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Authors

Adams, Andrea J
Bushell, Jessie
Grasso, Robert L

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ARTICLE

Disease Ecology

To treat or not to treat? Experimental pathogen exposure, treatment, and release of a threatened amphibian

Andrea J. Adams[1,2] | **Jessie Bushell**[3] | **Robert L. Grasso**[1]

[1]Resources Management and Science Division, Yosemite National Park, El Portal, California, USA

[2]Earth Research Institute, University of California, Santa Barbara, California, USA

[3]San Francisco Zoo, San Francisco, California, USA

Correspondence
Andrea J. Adams
Email: andrea_adams@ucsb.edu

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Abstract

Reintroductions may mitigate the effects of severe amphibian declines, but chytridiomycosis—the often-deadly disease caused by the fungal pathogen *Batrachochytrium dendrobatidis* (Bd)—can hinder these efforts. Exposing amphibians to the pathogen ex situ may improve disease outcomes post-release. We experimentally inoculated federally threatened California red-legged frogs (*Rana draytonii*) ex situ, cleared Bd infections, and then released them to the wild for monitoring in an area where Bd is present. Ex situ, previously exposed frogs had lower Bd loads than previously uninfected frogs, and all exposure groups, including previously uninfected frogs, naturally reduced their Bd loads in two independent experiments. After release to the wild, we observed no differences in field-contracted Bd infection among exposure groups. Treating post-metamorphic *R. draytonii* ex situ may not confer additional post-release benefit to this species in terms of chytridiomycosis burden. Our results, however, do not necessarily mirror Bd susceptibility throughout the amphibian life cycle, with other source populations that may have different Bd infection histories, or responses to different Bd strains. These results provide a clearer understanding of post-release disease responses of a threatened, Bd-susceptible species with prior pathogen exposure. Following ex situ experiments with in situ applications can provide a more comprehensive understanding of threat outcomes for declining species in reintroduction programs and strengthen critical links between ex situ and in situ conservation partners.

KEYWORDS
amphibian declines, disease mitigation, endangered species, ex situ conservation, frog conservation, immunization, itraconazole, translocations

INTRODUCTION

Half of all amphibian species are at risk of extinction (González-del-Pliego et al., 2019), and nearly 3% are in captive breeding and reintroduction programs (Harding et al., 2016). Ineffective or nonexistent links between captive conservation programs and in situ partners can hamper reintroduction success (Karlsdóttir et al., 2021),

and many amphibian reintroductions report failure, especially when disease effects are unmitigated (Fellers et al., 2007; McFadden et al., 2010; Stockwell et al., 2008). Chytridiomycosis, a disease caused by the fungal pathogen *Batrachochytrium dendrobatidis* (Bd), is among the primary causes of amphibian declines globally (Collins et al., 2009). Although chytridiomycosis can be devastating to susceptible amphibian populations, evidence of persistence and recovery provides hope (Catenazzi et al., 2017; Knapp et al., 2016). Determining the factors driving persistence versus extirpation, and exploiting those factors in reintroduction programs, is key to developing disease mitigation strategies that can facilitate the recovery of Bd-susceptible species (Mendelson et al., 2019).

Chytridiomycosis susceptibility varies widely between species (Fisher et al., 2009) and among different populations of the same species (Joseph & Knapp, 2018; Rosenblum et al., 2010), ranging from extirpation upon initial arrival to persistence with sublethal infections in an enzootic state (Briggs et al., 2010; Catenazzi et al., 2017; Retallick et al., 2004). In highly Bd-susceptible ranids, across-year adult survival is the most important mechanism for population persistence, and small changes in host resistance and tolerance can have dramatic effects on individual survival and population persistence (Briggs et al., 2005; Wilber et al., 2017).

