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In situ treatment of juvenile frogs for disease can reverse population declines

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Abstract

Effective management of wildlife populations threatened by disease requires accurate predictions about the consequences of intervention. However, generating such predictions is challenging, especially for organisms with complex life histories that are also threatened by climate change, such as montane amphibians. Cascades frogs (*Rana cascadae*) in northern California have experienced dramatic declines associated with the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*), and remnant populations are also threatened by changing climate conditions. We evaluated the population-level impacts of treating Cascades frog metamorphs with the antifungal chemical itraconazole using a field experiment and population simulations. We explored the influence of larval habitat on these treatment effects by including metamorphs from different larval habitat types. We found that frogs treated with itraconazole were more than four times more likely to survive their first winter than untreated controls and had reduced *Bd* infection intensity compared to other surviving frogs from the same cohort in the following year. We also found an effect of larval habitat type on *Bd* infection in recently metamorphosed frogs, with the lowest levels of infection occurring in frogs emerging from larval habitats that tend to be intermediate in temperature and drying rate. Applying the differential apparent overwinter survival of treated and untreated metamorphs to population projections suggests that intermittent antifungal treatment of metamorphs has the potential to restore population viability. Our results indicate that in situ treatment of individual hosts may be a useful component of a comprehensive management strategy to reduce the risk of pathogen-mediated population declines and extirpations.

KEYWORDS

Batrachochytrium dendrobatidis, chytridiomycosis, itraconazole, *Rana cascadae*, stage-structured population model, wet meadow

Kimberly Cook and Jonah Piovia-Scott contributed equally.

1 | INTRODUCTION

Wildlife disease has emerged as a conservation threat of global importance (Daszak et al., 2000; Smith et al., 2006), yet the effect of disease often varies among species, populations, and life stages. Developing and prioritizing disease management interventions that account for such variation is critical to saving imperiled biota (Langwig et al., 2015). Amphibians provide a stark example of the challenges associated with disease management in at-risk species. One of the principal causes of amphibian declines is an emerging infectious disease – chytridiomycosis – caused by fungal pathogens in the genus *Batrachochytrium*. One of these species, *B. dendrobatidis* (*Bd*) has caused the most dramatic effects on biodiversity of any pathogen in recorded history (Skerratt et al., 2007; Scheele, Pasmans, et al., 2019). Effective decision-making for the conservation of amphibian populations affected by *Bd* requires robust information about population-level consequences of intervention or lack thereof (Canessa et al., 2019; Brannelly et al., 2021). However, accurate predictions about these consequences are difficult to make because changing climate conditions can alter the effects of chytridiomycosis (e.g., Cohen et al., 2017, 2019; Sauer et al., 2020) and there is substantial variation in susceptibility to chytridiomycosis across species, populations, and life stages (Garner et al., 2016; Scheele et al. 2019; Fisher & Garner 2020; Sauer et al. 2020; Brannelly et al. 2021).

The most pronounced effects of *Bd* on survival are often associated with early post-metamorphic life stages (reviewed in Fisher et al. 2009; Rollins-Smith et al. 2011; Sauer et al. 2020). In addition, the breeding habitat is particularly vulnerable to changing environmental conditions, in part because amphibians often breed in water bodies that are prone to drying but serve as a refuge from aquatic vertebrate predators such as fish (e.g., Ryan et al. 2014). For example, in drought years, ponds may dry before larvae metamorphose resulting in recruitment failure due to desiccation (Lee et al. 2015), while in high snow years, larvae may face time constraints to reach metamorphosis before freezing recurs (e.g., Laurila et al. 2001). Some amphibians can speed metamorphosis in response to environmental cues (Denver et al. 1998), but often with the tradeoff of decreased body size (Loman & Claesson 2003) and reduced immunocompetence (Gervasi & Foufopoulos 2008). Thus, larval environmental conditions may have important consequences for the management of chytridiomycosis in at-risk populations of amphibians.

Once *Bd* becomes established in populations of at-risk amphibians, there is a limited set of management options available, including translocation, habitat manipulation, and treatment of hosts or environments with chemical or biological antifungal agents (Woodhams et al. 2011; Scheele et al. 2014, 2021; Langwig et al. 2015; Garner et al. 2016; Brannelly et al. 2021). While eradication of the pathogen is possible (Bosch et al. 2015), it is unlikely to be feasible in most situations, leading to a renewed focus on facilitating coexistence with *Bd* (Scheele et al. 2019; Brannelly et al. 2021). In situ treatment of amphibian hosts with antifungal chemicals, such as itraconazole, has emerged as one of the more reliable interventions for reducing *Bd* infections in individuals (e.g., Hudson et al. 2016; Geiger et al. 2017; Knapp et al. 2022). However, these studies have generally found only short-term benefits of this intervention, leading many researchers to conclude that benefits to populations will be minimal unless treatments are sustained (Garner et al. 2016; Fisher & Garner 2020). Furthermore, the effects of these in situ treatments are likely to vary with environmental conditions, a topic that has received little attention in field studies. In this study, we connect field itraconazole interventions to likely population-level outcomes by investigating the impact of itraconazole treatments on the overall population growth rate of remnant populations of an at-risk amphibian. We place these outcomes in the context of environmental variation by probing the influence of larval habitat on these treatment effects.

Chytridiomycosis is thought to be one of the main factors associated with the decline in the Cascades frog (*Rana cascadae*) in the southern Cascade Range, where this species is thought to have disappeared from >90% of its historical range (Fellers & Drost 1993; Fellers et al. 2007; Pope et al. 2014; de León et al. 2017). *Bd* prevalence is low in larval Cascades frogs, but newly metamorphosed Cascades frogs experience rapid increases in *Bd* infection (e.g., from ~10% to ~70% prevalence in 5 weeks; Hardy et al. 2015) and high mortality resulting from *Bd* infection (Piovia-Scott et al. 2015), resulting in repeated recruitment failure in remaining populations (Pope et al. 2014; Hardy et al. 2015; Piovia-Scott et al. 2015). In contrast, adult Cascades frogs have lower *Bd* infection intensities than subadult frogs (Piovia-Scott et al. 2011) and may experience lower levels of chytridiomycosis-related mortality (Piovia-Scott et al. 2015). A previous experiment has shown that treating recently metamorphosed frogs with the antifungal drug itraconazole decreases *Bd* infection intensity and increases overwinter survival in the wild (Hardy et al. 2015), suggesting that in situ treatment with itraconazole may represent a viable tool for disease management in this species.

Many of the remnant Cascades frog populations in the southern Cascades occur in wet meadow habitats where pond drying associated with climate change represents a substantial threat (Pope & Larson 2013; Pope et al. 2014). Meadow breeding habitats vary substantially in water temperature and permanency even within a single meadow, ranging from permanent spring-fed pools with constant cold-water temperatures (5.5–6.5°C) to shallow surface water pools that can range from 7°C to 32°C within a 24 hour period (Pope et al. unpublished data). This variation provides an opportunity to assess the impact of larval habitat on *Bd* effects in early post-metamorphic life stages.

