauri et al. 2014), with potential implications for amphibian species that depend on water for breeding and larval development (Carey and Alexander 2003; Ryan et al. 2014; Miller et al. 2018). Increases in warmer and drier conditions are expected to shorten the duration of seasonally available water (i.e., hydroperiod) in some wetland habitats (Hamlet et al. 2007; Lee et al. 2015), which can affect amphibian breeding, development, physiology, behavior, and fecundity (Carey and Alexander 2003; Parmesan 2006; Blaustein et al. 2010; Li et al. 2013; Walls et al. 2013), and in turn amphibian fitness and survival (Semlitsch and Wilbur 1988; Berven 1990; McMenamin et al. 2008). Further, warmer and drier conditions could exacerbate disease-related declines by affecting host immune function, physiology, behavior, and life-history traits in ways that increase host susceptibility (Alford et al. 2007; Rohr and Raffel 2010; Rohr et al. 2011; Altizer et al. 2013; Cohen et al. 2018).

Chytridiomycosis and reduced hydroperiod threats may differentially affect amphibians across life stages (Kohli et al. 2019). Due to differences in the pathophysiology of chytridiomycosis, post-metamorphic amphibians are at greater risk of mortality from *Bd* infection compared to the tadpole life stage (Bosch et al. 2001; Rachowicz and Vredenburg 2004; Rachowicz et al. 2006). In post-metamorphic amphibians, *Bd* infects the epidermis and can lead to electrolyte imbalance and lethal cardiac arrest (Voyles et al. 2009). Recently metamorphosed individuals may be particularly vulnerable to disease (Bakar et al. 2016) due to a substantial reconstruction of the immune system and a brief period of immunosuppression during metamorphic climax (Rollins-Smith et al. 2011). For larval amphibians, *Bd* infection is restricted to keratinized mouthparts (Fellers et al. 2001; Rachowicz

and Vredenburg 2004; Marantelli et al. 2004; Knapp and Morgan 2006). While mouthpart infection may decrease feeding and therefore growth in tadpoles (Parris and Cornelius 2004; Garner et al. 2009), mortality from *Bd* pathogenesis at the larval stage has not been widely observed (Bosch et al. 2001; Rachowicz and Vredenburg 2004; Garner et al. 2009). Conversely, tadpoles are highly vulnerable to mortality associated with rapidly drying habitats (Newman 1992; McMenamin et al. 2008; Walls et al. 2013). The threat of desiccation may be amplified for larval amphibians in warming and drying regions associated with a changing climate (McMenamin et al. 2008; Ryan et al. 2014; Kissel et al. 2018). As such, a reduced hydroperiod may directly or indirectly threaten amphibians during the larval stage, while chytridiomycosis is more likely to cause mortality in post-metamorphic amphibians.

The combined threats of drought and disease across life stages may interact to put amphibians at a greater risk of mortality than would be expected with one of these threats alone. For larvae that metamorphose before their habitat evaporates, development in a rapidly drying water body may have long-term impacts that predispose individuals to lethal disease outcomes (Gervasi and Foufopoulos 2008). Some species exhibit developmental plasticity and can accelerate metamorphosis to escape a drying habitat (Alford and Harris 1988; Newman 1992; Denver 2009), but accelerated metamorphosis is frequently associated with the costs of smaller body sizes and decreased immune function that reduce fitness later in life (Rollins-Smith 1998; Altwegg and Reyer 2003; Gervasi and Foufopoulos 2008; Garner et al. 2009; Crespi and Warne 2013). Drought-induced premature metamorphosis may thereby increase susceptibility to pathogens due to incomplete immune development, increased relative surface area for infection, altered sloughing rates, and differences in skin microbiome diversity (Rollins-Smith et al. 1988; Rollins-Smith 1998; Chammas et al. 2015; Bates et al. 2018; Wu et al. 2018). Therefore, the combined threats of reduced hydroperiod and chytridiomycosis may increase the likelihood of mortality and possibly exacerbate population declines.

Chytridiomycosis and reduced hydroperiod may be contributing to declines in distribution and abundance of the Yosemite Toad (*Anaxyrus canorus*), a federally listed threatened species that has experienced enigmatic declines over the past five decades (Sherman and Morton 1993; Green and Sherman 2001; U.S. Fish and Wildlife [USFWS] 2014; Brown et al. 2015). Field surveys conducted from 2004 through 2012 found metamorph, subadult, and adult *A. canorus* infected with *Bd* across the species range (Celeste Dodge, pers. comm.), and infection has been detected in *A. canorus* tadpoles at select sites (Fellers et al. 2011). In addition, historical sampling from museum specimens suggests that *A. canorus* adults were infected with *Bd* during the first witnessed decline of the species in the 1970s (Green and Sherman 2001; Celeste Dodge, pers. comm.). Not all amphibian species develop lethal chytridiomycosis (Daszak et al. 2004; Kriger and Hero 2007), however, and the susceptibility of this species has not yet been formally tested. Thus, empirical evidence of chytridiomycosis threats on *A. canorus* survival is currently lacking.