Immune defenses are likely among the most significant factors affecting Bd susceptibility and resistance/tolerance, disease progression, and population recovery (Rollins-Smith, 2017). Both adaptive (delayed, pathogen-specific) and innate (first-line, rapid, pathogen non-specific) immunity are likely important for fighting chytridiomycosis infection (Richmond et al., 2009; Savage & Zamudio, 2011; Woodhams et al., 2007). Adaptive immunological responses to repeated experimental Bd exposures have been treatment and species dependent (McMahon et al., 2014; Stice & Briggs, 2010; Venesky et al., 2014). The effect of prior Bd infection on disease outcome ranges from no effect on mortality (Cashins et al., 2013; DiRenzo et al., 2014) to increased survival under certain environmental conditions (Murphy et al., 2011) and reduced pathogen burdens coinciding with increased survival (McMahon et al., 2014).

The application of antifungal treatments to susceptible hosts both ex situ and in situ may improve immediate survivorship probabilities and disease outcomes. In the case of reintroductions after populations have been completely extirpated, tolerant hosts and environmental reservoirs remain (Daszak et al., 1999; Garmyn et al., 2012; Hudson et al., 2019; Mazzoni et al., 2003; Mitchell et al., 2008), providing a pathogen reservoir for future infections and disease outbreaks after animals are released into the wild. Therefore, using ex situ infection trials to activate immune responses prior to release may improve post-release disease and survivorship outcomes and thus long-term survival and recovery (Brannelly et al., 2016; Knapp et al., 2021; Scheele et al., 2014; Waddle et al., 2021; Woodhams et al., 2011). Despite growing research in Bd immunity mechanisms, studies that explicitly use field trials immediately relevant to current management decisions, including reintroductions, are rare but imperative (Canessa et al., 2019).

The federally threatened California red-legged frog (*Rana draytonii*; Figure 1) has declined from over 70% of its former range (U.S. Fish and Wildlife Service, 1996), prompting a call for pilot captive rearing and reestablishment efforts (U.S. Fish and Wildlife Service, 2002). Habitat loss, overharvest, and non-native aquatic predators and competitors have been identified as the primary drivers of the species' decline (Jennings & Hayes, 1994; U.S. Fish and Wildlife Service, 2002). Chytridiomycosis may have also played a role; however,

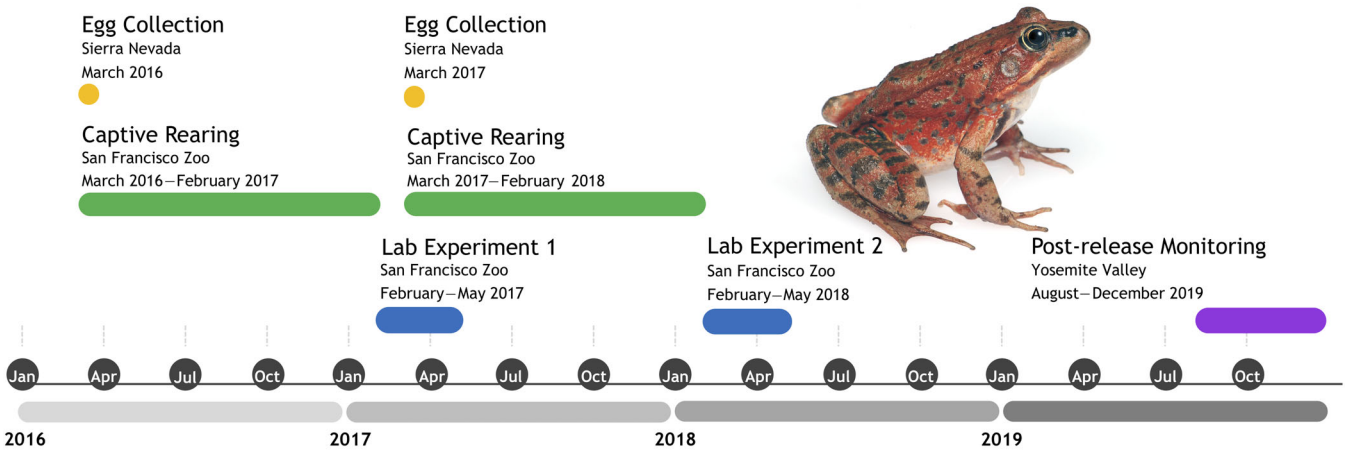

FIGURE 1 Study timeline. California red-legged frog (*Rana draytonii*) photo credit: Robert Hansen.