We used a field itraconazole treatment experiment featuring Cascades frog metamorphs to investigate the effects of treatment for *Bd* and larval habitat on *Bd* infection, apparent overwinter survival (we use the term "apparent survival" throughout because our approach does not distinguish between mortality and permanent emigration), and body size. We used the results of this experiment, along with data from long-term population monitoring, to parameterize population models for these remnant populations. These models were used to evaluate how the effects of *Bd* on metamorph apparent overwinter survival influences population growth rates and the potential for treatment-based management intervention to enhance population viability. We hypothesized a priori that treatment with itraconazole would reduce *Bd* prevalence and load, increase apparent overwinter survival, and increase Cascades frog population growth rate. We also expected larval habitat to influence *Bd* infection in recently metamorphosed frogs with potential consequences for apparent overwinter survival. Finally, to explore the long-term consequences of itraconazole treatment, we report recapture rates for frogs included in a previous itraconazole treatment experiment conducted by Hardy et al. (2015) at one of our study sites.

2 | METHODS

2.1 | Study system

Cascades frogs are highly aquatic and breed in lentic habitats (e.g., lakes, ponds, and pools in wet meadows) in mountainous areas (primarily in the Cascades Range) in northern California, Oregon, and Washington, USA; elevation range is roughly 400–2500 m, with the lower elevational limit being higher at the southern end of the range (Lannoo 2005). Eggs are laid shortly after snowmelt and larvae almost always metamorphose in the same year, adulthood (i.e., reproductive age) is reached in roughly 3 to 4 years, and the total life span can exceed 10 years; post-metamorphic life stages overwinter in aquatic habitats that do not freeze solid (Pope et al. 2014). Cascades frogs are thought to have been historically abundant at the southern end of the Cascades Range in northern California, but precipitous declines became apparent in the 1990s and only a handful of populations remain in the region; most of these remnant populations are declining and at risk of extirpation (Pope et al. 2014; Hardy et al. 2021).

Our study focuses on remnant Cascades frog populations at three wet meadows in northern California: Carter Meadow, Old Cow Creek Meadow, and Childs Meadow (Figure S1). *Bd* has been present at all three meadows at least as far back as 2008 (Pope & Larson 2013). Each meadow contains multiple Cascade frog breeding areas that can be classified based on their shape and primary water source: "springs" are steep-sided pools directly fed by groundwater, "fens" are shallow, groundwater-supported areas without distinct edges, and surface-water pools (hereafter "pools") are still-water depressions that receive input from surface-water channels. Detailed spring and summer hydrological measurements at our study sites in previous years have shown that springs tend to feature consistently cool water and low dry-down rates, and pools tend to feature warmer water and faster dry-down rates, while fens occupy a middle ground. The rate of larval and embryonic development in Cascades frogs generally increases with water temperature, but high summer water temperatures in our study area (which have been measured as high as 38°C in pools with Cascades frog larvae) appear to approach the thermal tolerance of the frogs (Pope & Larson 2013; Pope et al. 2014) – and exceed those of *Bd* (Piotrowski et al. 2004; Stevenson et al. 2013; Voyles et al. 2017).

Our study focuses on the 10 breeding areas used by Cascades frogs in our study meadows in 2018: 5 fens (4 at Carter and 1 at Old Cow), 3 pools (1 at Carter and 2 at Childs), and 2 springs (1 at Carter and 1 at Childs) (see Table S1 for details; representative photos are in Figure S2). Breeding areas within each meadow were separated by ~30–500 m. Thus, metamorphs could reliably be attributed to a particular breeding area shortly after metamorphosis (i.e., within the first ~20 days), but frogs had the capacity to move between breeding areas after that.

2.2 | Experimental design and data collection

In order to evaluate the effects of *Bd* on early post-metamorphic life stages of Cascades frogs and assess the efficacy of antifungal treatment as a management tool, we treated recently metamorphosed Cascades frogs from different larval habitats with the antifungal drug itraconazole and tracked their apparent survival, growth, and *Bd* infection intensity for a year. To generate inference about the effects of itraconazole treatment, we compared four treatment groups: (1) "itraconazole-treated" frogs; (2) "experimental controls", which underwent the same process as the itraconazole-treated animals, but received a sham treatment; (3) "field controls", which were captured and marked the day after the experimental frogs at each meadow, but were immediately released back into the field; and (4) "other frogs" from the same cohort that were only captured after the treatment period. Experimental controls were used to directly evaluate the effects of itraconazole, as they experienced the same captivity and treatment regime as treated frogs, but without the antifungal drug; field controls were used to evaluate the effect of the entire itraconazole treatment regimen, as they did not experience the associated captivity and holding period (see below for details). All four treatment groups were present at each study meadow (see below for details).

In September 2018, shortly after metamorphosis, when recently metamorphosed frogs could still be reliably associated with the breeding areas from which they emerged, we collected 186 newly metamorphosed Cascades frogs (as many as we could catch in a single day at each site: 115 from Carter Meadow, 27 from Childs Meadow, and 44 from Old Cow Meadow; see Table S1 for details) for use in our experiment by searching each breeding area and capturing frogs by hand or dipnet. Frogs were placed in individual containers stored in a cooler for transportation to a campground central to the three meadows. These experimental frogs likely represent <25% of the breeding output for each breeding area.

At the central location, we measured body size (snout–urostyle length) and transferred the frogs to 28-L plastic bins tilted to provide aquatic and terrestrial habitat. Each bin contained five to ten similar-sized individuals from the same breeding area. The number of animals per bin depended on how many animals were in each size class (small, medium, and large); there were at least two bins for each size class in each meadow. We then randomly assigned bins with similar-sized frogs from each breeding area to itraconazole treatment or sham treatment groups (totals: 97 itraconazole-treated frogs and 89 experimental controls). Frogs were given individually identifying visual implant elastomer tags (VIE) indicating breeding area and itraconazole versus sham treatment group. Experimental frogs were fed fruit flies ad libitum and each bin was cleaned (rinsed thoroughly and water replaced) during the daily treatments. Experimental frogs were swabbed for *Bd* on the day of capture and again just after the final treatments were completed. Frogs were swabbed five times on their feet and each thigh with a sterile swab (Medical Wire & Equipment, MW113), which was dried in the field and then stored in a cooler until it could be transported to the lab.