Drought is also regarded as a potential mechanism of *A. canorus* declines (Sherman and Morton 1993; Brown et al. 2015). Warmer and drier periods have been shown to reduce the hydroperiod of the shallow water bodies used by *A. canorus* for breeding and development, resulting in *A. canorus* egg and tadpole mortality from desiccation (Cunningham 1963; Sherman and Morton 1993; Brown et al. 2012). With climate projections across the species range for decreases in annual snowpack, earlier snow melt-out dates, and increased frequency and intensity of drought (Hamlet et al. 2007; Point Blue Conservation Science 2011; Godsey et al. 2014), investigators predict increases in *A. canorus* larval mortality rates from desiccation (Viers et al. 2013; Brown et al. 2015). While the direct effects of drought on larval mortality have been anecdotally observed (Cunningham 1963; Sherman and Morton 1993; Brown et al. 2012), the indirect effect of larval development under drought conditions on disease susceptibility post-metamorphosis remains unknown. Studying the interaction between chytridiomycosis and climate change induced shifts in water availability in *A. canorus* may help inform conservation efforts for this species.

To assess the interaction between reduced hydroperiod and *Bd* infection across life stages, we collected *A. canorus* eggs, reared tadpoles under drying conditions, and infected metamorphosed toads with *Bd*. We hypothesized that larval development under a reduced hydroperiod treatment would increase susceptibility to *Bd* infection and chytridiomycosis following metamorphosis. We predicted that (1) tadpoles reared under a shortened hydroperiod would metamorphose more rapidly and have smaller body sizes as compared to animals reared in tanks with stable water levels; (2) toadlets infected with *Bd* would have higher mortality rates than control toadlets not exposed to the pathogen, and mortality would be associated with an increase in *Bd* infection intensity and clinical signs and symptoms of chytridiomycosis; and (3) toadlets reared under a shortened hydroperiod would have higher disease-induced mortality rates than toadlets reared under stable water levels. Understanding the combined effects of reduced hydroperiod and chytridiomycosis on amphibian survival may improve understanding of drivers of decline and thus help to inform threat mitigation strategies and recovery efforts for this species.

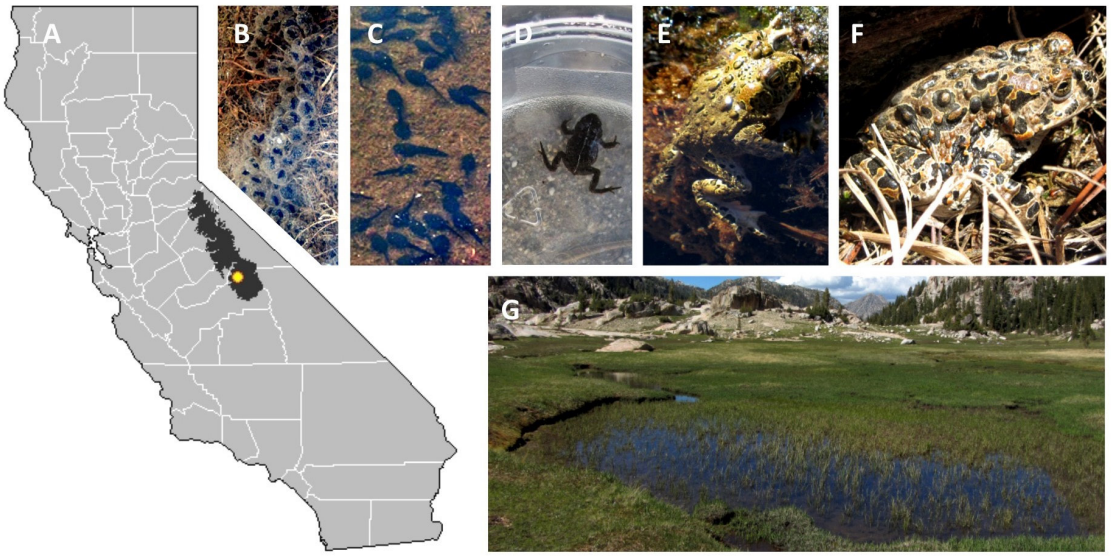

Figure 1. (A) Yosemite Toad (*Anaxyrus canorus*) range in the central Sierra Nevada (black polygon, adapted from Brown et al. 2012). We collected eggs from the southern range of the species (A, yellow dot). (B) *Anaxyrus canorus* lay eggs in ephemeral and intermittent water bodies where (C) tadpoles then develop over the course of a summer. (D) Metamorphosed toadlets take 2–4 y to reach sexual maturity (E, adult male; F, adult female). (G) An ephemeral water body used by *A. canorus* in Yosemite National Park, California, USA. Maps were generated using R packages ggmap and rgdal. (Photographed by Alexa Lindauer).

Materials and Methods

Species description.—*Anaxyrus canorus* are found exclusively in the Sierra Nevada of central California, USA, between 1,950–3,500 m (Karlstrom 1962). Adults and tadpoles are commonly found in high elevation meadows where snowmelt runoff and groundwater recharge create intermittent water bodies essential for breeding and tadpole development (Karlstrom 1962; Sherman and Morton 1993; Wang 2012). The species overwinters in rodent burrows or other subterranean hibernacula, and adults emerge at snowmelt to breed in flooded meadows, ephemeral pools, or shallow ponds (Mullally 1953; Karlstrom 1962; Fig. 1). Females lay strands of 1,500–2000 eggs which take 40–60 d to complete metamorphosis (Mullally 1953; Karlstrom 1962; Fig. 1).