Bd susceptibility in *R. draytonii* is poorly understood. In situ, one chytridiomycosis-induced mortality was documented in an early post-metamorphic individual (Adams et al., 2020). Ex situ, individuals with wild-contracted infections of unknown intensity exhibited no mortality during a brief observational study (Padgett-Flohr, 2008). High estimated Bd prevalence in *R. draytonii* has been associated with lower survival (Russell et al., 2019); therefore, chytridiomycosis may be affecting populations despite infrequent observation of mortality events (Fellers et al., 2011; Piovia-Scott et al., 2015). Extant *R. draytonii* populations in California may exist in a Bd-enzootic system: the earliest detection of the pathogen was in 1915 in California (Adams, Pessier, & Briggs, 2017) and in the 1970s in the Sierra Nevada (Green & Kagarise Sherman, 2001). Persistent mortality, however, can negatively affect populations in a post-epizootic state (Catenazzi et al., 2017). Inoculation may be a viable intervention for translocated individuals if *R. draytonii* is Bd susceptible and prior pathogen exposure improves disease outcomes post-release.

We conducted ex situ inoculation trials with post-metamorphic *R. draytonii* through Bd exposure and subsequent Bd clearance using an antifungal drug ("treatment"), and then released these frogs into the wild where Bd is present in order to address the following questions: (1) To what extent is *R. draytonii* susceptible to Bd infection? (2) Does treatment reduce subsequent Bd infection burden? (3) Does such a response last at least one year? (4) Does treatment affect Bd infection after release? and (5) Are treatments a necessary aspect of conservation translocation efforts for *R. draytonii*? We hypothesized that ex situ treatment would lead to a decrease in subsequent Bd infection burden, and that this effect would persist for at least 1 year, leading to higher disease resistance after release. If the species is highly Bd susceptible and inoculations reduce subsequent infection loads, treatments may minimize the risk of post-release chytridiomycosis burden.

METHODS

We collected *R. draytonii* eggs from the wild and reared them in captivity. Once they metamorphosed, we conducted two ex situ infection experiments on first- and second-year frogs (one each in 2017 and 2018). After these experiments, 2- and 3-year-old frogs were released into the wild in Yosemite Valley, CA, USA, in 2019 and monitored for an additional field experiment (Figure 1). For clarity, we restrict the use of terms as follows: "laboratory experiment" refers to the full course of each of the two ex situ Bd exposure experiments; "exposure" refers to experimental exposure of frogs to the Bd pathogen; "antifungal treatment" refers to the application of an antifungal drug to clear Bd from frog skin; and "exposure group" refers to groups of frogs by Bd exposure type. Exposure groups include frogs that were exposed to Bd once within each experiment ("1×"), those that were exposed, cleared with an antifungal drug, and re-infected ("2×"), 2×-infected frogs that were exposed to the pathogen a third time ("3×"), and sham controls.

Study species

R. draytonii currently ranges from Mendocino County, California in the north (Shaffer et al., 2004) to northern Baja California, Mexico in the southernmost portion of the range (Peralta-Garcia et al., 2016). The translocation of the species to Yosemite Valley was facilitated by the successful eradication of non-native American bullfrogs (*Lithobates catesbeianus*, a *R. draytonii* predator and competitor) from the site (Kamoroff et al., 2020). Despite the lack of definitive *R. draytonii* records from Yosemite Valley (Adams et al., n.d.), the conservation translocation of the species provides a safe harbor in a protected area for future reintroductions in the greater Sierra Nevada region.