Frogs assigned to the itraconazole treatment group were bathed in a 0.0025% itraconazole (Sporanox 10 mg/mL oral solution; Janssen) solution for 5 minutes on 6 consecutive days (Brannelly 2014; Hardy et al. 2015). Treatment occurred in a 6-L container with 1 L of treatment solution. For each daily treatment, all frogs from each housing bin were treated simultaneously in a clean container with a fresh treatment solution. The solution was deeper than the height of the animals – frogs generally swam around the container during the treatment period (~2/3 submerged) and were gently coaxed back into the solution if they tried to climb the walls of the container. Frogs were immediately returned to their housing bins following each day's treatment and were released back into their respective meadow after the 6th day of treatment. This experiment was placebo controlled by including a sham treatment ("experimental controls") in which clean water was used in place of the itraconazole solution.

On the first day of the treatment period, we returned to our study meadows and captured an additional 146 metamorphs (as many as we could catch in a single day at each site; Carter: 87, Childs: 33, and Old Cow: 26) to serve as "field controls." Field controls were not removed from the meadow but were given a single cohort-identifying VIE mark, measured (snout–urostyle length), swabbed for *Bd*, and released at the site of capture.

Each meadow was surveyed twice more in the fall of 2018 after treated frogs and experimental controls were released. These surveys occurred ~3 weeks after release (October 17–19) and ~ 6 weeks after release (November 6–9). All metamorphs encountered were captured to identify their treatment group (treated, experimental control, field control, or other), measure them, and swab for *Bd*.

Each meadow was surveyed four times in 2019 (approximately once a month between June and September, which encompasses most of the active period for Cascades frogs in this region) to determine apparent overwinter survival and *Bd* infection intensity of treated and untreated frogs. All aquatic habitats were searched for frogs. Young of the previous year (i.e., the cohort that included our experimental frogs and field controls) were measured, swabbed for *Bd*, and checked for VIE marks; untagged frogs received a cohort-identifying VIE mark. In addition, we report recaptures of individuals from a previous smaller-scale itraconazole treatment experiment (28 itraconazole-treated frogs and 28 experimental controls; 4-day treatment period with 10 minutes in 0.01% itraconazole on each day) conducted at Carter Meadow in 2012 (Hardy et al. 2015). This reporting includes recaptures that took place between 2014 and 2019 (including the surveys described above).

We extracted DNA from swabs using established protocols (Boyle et al. 2004; Hyatt et al. 2007). Briefly, samples were extracted with PrepMan Ultra (Life Technologies) and extracted samples were diluted 1:10 with molecular-grade water prior to PCR. Extracted samples were tested in singlicate for the presence and intensity of *Bd* infection using quantitative PCR (qPCR) with a CFX real-time PCR system (Bio-Rad Laboratories). Each reaction well contained a total of 12.5 μl: 2.5 uL of sample (or standard or negative control), 6 uL of master mix (SsoAdvanced Supermix; Bio-Rad Laboratories), and 0.6 uL of primer-probe mix (TaqMan MGB probe; ThermoFisher); we ran each reaction for 50 cycles. To determine infection intensity, we used oligonucleotide standards (gBlocks, Integrated DNA Technologies) to estimate how many copies of the internal transcribed spacer (ITS) were present on each swab; four levels of standards (1, 10, 100, and 1000 copies) were run on each plate, with three replicate wells for each level, along with three negative controls. We converted copy numbers to zoospore equivalents (ZE) using values obtained by running oligonucleotide standards and zoospore standards (derived from *Bd* isolated from Cascades frogs in northern California) on the same qPCR plate. *Bd* infection intensities (in ZE) are presented on a log scale in figures, and raw data means and standard errors are derived from log-transformed data (with 1 added to raw ZE values to avoid taking the log of 0). All data collection was approved by IACUC and the California Department of Fish and Wildlife.

2.3 | Statistical analyses

We evaluated the effects of larval habitat on body size at metamorphosis using linear mixed-effects models (LMM) fit to data collected at the time of initial capture for the frogs used in the itraconazole experiment. The response variable was snout–urostyle length (SUL), larval habitat (i.e., spring, fen, or pool) was a fixed effect, and meadow and breeding area were random effects.

We evaluated the effects of larval habitat on *Bd* infection intensity in recently metamorphosed frogs using pre-treatment infection data for experimental frogs with a zero-inflated negative binomial (ZINB) mixed model. Larval habitat (spring, fen, or pool) was a fixed effect, body size (SUL) was a continuous covariate, and meadow and breeding area were random effects. We used a ZINB mixed model, as we reasoned that the zeros resulted from a binomial process (*Bd* present on a frog or not) and a count process (*Bd* zoospore establishment and survival) (Zuur et al. 2009). Zeros may also be the result of imperfect detection (i.e., false negatives), which is particularly likely at low infection intensities, and positives may be the result of *Bd* DNA that is not associated with live *Bd* cells.

We also used ZINB mixed models to assess the effect of itraconazole treatment on *Bd* infection during the 6-day treatment period in two ways. First, we compared pre- and post-treatment infection intensity in itraconazole-treated animals: time (before vs. after treatment) was a fixed effect of interest; and meadow, breeding area, and metamorph identity were random effects. Second, we compared post-treatment infection intensity between treated animals and experimental controls: treatment group (itraconazole treated vs. experimental control) was a fixed effect of interest, log(pre-treatment *Bd* infection+1) was a continuous covariate, and meadow and breeding area were random effects.

For both ZINBs described above, the zero-inflation component of the model included all fixed effects and continuous covariates. Likelihood ratio tests were used to estimate the significance of fixed effects; for ZINBs, we conducted the likelihood ratio test by removing the effect of interest from both the negative binomial and zero-inflation parts of the model. Three frogs missing *Bd* data were removed from the analyses.

We used a Bayesian hierarchical Cormack–Jolly–Seber (CJS) model to test for differences in apparent overwinter survival among itraconazole-treated frogs, experimental controls, and field controls using code developed by Kéry and Schaub (2011). Capture histories included the initial capture at the time of treatment in 2018 and the four surveys conducted in 2019; we did not include the late fall 2018 surveys as our focus was on apparent overwinter survival, and some frogs had likely begun overwintering by the time these surveys were conducted. Capture histories were straightforward for itraconazole-treated frogs and experimental controls, as they received VIE marks at the time of the first capture that was specific to each individual. Field controls were more complicated, as they received a batch mark at the time of initial capture in 2018. We assembled capture histories for field controls as follows. Upon initial capture, in 2019, we assigned each field control to a random capture from the same site in 2018 allowing us to build capture histories that span the winter. Captured frogs were given survey-specific VIE marks in 2019 (and one grew large enough to receive a PIT tag), which allowed us to assemble 2019 capture histories. Our CJS model included effects of treatment group (itraconazole treated, experimental control, or field control), time (intervals between surveys), and meadow (as a random effect) on apparent survival. Time was constrained to two parameters: apparent overwinter survival (fall 2018 to first 2019 survey) and apparent summer 2019 survival (three intervals among four summer 2019 surveys). In other words, we assumed apparent survival was constant for the three time periods between the summer 2019 surveys in order to reduce the number of parameters and produce a more robust inference for the key parameter of interest – apparent overwinter survival. Detection probability was assumed to be constant. Confidence in the difference in probability of apparent survival between treatment groups was determined by the degree of overlap in the posterior distribution of the parameters. We used wide non-informative priors for all parameters. The model was run for 200,000 iterations with a 50,000 burn-in and thinning every 100 samples. We checked for convergence of three parallel MCMC chains using the Gelman–Rubin diagnostic (Gelman 2004).