Egg collection.—We collected 200 live *A. canorus* eggs on 21 June 2017 from Kaiser Meadow in the Sierra National Forest, Fresno County, California (Fig. 1A, yellow dot), a site with a large, stable, yearly breeding population with a history of long-term monitoring (Stephanie Barnes, pers. comm.). Eggs are an ideal stage to collect because they are the least likely to carry *Bd* and their removal is least likely to impact population dynamics given the high mortality of eggs and larvae in the wild (Sherman 1980; Sherman and Morton 1993; Biek et al. 2002; Scherff-Norris et al. 2002). We collected 40 eggs from five distinct clutches of unknown parentage and transported the eggs to the University of Nevada, Reno, USA, following U.S. National Park Service protocols for the transportation of threatened and endangered amphibian species (Rob Grasso, pers. comm.).

Husbandry.—Amphibians commonly have high mortality rates during hatching and metamorphosis in both natural and controlled settings (Biek et al. 2002; Scherff-Norris et al. 2002); however, our rearing and husbandry practices were largely successful. Of the 200 eggs collected, we had 75% survival from egg collection to hatching and 79% survival from hatching to metamorphosis. After hatching, we randomly assigned tadpoles from different clutches to tanks. During the early tadpole stages preceding initiation of drying treatments, we housed animals at low densities (approximately 1 tadpole per L) in disinfected polycarbonate tanks (Cambro, 53 cm length × 33 cm width × 20 cm height) filled to a depth of 14 cm with aged tap water on constant aeration. While *A. canorus* tadpoles select water body depths between 4 – 5 cm (Liang et al. 2017; Karen Pope et al., unpubl. report), a 14 cm water depth is within the range of pool depths used by *A. canorus* tadpoles in the wild (Karen Pope et al., unpubl. report; Steven Lee, pers. comm.). We selected this water depth to reduce cannibalism and improve water quality by decreasing tadpole densities (Scherff-Norris et al. 2002). We placed tanks under UVB lights on a 12:12 L/D cycle and maintained room temperature at 21–22° C, a temperature within the *Bd* thermal optimum (Piotrowski et al. 2004; Voyles et al. 2012; Stevenson et al. 2013; Voyles et al. 2017) and within the range of *A. canorus* pool temperatures (Roche

et al. 2012; Maier 2018; Karen Pope et al., unpubl. report). We fed tadpoles a complex diet of amphibian gel (Mazuri, Land O' Lakes, Inc., St. Louis, Missouri, USA), ground spirulina and shrimp flakes, Sera Micron Growth Food (sera, Heinsburg, Germany), and dark leafy greens (excluding spinach) *ad libitum* (Scherff-Norris et al. 2002; Jessie Bushell, pers. comm.). Once tadpoles reached metamorphosis, we placed perches in tanks to allow metamorphosing toadlets to climb out of the water.

After metamorphosis, we housed toadlets separately in individual containers (17.3 cm length × 12.2 cm width × 5.6 cm height) to track individuals and to help control *Bd* zoospore concentration after exposure (Carey et al. 2006). We fed juvenile toads fruit flies coated in vitamin D3 and calcium (Rep-Cal Research Labs; Los Gatos, California, USA). We provided aquatic and moist terrestrial habitats in each enclosure to mimic *A. canorus* habitat needs in the wild. Before beginning the susceptibility trial, we allowed toadlets to acclimate to their new containers for a minimum of 9 d. After the initiation of the susceptibility trial, we replaced aged tap water with Holtfretter's artificial pond water (20% Holtfretter's solution: 250 mL, in mMol: 6.0 NaCl, 0.06 KCl, 0.09 $CaCl_2$, 0.24 $NaCO_3$; pH 7.0) to control for potential effects of tap water on *Bd* infection (Voyles et al. 2009).

Larval response to reduced hydroperiod.—To examine the effects of reduced hydroperiod on body size and time to metamorphosis, we simulated pool drying by applying three hydroperiod treatments to tadpole tanks. After tadpoles reached a median Gosner stage of 36 (Gosner 1960), we haphazardly assigned tadpoles (n = 108) to one of three hydroperiod treatments (fast-drying, slow-drying, or no drying control), with six replicate tanks per treatment. We reduced water depth by 2 cm per day in the fast-drying treatment, reduced water depth by 1 cm per day in the slow-drying treatment, and maintained a 14 cm water depth in control treatments. Once water levels reached a depth of 2 cm in fast and slow treatment tanks, we maintained a constant water depth of 2 cm for individuals that had not yet completed metamorphosis.

To track tadpole response to a drying environment, we measured tadpole size and development stage. Before assignment to an experimental tank and at one-week intervals after initiation of the drying treatment, we weighed individuals to the nearest 0.1 g, measured snout-vent-length (SVL) to the nearest 0.1 mm using calipers, and visually determined development stage of each tadpole using the Gosner system (Gosner 1960). In addition, we measured individual mass and SVL at metamorphosis completion and recorded date of metamorphosis.

During development between Gosner stages 25–30, some tadpoles developed axial malformations including scoliosis (abnormal lateral curvature of the spine), kyphosis (abnormal backward curvature of the spine), or kinked or S-shaped tails. Of the 93 toadlets used in the susceptibility trial, 17 exhibited severe axial deformities, as determined by visual observation. Using Chi-square, we found that incidence of axial deformities was independent of drying treatment. ($\chi^2 = 2.27$, df = 4, $P = 0.685$).