We used individuals recaptured the summer following the experiment (2019) to evaluate the longer-term effects of antifungal treatment and larval habitat on body size (using LMMs) and *Bd* infection intensity (using ZINB mixed models) for those frogs that survived the winter. Because only one experimental control survived its first winter, we were unable to use experimental controls in these models. However, we were able to use field controls and “other frogs” from the same cohort, with the primary difference between the two groups being that field controls were known to have metamorphosed at approximately the same time as treated frogs, while other frogs could have metamorphosed later. Treatment group (itraconazole-treated, field control, or other frogs) and survey (which corresponds to the four different site visits over the course of the 2019 field season) were included as fixed effects; meadow was included as a random effect. Larval habitat was not included as a predictor because it was not known for field controls and other frogs. If an individual was captured more than once in 2019, we only included its first capture in these analyses to avoid pseudoreplication; two frogs were removed from the *Bd* infection analysis due to missing *Bd* data.

All LMMs and ZINBs were performed in R 3.6.1 (R Core Team 2019). LMMs were fit using the “lme4” package (Bates et al. 2015), ZINBs were fit using the “glmmTMB” package (Brooks et al. 2017), and post-hoc tests were performed using the “emmeans” package (Lenth 2018). We used a type III analysis of variance with Satterthwaite's approximation to determine significance of fixed effects in LMMs (Kuznetsova et al. 2017). Models were consistent with assumptions, as determined by evaluating diagnostic plots generated using the DHARMa package (Hartig 2022). Cormack–Jolly–Seber models were fit using JAGS (version 4.3.0, Plummer 2003) using R and the R2jags wrapper package (Su & Yajima 2015).

2.4 | Population simulations

We used simulations based on a stage-structured population model (Figure S3; Table S2) to evaluate: (1) the impacts of pathogen-driven overwinter mortality in metamorphs on population dynamics, and (2) the potential for itraconazole-based management interventions to enhance population growth. We estimated metamorph overwinter mortality rates using apparent overwinter survival for itraconazole-treated and field control frogs from the current study. The remaining parameters (apparent adult survival, apparent subadult survival, growth rate, and per female fecundity rate) were estimated using a Bayesian Jolly–Seber model on up to 12 years of population monitoring data from four populations of Cascades frogs in the study region, including the three meadows used in this study (see Supporting Information for details). For each simulation, we started with an adult population size of 100 and ran 1000 simulations of population dynamics for 10 years. We then calculated the geometric population growth rate (λ) for adults by taking the geometric mean of each annual λ during the simulation. In order to assess the effect of *Bd* on population dynamics, we compared a model in which all metamorphs experienced the itraconazole-treated apparent overwinter survival rate to a model in which all metamorphs experienced the apparent overwinter survival rate estimated for animals that did not receive itraconazole treatment. To evaluate the efficacy of different management strategies, we compared models that varied in the proportion of metamorphs that received the itraconazole treatment and the frequency of itraconazole treatment (i.e., the proportion of years in which treatment is performed).

3 | RESULTS

3.1 | Effects of larval habitat on metamorph size and *Bd* infection

There was substantial variation in environmental conditions between the 10 breeding areas used in our experiment. The mean maximum temperature was 15.6°C in springs ($n = 2$), 25.7°C in fens ($n = 5$, one missing temperature data), and 37.42°C in pools ($n = 2$, one missing temperature data). Mean volume lost from May to June was 4.6% in springs (one missing site), 21.4% in fens (two missing sites), and 23.3% in pools (see Table S1 for details). In spite of this variation, body size did not differ between larval habitats (spring vs. fen vs. pool) for the 186 metamorphs used in the itraconazole exposure experiment ($F_{2,6.3} = 0.97$, $p = .43$). The infection intensity in recently metamorphosed frogs from fens was at least an order of magnitude lower than that of frogs from pools and springs (means – fen: 52.8 ZE, pool: 918.0 ZE, spring: 1965.8 ZE; larval habitat effect: $p = .007$; Figure 1a, b; Table S3 and S4).

3.2 | Effects of itraconazole treatment on *Bd* infection and apparent overwinter survival

By the end of the 6-day treatment period, the mean *Bd* infection intensity for itraconazole-treated frogs was 5.1 ZE, which was approximately an order of magnitude lower than that of the same animals prior to treatment (pre-treatment mean: 101.8 ZE; time effect: $P < .001$; Figure 1b, d; Table S5A), and approximately two orders of magnitude lower than that of experimental controls after the treatment period (experimental controls post-treatment mean: 4024.5 ZE; treatment effect: $p < .001$; Figure 1b, d; Table S5B). Of the 95 treated frogs, 39% went from *Bd* positive to *Bd* negative during the treatment period, 21% remained *Bd* positive, 34% remained *Bd* negative, and 6% went from *Bd* negative to *Bd* positive; for the 88 experimental control frogs, 6% went from *Bd* positive to *Bd* negative, 61% remained *Bd* positive, 5% remained *Bd* negative, and 28% went from *Bd* negative to *Bd* positive. Relatively few frogs were recaptured in fall surveys approximately 3 and 6 weeks after the treatment period (5 treated, 8 experimental controls, and 5 field controls; Figure S4).

We recaptured 26 of the 97 frogs treated with itraconazole the summer following treatment, 9 of the 146 field controls, and only 1 of the 89 experimental control frogs. Our Cormack–Jolly–Seber model provided strong support for differences in apparent overwinter survival between treatment groups with an estimated apparent overwinter

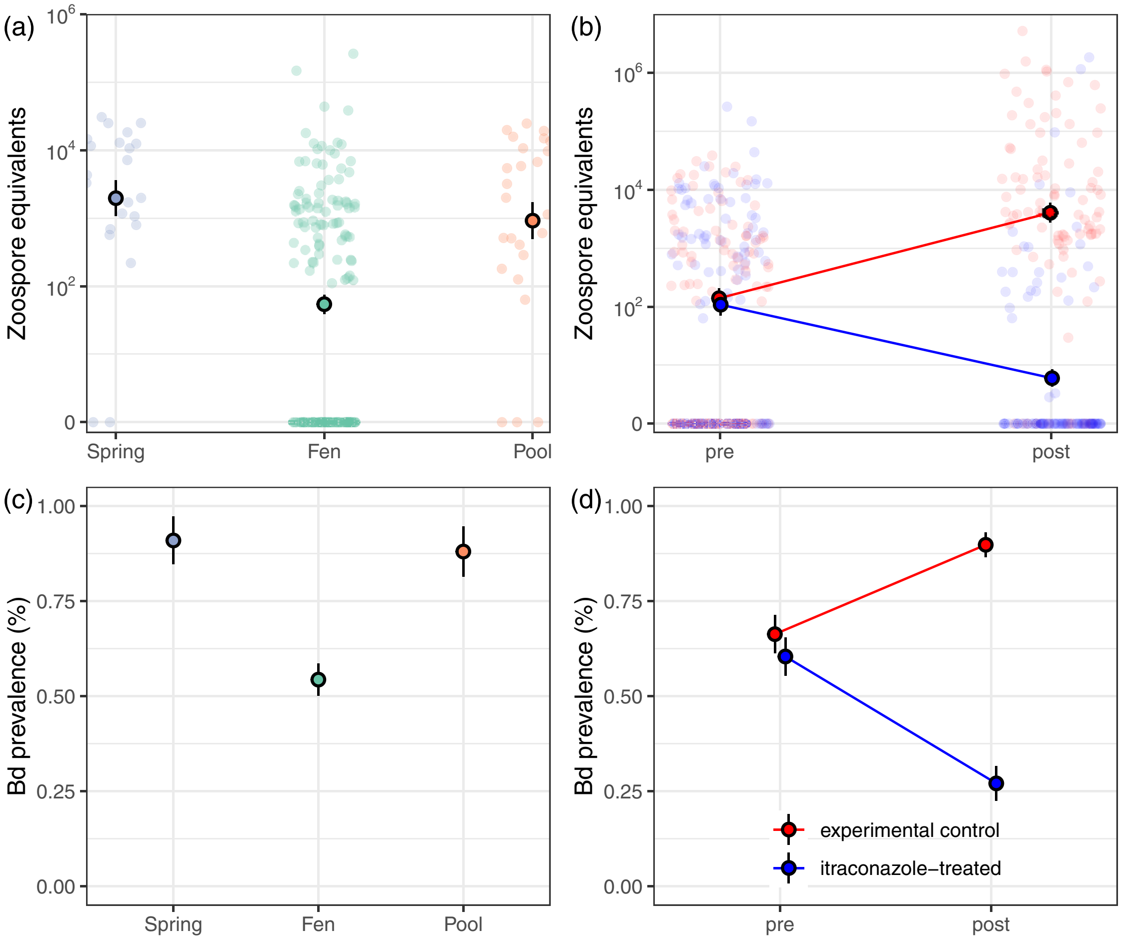

FIGURE 1 Effects of larval habitat and itraconazole treatment on *Bd* infection in recently metamorphosed Cascades frogs Infection intensity and *Bd* prevalence of recently metamorphosed Cascades frogs from different larval habitat types prior to treatment (panels A, C); panels B and D compare *Bd* infection and prevalence prior to treatment ("pre") and at the end of the 6-day treatment period ("post"). Raw data and associated means and standard errors are shown. Note log scale on y-axis in panels A and B; means and standard errors in these plots are based on log-transformed data.

survival probability for itraconazole-treated frogs (mean apparent overwinter survival estimate: 0.48, 95% credible interval: 0.21–0.86) that was 4.4 times greater than that of field controls (0.11, 0.03–0.29), which in turn was 5.5 times greater than that of experimental controls (0.02, 0.001–0.06) (Figure 2). For the posterior distribution samples of the apparent overwinter survival estimates, 100% of the posterior samples were higher for treated frogs than the field control frogs; this was also true for treated versus experimental control frogs. Field control apparent survival estimates were higher than experimental control frogs in 99% of posterior draws.

In 2012, 28 Cascades frog metamorphs were treated with itraconazole as part of a previous experiment. Surveys conducted between 2014 and 2019 revealed that most of the 7 treated frogs that survived their first winter survived to adulthood (5 of 7; 71%), while none of the 28 control animals were seen again after 2012.

3.3 | Surviving young in previous year

In 2019, the summer after treatment, infection intensity was approximately one order of magnitude lower in itraconazole-treated frogs than in other frogs from the same cohort that were not marked the previous year (means – treated: 30.1 ZE, other frogs: 311.7 ZE; treatment group effect: $\chi^2 = 9.8$, df $= 4$, $p = .046$; Figure 3; Table S6; treated vs. other frogs contrast: $p = .01$); field controls did not differ significantly from either of the other groups (mean: 190.2 ZE; contrasts with field controls: $p > .2$). There were no significant differences in body size between frogs from different treatment groups, although there was a marginally significant tendency for itraconazole-treated frogs to be larger (by 18%) than other frogs (Figure 3; Table S6; Tukey-adjusted contrast: $p = .06$).

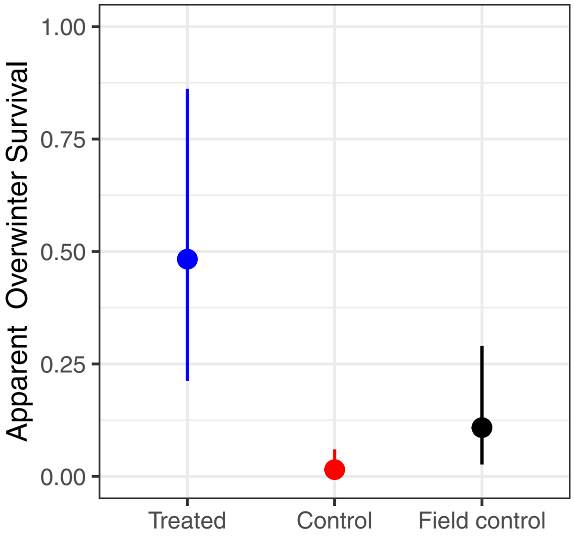

FIGURE 2 The effects of treatment with itraconazole on apparent overwinter survival of Cascades frog metamorphs Estimated apparent overwinter survival of recently metamorphosed frogs treated with itraconazole, experimental controls receiving a sham treatment, and field controls (marked and left untreated). Error bars represent 95% credible intervals derived from the Bayesian Cormack–Jolly–Seber model described in the text.

3.4 | Population dynamic implications of itraconazole treatment

Our population simulations show that an increase in metamorph survival consistent with that conferred by itraconazole treatment would increase the mean per capita population growth rate (λ) from 0.9 to 1.2 (Figure 4a). In addition, our simulations indicate that mean population growth rates are positive (i.e., $\lambda > 1$) when 40% of metamorphs are treated in 40% of years, with decreases in either variable requiring compensatory increases in the other to maintain positive population growth (Figure 4b).