***Juvenile susceptibility to* Bd**.—We revived a cryoarchived aliquot (Boyle et al. 2003) of a Sierra Nevada isolate, MYLF 16343, on agar plates following a standard protocol (Boyle et al. 2004). Once the cultures revived, we passaged *Bd* in TGhL liquid growth media (16 g tryptone, 4 g gelatin hydrolysate, 2 g lactose, in 1000 mL distilled water, autoclaved) in 75 cm^2 tissue culture flasks (20 mL TGhL, 4 mL *Bd* culture) at 18° C for 7–9 d until peak zoospore release (determined by visual inspection under an inverted microscope; Voyles 2011). At peak zoospore release, we harvested *Bd* zoospores for the exposure inoculum by filtering liquid *Bd* cultures through sterile filter paper to remove zoosporangia (Voyles 2011). Using a hemocytometer, we determined an inoculum zoospore concentration of $278 \pm 21 \times 10^4$ zoospores per mL (Carey et al. 2006; Voyles et al. 2009; Murphy et al. 2011). We generated a negative control solution by using an equal volume of sterilized TGhL that did not contain *Bd* zoospores.

We randomly assigned toadlets within each hydroperiod treatment group to either exposed (*Bd*+) or unexposed (*Bd*-) groups, splitting toadlets from hydroperiod treatments equally between *Bd* exposure groups. Before inoculation, we swabbed the skin of all toadlets for *Bd* using a standardized swabbing procedure to determine if toadlets were free of *Bd* (Boyle et al. 2004; Hyatt et al. 2007). We exposed 47 toadlets to *Bd* inoculum (1 mL *Bd* zoospore filtrate in TGhL to 4 mL of Holtfretter's artificial pond water) and 46 toads to a control solution (1 mL TGhL in 4 mL Holtretter's solution) via immersion bath in small exposure containers for 20 h (4.4 cm diameter × 3 cm H, 60 mL capacity). After the 20-h exposure period, we moved toadlets to clean containers with 15 mL 20% Holtfretter's solution.

We collected diagnostic skin swabs, measured toadlet body size, and noted toadlet health using the qualitative scoring system of Voyles et al. (2009) every two weeks over the course of the susceptibility trial and at day of death. We used the skin swabs to estimate infection intensity using quantitative polymerase chain reaction (qPCR; Boyle et al. 2004). We assumed that *Bd*-positive swabs with a cycle threshold greater than 38 were false-positives (Hyatt et al. 2007), and we excluded them from analyses.

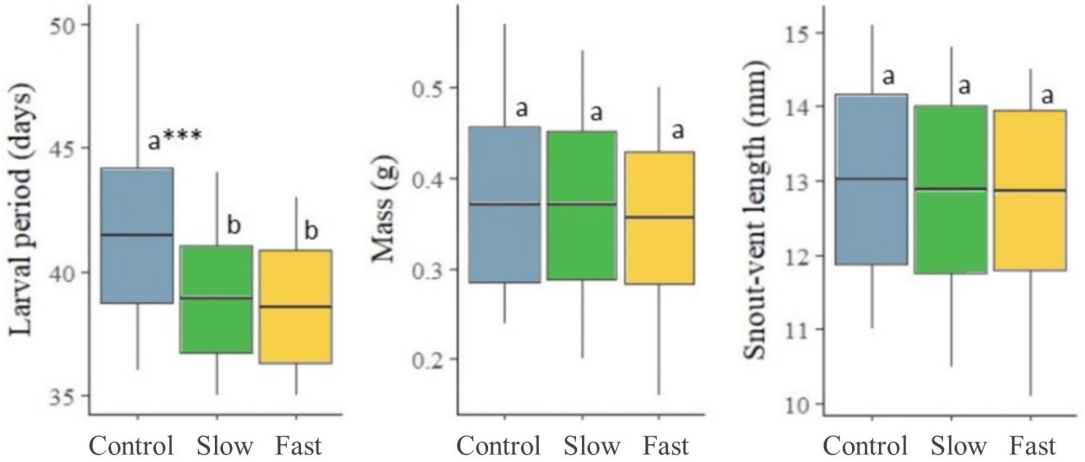

Figure 2. Time to and size at metamorphosis of Yosemite Toad (*Anaxyrus canorus*) in response to manipulation of hydroperiod (X axes). Blue bars represent *A. canorus* tadpoles from control tanks (n = 31) that experienced a constant water depth throughout development. Green bars represent tadpoles from slow-drying tanks (n = 30) that experienced a 1 cm reduction in daily water depth. Yellow bars represent tadpoles from fast-drying treatments (n = 33) that experienced a 2 cm reduction in daily water depth. Different letters represent statistically different groups; asterisks represent $P < 0.001$. Midline represents mean, boxes represent one standard error, and whiskers represent maximum and minimum values.

Statistical analysis.—We conducted all analyses using R v3.4.3 (R Core Team 2018). We tested all data sets for normality and transformed independent variables or used non-parametric tests if we found any evidence of assumption violation. Unless otherwise noted, summary statistics in figures and text represent mean $\pm$ standard error (SE), and the alpha level is 0.05. We assessed the effect of reduced hydroperiod on time to and size at metamorphosis by testing the difference in larval period, mass at metamorphosis, SVL at metamorphosis, and mass at Gosner stage 41 among different drying treatments. We used mixed model ANOVA with tank as a random variable using R packages lme4 v1.1.17 (Bates et al. 2015) and lmerTest (Kuznetsova et al. 2017). If the results suggested significant differences among hydroperiod treatments, we used Tukey's post-hoc test on mixed model ANOVA using the multcomp R package v1.4.8 (Hothorn et al. 2008).

To test for differences in survival between *Bd*-exposed and *Bd*-negative control toadlets, we used a Kaplan-Meier estimator for survival curves and log-rank tests with a Weibull distribution using the Survival package v2.41.3 (Therneau, T. 2015. A Package for Survival Analysis in S. version 2.38. Available from https://CRAN.R-project.org/package=survival. [Accessed 4 March 2018]). We removed four toadlets that died during the 20-h exposure period from all survival analyses, as their deaths were likely not caused by chytridiomycosis given the time required by *Bd* to encyst, replicate, and cause lethal damage to the epidermis (Longcore et al. 1999). Because loss of body mass has been associated with *Bd* infection (Peterson et al. 2013), we also tested for differences in mass two weeks post exposure both within and among *Bd* treatment groups using paired *t*-tests and Welch's *t*-tests, respectively. For *Bd*-exposed toadlets only, we tested differences in *Bd* load at time of death among drying treatments using ANOVA. We applied a $\log_{10}$ transformation to raw *Bd* genomic equivalents to reduce skewness. We tested for effects of drying treatment on survival using a Cox proportional hazards model with the Survival package and assessed model significance using a Wald test (we note that likelihood ratio and score tests produced similar results). Models met assumptions of proportional hazards.

Results

Larval response to reduced hydroperiod.—Tadpoles exposed to drying treatments metamorphosed earlier than control tadpoles. Compared to tadpoles from control tanks, tadpoles from slow- and fast-drying tanks completed metamorphosis 2.55 $\pm$ 0.61 and 2.88 $\pm$ 0.60 d earlier, respectively (slow-drying, $t = -4.15$, df = 91, $P < 0.001$; fast-drying, $t = -4.789$, df = 91, $P < 0.001$; Fig. 2A). We found no significant difference in time to metamorphosis between fast- and slow-drying treatment groups (Tukey's HSD, fast-control $P < 0.001$, slow-control $P < 0.001$, fast-slow $P = 0.592$; Fig. 2A).

While a reduced hydroperiod accelerated the time to metamorphosis in *A. canorus* tadpoles, tadpole and metamorph body size did not differ at metamorphosis. We found no significant differences in snout-vent length (fast-drying: $t = -0.549$, df = 91, $P = 0.584$; slow-drying: $t = -0.477$, df = 91, $P = 0.635$) or body mass (fast-drying: $t = -0.709$, df = 91, $P = 0.480$; slow-drying: $t = -0.016$,

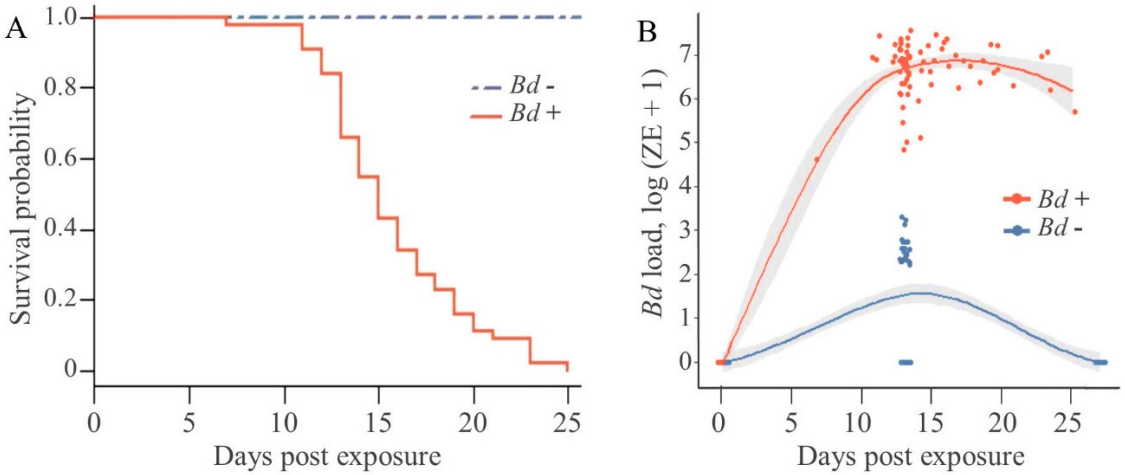

Figure 3. Survival probability and *Bd* load in juvenile Yosemite Toads (*Anaxyrus canorus*) exposed to *Bd* inoculum or *Bd*-negative control solution. (A) *Bd*-exposed A. canorus toadlets (orange solid, n = 44) experienced 100% mortality by 25 d after exposure, while the *Bd*-negative toadlet group (grey dashed, n = 45) had zero mortality over the same four weeks (Kaplan-Meier, log-rank test, $P < 0.001$). (B) Mortality in *Bd*-exposed toads was associated with increases in *Bd* load over time, with *Bd* loads reaching a median 7.5×10^6 zoospores. *Bd*-positive qPCR results from skin swabs in *Bd*-negative control toads (grey), are likely due to contamination; all *Bd*-negative control toads had *Bd*-negative qPCR results at four weeks post initiation of the susceptibility trial.