4 | DISCUSSION

Our study showed that a single course of antifungal treatment in newly metamorphosed frogs from wild populations affected by the fungal pathogen *Bd* can dramatically increase an individual's probability of apparent survival through their first winter, and potentially decrease their infection intensity the following summer. Applying the differential apparent survival of treated and untreated metamorphs to population projections suggests that a management program based on the antifungal treatment of metamorphs has the potential to restore population viability. We also found that *Bd* infection intensity was lower in recently metamorphosed frogs emerging from larval habitats that tend to be intermediate in temperature and drying rate.

4.1 | Effects of larval habitat on *Bd* infection

This study demonstrated the effect of larval habitat on pathogen infection intensity after metamorphosis. Specifically, *Bd* infection in recently metamorphosed frogs tended to be higher for springs (which tend to be cool and hydrologically stable) and pools (which tend to be warm and have high drying rates) and lower in fens

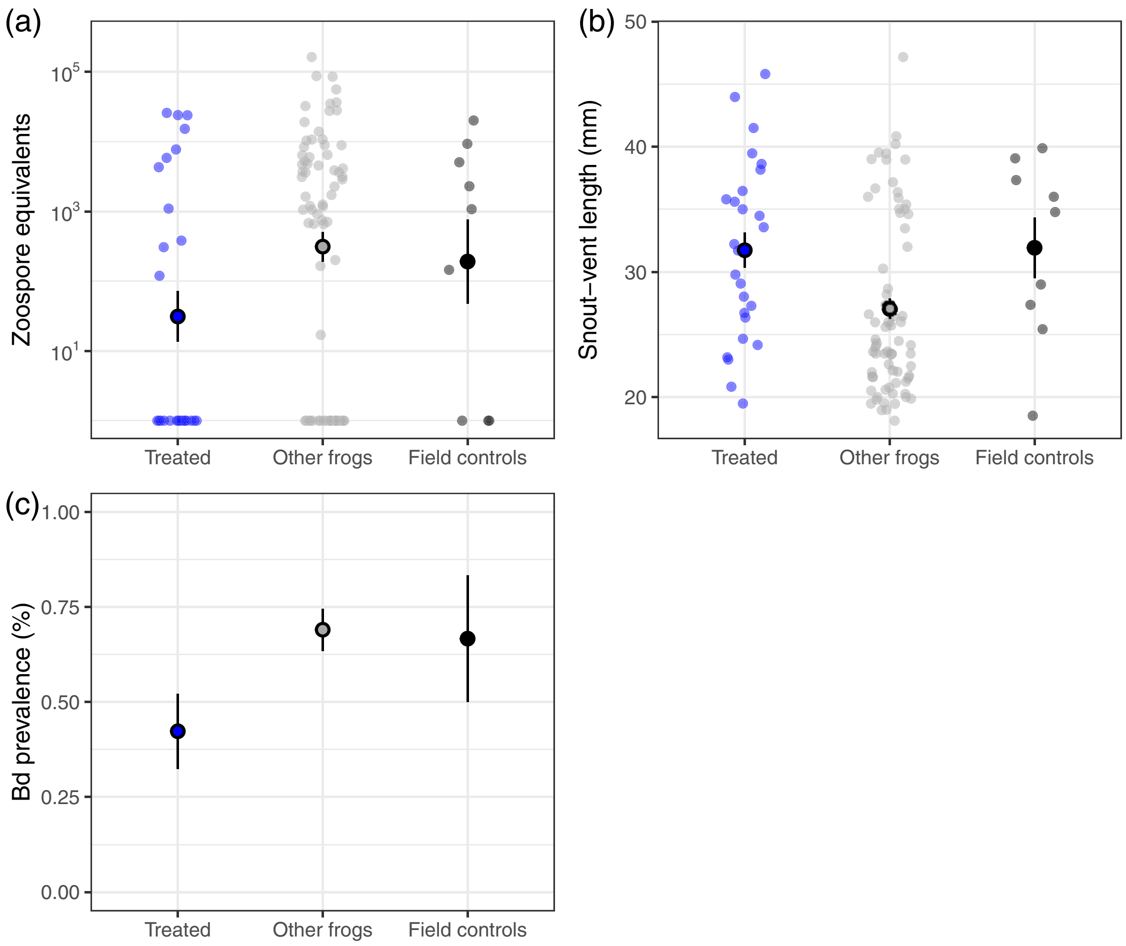

FIGURE 3 The effects of treatment with itraconazole on (a) *Bd* infection intensity, (b) body size, and (c) *Bd* infection prevalence in young of previous year Cascades frogs the summer following treatment Frogs captured in 2019 include frogs treated with itraconazole ($n = 26$), field controls ($n = 9$), and other young of the previous year (other frogs; $n = 73$) which are from the same cohort as treated and field control frogs but were not part of the experiment. Raw data and associated means and standard errors are shown. Note log scale on y-axis in panel A.

(which tend to be intermediate in temperature and drying rate). These results suggest a "Goldilocks effect" of larval environmental conditions on *Bd* infection intensity. While the effect of larval habitat on *Bd* infection could be driven by many factors, we hypothesize that metamorphs emerging from spring habitats had higher *Bd* infection intensity than those emerging from fens because water temperatures in springs (see Table S1) did not exceed the critical range for *Bd*, which is thought to be 4–27°C (Piotrowski et al. 2004; Stevenson et al. 2013; Voyles et al. 2017), and because host immune capacity, and thus ability to resist *Bd* infection, may be reduced at lower temperatures (Rollins-Smith et al. 2011). In addition, we hypothesize that metamorphs emerging from surface-water pools may be more susceptible to infection than those emerging from fens because temperatures in pools may regularly exceed the frog's thermal optimum (i.e., the thermal mismatch hypothesis for cool-adapted species; Cohen et al. 2017; Sauer et al. 2020), and because rapid dry down of pools may decrease post-metamorphic immune function (Gervasi & Foufopoulos 2008; Rollins-Smith et al. 2011; Kohli et al. 2019). However, it is also possible that larval habitats differed in other factors that could be related to *Bd* infection, including density and contact rates (Rachowicz & Briggs 2007; Briggs et al. 2010; Bosch et al. 2020) and host genetic factors related to *Bd* resistance (e.g., Savage & Zamudio 2011, 2016; Bataille et al. 2015).

Such effects of larval habitat may play an important role in determining the impact of climatic change on interactions between amphibians and *Bd*. Years in which optimal larval habitat is not available in wet meadows (e.g., drought years) are expected to become more common based on climate projections for western North America (Cook et al. 2004). Thus, the effects of climate change on environmental conditions during early life

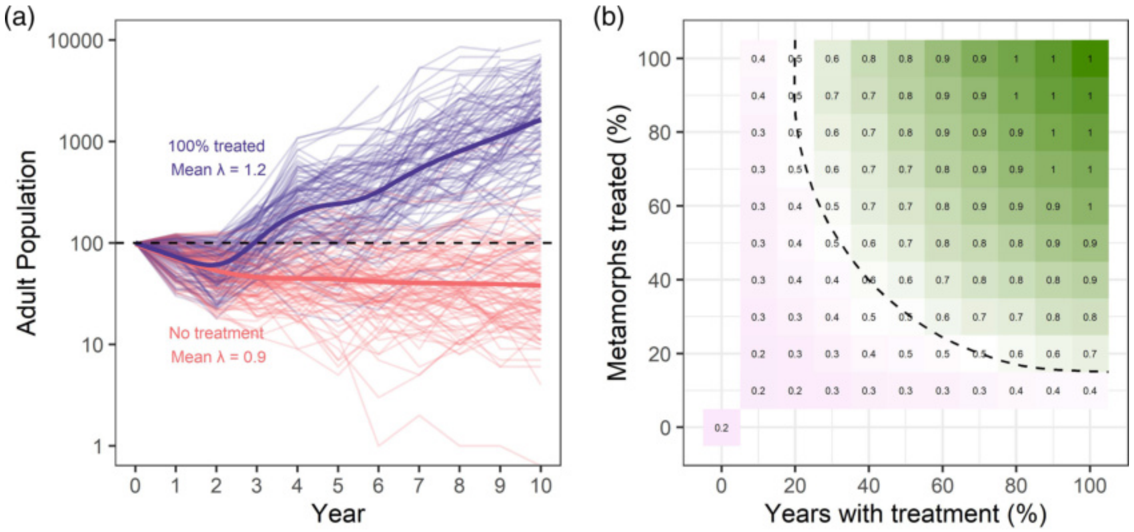

FIGURE 4 Projected population-level consequences of itraconazole treatment (a) Adult population size in simulations without any itraconazole treatment (red) and with treatment of all metamorphs in each year over a 10-year period (blue); heavy lines show the mean across simulations and faint lines show the results of 100 individual simulations. (b) The effect of treatment frequency and proportion of metamorphs treated on population growth rate (λ). The dashed line shows the contour representing the difference between population increase (mean λ > 1) and population decline (mean λ < 1). The numbers within the boxes represent the proportion of simulations with positive population growth.

stages may exacerbate the impact of *Bd* on Cascades frog populations in northern California. Habitat modification oriented toward improving breeding habitat, either alone or in concert with in situ treatment, may represent a promising management intervention in scenarios such as these (Brannelly et al. 2021).

4.2 | Effects of treatment on early post-metamorphic life stages

We detected dramatic reductions in *Bd* infection intensity after a single course of treatment with itraconazole and a dramatic increase in apparent overwinter survival of itraconazole-treated frogs compared to controls, presumably due to a reduction in chytridiomycosis-related mortality. This treatment effect is consistent with previous field-based itraconazole treatment studies in Cascades frogs (Hardy et al. 2015) and other amphibian species (e.g., Hudson et al. 2016; Geiger et al. 2017; Knapp et al. 2022). It is likely that itraconazole treatment enhanced apparent survival by helping to maintain infection intensity below a critical threshold for disease-induced mortality (Vredenburg et al. 2010) during the overwintering period, which is when *Bd* infection and *Bd*-related mortality peak for many species (Rachowicz & Briggs 2007; Kinney et al. 2011; Stevenson et al. 2013; Brannelly 2014; Rumschlag & Boone 2018). It is also possible that reduced *Bd* infection facilitates apparent overwinter survival by allowing frogs to maintain cold tolerance (Siddons & Searle 2021).

The persistent reduction in *Bd* infection intensity in treated frogs the summer after metamorphosis (Figure 3a, c) suggests that the application of an antifungal treatment shortly after metamorphosis can have persistent effects on immune capacity and the progression of infection. These results are supported by further analysis of long-term apparent survival rates from a previous single itraconazole exposure experiment at Carter Meadow. In 2012, 28 metamorphs were treated with itraconazole and 7 of the treated frogs survived their first winter compared to none of the controls (Hardy et al. 2015). Follow-up surveys revealed that 71% of the treated frogs that survived their first winter survived to adulthood, which is greater than the estimated juvenile to adult apparent survival rates for our study system (see Supplementary Materials). It is unlikely that itraconazole persists in amphibian skin (Stice & Briggs 2010; Cashins et al. 2013), so these longer-term reductions in infection are likely the result of the persistent enhanced immune capacity to prevent *Bd* proliferation. There are many possible explanations for such persistent enhanced immune capacity, including both adaptive immunity and priming of the innate immune system (Grogan et al. 2018; Waddle et al. 2021).

Our results differ from previous field studies that did not find evidence of persistent effects of itraconazole treatment on apparent survival or *Bd* infection (Bosch et al. 2015; Hudson et al. 2016; Geiger et al. 2017; Knapp et al. 2022 [this study found persistent effects of treatment for adults, but not for earlier life stages]). Our treatment targeted a specific period (shortly after metamorphosis) when Cascades frogs are highly susceptible to *Bd* and experience dramatic increases in *Bd* prevalence (Hardy et al. 2015). Our reasoning was that we might be able to slow infection at a critical time to allow for priming of the innate immune system (Grogan et al. 2018). A similar targeted treatment strategy may help achieve persistent treatment effects in other at-risk species and populations.

It is possible that the positive effects of itraconazole treatment on apparent overwinter survival of metamorphs are not solely due to the deleterious effects of itraconazole on *Bd*. For example, other fungal pathogens could also be suppressed by itraconazole. However, multiple lines of evidence point to *Bd* as the primary cause of enhanced mortality in untreated individuals. First, *Bd* has been shown to have dramatic effects on the survival of recently metamorphosed Cascade frogs in controlled laboratory exposures of captive-reared *Bd*-naïve frogs (Piovia-Scott et al. 2015). Second, *Bd* outbreaks in wild Cascades frog populations are associated with dramatic reductions in recruitment to the subadult life stage, suggesting that *Bd*-related mortality of early post-metamorphic life stages is also important outside of the laboratory (Piovia-Scott et al. 2015). Finally, a study of *Bd* in Cascades frog museum specimens from northern California suggested that the timing of *Bd*'s arrival in the region is consistent with the onset of regional Cascades frog declines (de León et al. 2017).

Unlike some previous studies (e.g., Hardy et al. 2015), itraconazole treatment did not appear to decrease the growth of treated frogs (Figure 3b). While previous itraconazole studies identified the potential for negative side effects (Baitchman & Pessier 2013; Hardy et al. 2015), our results suggest that the lowest concentrations shown to reduce *Bd* in the lab (Brannelly 2014) can translate into increased apparent survival in the field with limited negative side effects. We hypothesize that the negative effects of itraconazole treatment on growth found in a previous study in this system (Hardy et al. 2015) were due to the use of a higher itraconazole concentration.