df = 91, $P = 0.988$) at time of metamorphosis between different drying treatment groups (Fig. 2). Similarly, we found no significant difference in snout-vent length (fast-drying: $t = 0.107$, df = 79, $P = 0.915$; slow-drying: $t = -0.968$, df = 79, $P = 0.336$) or mass (fast-drying: $t = 0.367$, df = 79, $P = 0.715$; slow-drying: $t = 1.589$, df = 79, $P = 0.116$) between drying treatments at Gosner stage 41, the developmental stage before metamorphic climax. Mean mass and SVL of toadlets across all treatments at completion of metamorphosis was 0.37 ± 0.01 g and 12.9 ± 0.11 mm, respectively. Tank did not explain variance in any model.

***Juvenile susceptibility to* Bd**.—Survival results were strikingly different between *Bd*-exposed and *Bd*-negative (control) toadlets (Kaplan-Meier, log-rank test, $\chi^2 = 106$, df = 1, $P < 0.001$; Fig. 3A). None of the 45 toadlets serving as *Bd*-negative controls developed signs and symptoms of chytridiomycosis, and none died over the four-week period of the susceptibility trial. In contrast, all 44 toadlets exposed to *Bd* became infected, developed clinical signs of severe chytridiomycosis, and died within 25 d of exposure (Fig. 3A). Progression of disease from onset of visible clinical signs to death was rapid, with individuals dying a mean of 3 d after first observed signs of lethargy or inappetence. One individual died within 7 d of exposure with a load of 4.2×10^4 GE (genomic equivalents), and the remaining toadlets died within 11–25 d of exposure (median = 15 d) with zoospore loads ranging from 1.5×10^5 to 2.8×10^7 GE with a median of 7.5×10^6 GE (Fig. 3B). For toadlets exposed to *Bd*, we did not find an effect of drying treatment on survivorship ($W_T = 1.52$, df = 2, $P = 0.469$; Fig. 4) or a difference in *Bd* load among fast-drying, slow-drying, and no drying control treatments ($F_{2,41} = 0.668$, $P = 0.518$).

Toadlets exposed to *Bd* that survived the first two weeks post exposure lost body mass during that time interval ($t = 4.95$, df = 36, $P < 0.001$), while toadlets in the *Bd*-negative control group neither gained nor lost mass during the first two weeks of the susceptibility trial ($t = 1.89$, df = 44, $P = 0.065$). Toadlet change in mass over the first two weeks was significantly different between *Bd*-exposed and *Bd*-negative control individuals ($t = 3.56$, df = 54, $P < 0.001$), with *Bd*-exposed toadlets losing an average of 0.04 ± 0.01g in two weeks, which

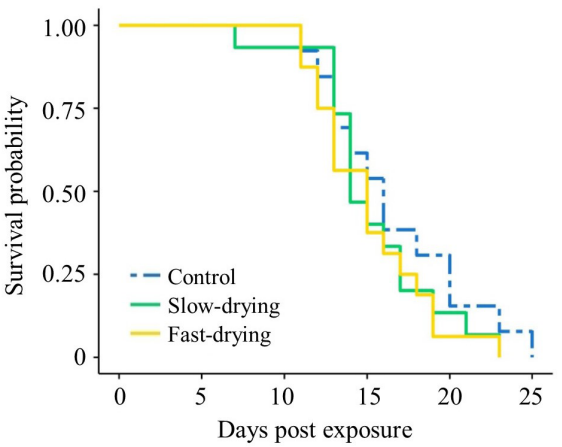

Figure 4. Survival probability of *Bd*-positive Yosemite Toad (*Anaxyrus canorus*) toadlets reared under control (blue dashed, n = 13), slow-drying (green solid, n = 15), or fast-drying (yellow solid, n = 16) hydroperiod treatments.

is approximately 10% of their mass at the initiation of the susceptibility trial.

Quantitative PCR results suggest that some toadlets in the *Bd*-negative control group were weakly *Bd*-positive (mean 471 ± 92 GE) two weeks after initiation of the susceptibility trial (Fig. 3B). All control toadlets had *Bd*-negative qPCR results four weeks post exposure and at follow-up swabbing events over the following month. The *Bd*-positive qPCR results from control toadlets early in the susceptibility trial are likely false positives resulting from contamination during swabbing, DNA extraction, or qPCR. Although we cannot determine the precise cause of this result, false positives may be explained because we used ethanol to decontaminate some surfaces and instruments during swabbing, when some of the *Bd*-positive toadlets had high infection intensities. Because ethanol kills *Bd* but does not fully degrade DNA, this may have been the source of contamination.