It is worth noting that the experimental controls experienced dramatic increases in *Bd* infection during the 6-day treatment period (Figure 1b, d), and reduced apparent overwinter survival compared to field controls. This may be at least partially attributable to increased *Bd* transmission in the absence of treatment under the experimental housing conditions – experimental controls (like intraconazole-treated frogs) were housed in groups of 5–10. Future treatment studies and management interventions should consider the potential effects of housing conditions on disease transmission, especially when control animals are used.

4.3 | Population dynamic implications of itraconazole treatment

Our population simulations suggest that itraconazole treatment of recently metamorphosed Cascades frogs in populations with endemic *Bd* could shift population growth trajectories from negative to positive. This is somewhat surprising, as population dynamics of pond-breeding amphibians with explosive breeding potential are thought to be relatively insensitive to changes in the survival of early life stages (Biek et al. 2002). However, other simulation studies have suggested that Bd-related overwinter mortality of juvenile pond-breeding frogs can drive population declines (Rumschlag & Boone 2018). It appears that the effects of *Bd* on early post-metamorphic life stages are severe enough to cause population declines in multiple species. Fortunately, the efficacy of itraconazole treatment at this life stage (at least for Cascades frogs) suggests that population recovery may be achievable through treatment. Importantly, our study does not indicate that treating early life stages is more effective at restoring population viability than treating later life stages. Rather, we find that treating early life stages may be sufficient to achieve positive population growth rates.

In some amphibian populations undergoing high mortality due to *Bd*, compensatory recruitment, whereby increased reproductive investment counters increased mortality, has been shown to stabilize or even reverse declining population trends (Muths et al. 2011; Brannelly et al. 2021). However, in cases where metamorphs experience high *Bd*-related mortality, compensatory recruitment may not counter increased mortality without additional disease management intervention.

It is possible that our simulations underestimate the effect of our itraconazole treatment on Cascades frog populations and the potential increase in population growth rates resulting from itraconazole treatment. We only considered the effects of treatment on survival during the first winter, and the survival of treated frogs during later life stages may be higher than the estimates used in our model, which are inferred from long-term population monitoring data (see discussion of persistent effects of itraconazole treatment in the previous section). Thus, it is possible that positive population growth could be achieved with less ambitious management plans than those depicted in Figure 4b. Under conditions of a known *Bd* outbreak, we recommend including older life stages in treatments to counter the expected decreased survival of these important life stages. It is worth noting that our estimates for mean population growth rates mask substantial variation in outcomes between simulation runs (Figure 4a). In addition, our simulations likely underestimate the impact of environmental stochasticity, which only influences adult survival in our model, and thus does not have any direct influence on the impact of *Bd* on Cascades frog populations.

4.4 | Conclusions and management implications

We found that treating recently metamorphosed frogs with itraconazole can be an effective management tool for maintaining the viability of at-risk amphibian populations threatened by chytrid fungal pathogens. Previous studies have emphasized that the effects of treatment are likely to be short term, requiring frequent, ongoing treatment to achieve long-term management goals (Langwig et al. 2015; Garner et al. 2016; Knapp et al. 2022). Our study suggests the potential for longer-term effects of treatment – also observed for adult mountain yellow-legged frogs by Knapp et al. (2022) – which may allow for the development of intermittent treatment plans to achieve long-term management goals.

Our study provides additional insights into the effects of antifungal treatment that may be applicable in other systems. First, there appear to be persistent reductions in pathogen infection and apparent survival rate in treated individuals, despite continued exposure to the pathogen. Second, the treatment appears to facilitate the passage of individuals through a critical early-stage life-history transition, and long-lived adults (which appear to be less likely to succumb to disease) may buffer at-risk populations from extirpation. It is worth noting that regularly implementing in situ treatment with antifungal agents could hinder the selective processes that promote *Bd* resistance or tolerance, the development of which is thought to underly the best-documented cases of recovery in the face of ongoing *Bd* exposure (Knapp et al. 2016; Voyles et al. 2018). However, these selective processes only occur in extant populations, so antifungal treatments that prevent population extirpation may still increase the chances of such evolutionary rescue. Further research is needed to evaluate the impacts of different treatment strategies on the potential for evolutionary rescue in species affected by *Bd* (Converse et al. 2017; Christie & Searle 2018; DiRenzo et al. 2018; Canessa et al. 2019; Scheele et al. 2019).

We expect treatment of hosts to be most beneficial when host populations have a long-lived life stage that is relatively resistant to disease and when treatment can be targeted at times and places where environmental conditions and host life history lead to enhanced disease susceptibility. In situations where all life stages are highly susceptible to disease (e.g., Sierra Nevada Yellow-legged frogs), treatment may not be an effective disease mitigation strategy, and management efforts should focus on other approaches such as expanding populations that are coexisting with *Bd* in an enzootic state (Knapp et al. 2022). However, many amphibian species experience reduced *Bd* susceptibility in later life stages (Fisher et al. 2009; Rollins-Smith et al. 2011; Sauer et al. 2020), and in these contexts, in situ treatment of susceptible early life stages may amplify the effects of other management actions, such as habitat manipulation. Furthermore, using in situ treatment to maintain populations on the landscape can provide additional source populations for future translocations or captive breeding programs. Finally, one of the main advantages of in situ treatment is that it features relatively low supply costs (see Table S7 for an estimate of treatment costs), and is likely to be less expensive, more logistically feasible, and feature fewer adverse side effects to other species than some other interventions, such as supplementing populations from captive breeding programs or environmental application of fungicides. Thus, in situ treatment of individual hosts may be a useful component of a comprehensive management strategy to reduce the risk of pathogen-mediated population declines and extirpations.

ARTICLE IMPACT STATEMENT

Integrating a field-based treatment experiment with population modeling demonstrates the potential to restore population viability.

AUTHOR CONTRIBUTIONS

Kimberly Cook, Karen Pope, and Jonah Piovia-Scott conceived and designed the study; Kimberly Cook led the field experiment and all authors contributed to field work; all authors contributed to data analysis; Adam Cummings and Karen Pope fit mark–recapture models to field data and performed population simulations; Kimberly Cook wrote the first draft of the manuscript; Jonah Piovia-Scott wrote subsequent drafts; and all authors contributed substantially to manuscript revisions.

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

SUPPORTING INFORMATION

Supplementary methods include a map of the study area, characteristics of the breeding areas used in the experiment and photographs of selected breeding areas, and a detailed description of the model used for population simulations. Supplementary results include tables of statistical results, supplementary data figures, and cost estimates for itraconazole treatment.

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DATE AVAILABILITY STATEMENT

Data and scripts used for analyses are available through the United States Forest Service Research Data Archive[1].

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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