Discussion

Changes in the environment can alter host morphology and physiology with potential fitness consequences (Pigliucci 2001; Dewitt and Scheiner 2004), including an increased risk of disease susceptibility (Rollins-Smith et al. 1988; Gervasi and Foufopoulos 2008; Rohr et al. 2013). For amphibian hosts with distinct habitat needs across life stages, an environmental change encountered during the aquatic larval stage may alter disease outcomes in adulthood (Gervasi and Foufopoulos 2008). In this study, we investigated the interaction between reduced hydroperiods and disease threats across *A. canorus* life stages. We found that *A. canorus* juveniles are highly susceptible to *Bd* infection and develop lethal disease, but reduced hydroperiod during tadpole development did not affect disease outcomes post-metamorphosis.

***Juvenile susceptibility to* Bd**.—We found that *A. canorus* juveniles were highly susceptible to chytridiomycosis in our laboratory exposure experiment. Juveniles exhibited clinical signs of disease, lost body mass over the course of disease progression, and carried high zoospore loads. Most importantly, exposure to *Bd* led to 100% mortality of *A. canorus* toadlets within 25 d. Time to death and pathogen load at death were similar to infection patterns in *Bd* susceptibility trials for juvenile *Anaxyrus boreas* (Carey et al. 2006), a closely related alpine toad with declines attributed to chytridiomycosis (Muths et al. 2003). High susceptibility to lethal chytridiomycosis, paired with evidence of *Bd* infection in museum specimens collected during declines of *A. canorus* beginning in the 1970s (Green and Sherman 2001), suggest that chytridiomycosis may have contributed to historical declines of *A. canorus* and could be a direct cause of mortality in wild populations.

Reduced hydroperiod did not alter toadlet susceptibility to *Bd*. Our results do not suggest a difference among drying treatments in toadlet survival, survival time, or *Bd* load at death. Reduced hydroperiod may not influence *Bd* susceptibility for this species, drying treatments may not have been severe enough to impose physiological costs, or high inoculation doses may have masked potential drying treatment effects. Lower inoculation doses paired with more extreme hydroperiod scenarios may tease apart the effects of drought and disease on survival rate. In addition, measuring other physiological parameters affected by a drying environment, such as immune function and glucocorticoid hormone levels (Gervasi and Foufopoulos 2008; Garner et al. 2009; Crespi and Warne 2013; Rollins-Smith 2017) may help assess the role that larval stress and developmental programming play in disease susceptibility at later life stages.

We found that juvenile *A. canorus* were highly susceptible to *Bd* infection in a controlled setting; however, disease dynamics in the wild are likely to be more complex and warrant further investigation to help guide management decisions. For example, the thermal microhabitats that *A. canorus* select in the wild may affect disease development. *Bd* grows within a defined temperature range (approximately 2–27° C; Johnson et al. 2003; Piotrowski et al. 2004; Woodhams et al. 2008), and viability drops dramatically above 27° C (Stevenson et al. 2013; Voyles et al. 2017). While adult *A. canorus* body temperatures most frequently fall within the *Bd* thermal range (Mullally and Cunningham 1956; Cunningham 1963), adult body temperatures have also been recorded above the *Bd* thermal maximum (28–33° C; Mullally and Cunningham 1956; Cunningham 1963). Wild *A. canorus* may be able to behaviorally regulate *Bd* infection by using warmer and drier microhabitats during summer months (Richards-Zawacki 2009; Daskin et al. 2011; Murphy et al. 2011; Hossack et al. 2013; Greenspan et al. 2017). Alternatively, cooler and wetter conditions experienced during toad hibernation and emergence at snow melt may represent times of increased infection prevalence and intensity (Piovia-Scott et al. 2011) from possible increases in pathogen fecundity (Woodhams et al. 2008; Voyles et al. 2012) or reductions in host immune function (Rollins-Smith and Woodhams 2012). Long-term monitoring of *Bd* prevalence and infection intensity on individuals in conjunction with measuring temperatures and water availability across different spatial (i.e., pool, meadow, elevation, latitude) and temporal (i.e., seasonal, annual) scales will help define the roles that *Bd* infection and the environment play in *A. canorus* declines.

Tadpole response to reduced hydroperiod.—Reduced hydroperiod decreased time to metamorphosis by 2–3 d. This result suggests that *A. canorus* tadpoles can accelerate their development time in response to a drying environment, which could potentially increase survival under some reduced hydroperiod scenarios. Our results, however, represent a decrease in development time under an experimentally manipulated drying regime, and the range in developmental plasticity of *A. canorus* tadpoles under more complex ecological conditions (i.e., water temperature and quality, predation, tadpole density, natural evaporation rates) remains unknown. Future studies on the ability of *A. canorus* tadpoles to escape drying pools within and among wild populations across the species range could better define drought threats for this life stage and potentially identify differences in drought tolerance among populations (Maier 2018).

We did not find an effect of drying treatment on toadlet body size at metamorphosis, which may be explained by multiple factors. First, we may not have observed a decrease in metamorph size due to the intensity of our hydroperiod treatments. Specifically, our ending experimental water depth of 2 cm in reduced hydroperiod tanks may not have been severe enough to elicit a response for this species. As ephemeral water body specialists, *A. canorus* tadpoles prefer specific water depths between 4–5 cm (Liang et al. 2017; Karen Pope et al., unpubl. report), and it is not uncommon to observe *A. canorus* tadpoles in water depths of 1–2 cm (Karlstrom 1962; Sherman 1980; Karen Pope et al., unpubl. report). We selected deeper starting and ending water depths that fall within the range of observed *A. canorus* tadpole pool depths (Karen Pope et al., unpubl. report; Steven Lee pers. comm.) to reduce cannibalism and improve water quality during husbandry (Scherff-Norris et al. 2002), but our drying treatments may not have been severe enough to increase physiological stress capable of affecting body size metrics.

Second, initiating our drying treatment during the later stages of metamorphosis may not have been early enough to elicit a tradeoff between larval development time and body size (Alford and Harris 1988; Gervasi and Foufopoulos 2008). Tadpole growth rates vary during early (premetamorphic) and late (prometamorphic) stages of tadpole development (Wilbur and Collins 1973), with a deceleration in tadpole growth preceding metamorphic climax (Alford and Harris 1988; Wilbur and Collins 1973). Environmental stressors, such as habitat desiccation, may be more likely to inhibit growth during premetamorphosis when growth rates are higher as opposed to during the later prometamorphosis stage, a developmental period characterized more by tissue differentiation than by growth (Denver 1997a; Denver 1997b; Gervasi and Foufopoulos 2008). Thus, earlier initiation of drying treatments may be more likely to induce a reduction in body size in response to a drying environment.

Third, a body size effect may be caused by other ecological factors associated with a reduced hydroperiod such as temperature, density, competition, predation, or food availability, (Wilbur 1987; Altwegg and Reyer 2003; Edge et al. 2016). Our results are consistent with O'Regan et al. (2014) who found faster developmental times without a body size cost in three anurans (Great Basin Spadefoot, *Spea intermontana*, Pacific Chorus Frog, *Pseudacris regilla*, and Northern Red-legged Frog, *Rana aurora*) in a warming and drying mesocosm experiment. O'Regan et al. (2014) suggest that increased temperatures led to increased food availability, which may have offset expected body size reductions. Because we fed our tadpoles ad libitum, we may not have detected an effect of drying treatment on body size at metamorphosis. Abiotic and biotic factors difficult to accurately replicate in laboratory or mesocosm experiments may be important cues in accelerating metamorphosis and decreasing body size in wild populations.

Selecting ecologically relevant and species-specific timing and intensity of drying regimes are likely important in eliciting age-body size tradeoffs in controlled experiments. The effect of shortened hydroperiod on age and size at metamorphosis is linked to species life history and breeding strategy, where species exposed to highly variable environments are more likely to express high levels of plasticity (Bradshaw 1965; Wilbur and Collins 1973; Van Buskirk 2002). Past studies present a range of outcomes resulting from experimentally reduced hydroperiod that are attributed to interspecific differences in plasticity range or other ecological factors that affect tadpole growth and development (Wilbur 1987; Newman 1989; Denver 1998; Gervasi and Foufopolous 2008; O'Regan et al. 2014). As well-adapted ephemeral pool breeders, *A. canorus* tadpoles likely exhibit developmental plasticity resulting in earlier metamorphosis under drying conditions, which may come with a body size cost in wild populations. We suggest follow-up experiments that measure pool depth and other factors (e.g., predation, temperature, density, food availability; Altwegg and Reyer 2003; reviewed in Edge et al. 2016) in natural breeding pools to determine ecologically relevant drivers of phenotypic plasticity and growth-development tradeoffs.

Implications for conservation.—In recent decades, *A. canorus* has experienced decreases in abundance at select sites and reductions in its distribution (Sherman and Morton 1993; Jennings and Hayes 1994; Drost and Fellers 1996; Green and Sherman 2001; Brown et al. 2012), leading to its current listings of threatened

by the USFWS and endangered by the International Union for the Conservation of Nature (IUCN). Despite efforts investigating invasive predators, pesticide use, and meadow grazing by pack stock and cattle as drivers of declines, no clear patterns have emerged (Grasso et al. 2010; Roche et al. 2012; McIlroy et al. 2013). Our results show that *A. canorus* juveniles are susceptible to *Bd* infection and develop lethal chytridiomycosis in a controlled setting, suggesting that chytridiomycosis may be a proximate cause of decline in this species. While reduced hydroperiod did not alter disease outcomes in this study, the effects of drought and disease should be considered in the development of conservation plans. Irrespective of disease, drought events have been observed to increase *A. canorus* mortality through egg and tadpole desiccation (Sherman and Morton 1993; Brown et al. 2015), which could negatively affect population structure and size (McMenamin et al. 2008; Matthews et al. 2013; Kissel et al. 2018). Additively, the potential effects of drought on toad physiology and behavior in the wild may exacerbate disease risk (Gervasi and Foufopoulos 2008; Blaustein et al. 2012). In the face of predicted increase in climate extremes, continued research examining the independent and additive effects of chytridiomycosis and drought on populations may help inform recovery efforts for imperiled amphibian species.

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Alexa Lindauer focuses on environmental factors driving wildlife disease. Using laboratory- and field-based approaches, Alexa examines the effects of temperature and water availability on pathogen virulence and host susceptibility. Her research currently focuses on the amphibian disease chytridiomycosis, which is caused by the fungus *Batrachochytrium dendrobatidis* (*Bd*) and is responsible for global amphibian species declines and extinctions. Conservation drives her research questions and she aims to provide land managers with empirical evidence that informs recovery efforts for at-risk amphibian species. (Photographed by Alexa Lindauer).

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