Egg collection and captive rearing

Bd infects the skin of metamorphosed individuals and the keratinized mouthparts of tadpoles (Berger et al., 1998); therefore, we collected eggs from the wild to reduce the risk of Bd transport. We collected approximately 1700 *R. draytonii* eggs from a lotic donor site (Bear Creek Watershed, Sierra Nevada Mountains, El Dorado County, CA, USA) in March 2016 and 2017 (Figure 1). Because the source population can affect Bd outcome (Brannelly et al., 2016), and acquired resistance can be confounded with selection for other forms of resistance (Venesky et al., 2014), we sourced all egg masses from the same population. We reared frogs in reverse osmosis water, divided tadpoles into six 75-L tanks, and then moved them to 150-L tanks at Gosner Stage 25 (Gosner, 1960). After metamorphosis, juvenile frogs were moved to terrestrial enclosures of 1300-L stock tanks with mesh lids and ultraviolet-B full-spectrum lighting. Tadpoles were offered plated algae and algae gel, and frogs were fed crickets and a variety of worms powdered with calcium D_3 supplementation. We implanted passive integrated transponder (PIT) tags when frogs reached 40 mm (Appendix S1: Supplemental Methods).

Experimental Bd exposure and antifungal treatment

Overview

We conducted two laboratory infection experiments—one each in 2017 (hereafter "Laboratory Experiment 1") and 2018 (hereafter "Laboratory Experiment 2"; Figure 1). All frogs were swabbed prior to the start of both experiments to confirm that they were Bd-negative. Briefly, in Laboratory Experiment 1, we experimentally exposed a subset of frogs to Bd, cleared all frogs with an antifungal drug, and then subsequently Bd-exposed all frogs (except sham controls) in order to observe if frogs infected a second time had lower pathogen burdens as compared to frogs infected for the first time (Table 1). We repeated the experiment in 2018 with a single Bd exposure (Laboratory Experiment 2; Figure 1).

Bd isolates

Bd produces high variability in virulence across different strains (Becker et al., 2017; Berger et al., 2005; Doddington et al., 2013; Farrer et al., 2011); therefore, we exposed frogs to four Bd isolates (TST77, CJB4, CJB5, and CJB7) to minimize any strain-specific differences in infection. All isolates were cultured from *Rana sierrae* or *Rana muscosa*, two closely related and highly Bd-susceptible *R. draytonii* congeners. Isolates TST77 and CJB4 were isolated from sites where *R. sierrae* is persisting with infections, while CJB5 and CJB7 were isolated from sites where *R. muscosa* has been nearly extirpated following chytridiomycosis outbreaks. We cultured Bd isolates as described in Jani and Briggs (2018) (Appendix S1). We used these four Bd isolates because their use in previous studies (Jani et al., 2021; Jani & Briggs, 2018) has standardized the protocol for their use. Frogs in all experimentally Bd-exposed groups were exposed to the same mix of four strains under identical laboratory conditions.

Bd exposure experiments

Laboratory Experiment 1

For the first experiment in 2017, we randomly assigned 60 frogs from the 2016-collected cohort (Figure 1) to three exposure groups of 20 individuals each: sham control, 1×, and 2×. The 2× group was the exposure group in which we expected to observe lower Bd loads in response to a second Bd exposure after initial exposure and antifungal treatment (Table 1).

Laboratory Experiment 2

In an effort to (1) repeat the results of Experiment 1, and (2) evaluate any difference in Bd loads between first-time infected frogs and frogs previously Bd-exposed a year earlier, we conducted a second experiment in 2018. For this experiment, we exposed 20 previously Bd-exposed frogs (10 each from the 2× and 1× groups), 20 Bd-naïve frogs from the 2016 cohort used as sham controls in the 2017 experiment, and 20 Bd-naïve frogs from a newly collected and captive-reared 2017 cohort. Twenty frogs (all from the new, 2017 collection cohort) served as sham-treated controls (Table 2).

Laboratory Experiments 1 and 2

Individual frogs were kept in separate enclosures containing 1.5 L of water for the duration of both laboratory experiments. We exposed frogs to 250,000 zoospores from each of the four isolates (10^6 zoospores total) per day for three consecutive days. We monitored frogs daily for clinical symptoms of chytridiomycosis, including lethargy, skin sloughing, loss of righting reflex, skin discoloration, and loss of appetite (Daszak et al., 1999; Nichols et al., 1998, 2001). Twenty-one days post-Bd exposure, we bathed all individuals, including sham controls, in itraconazole (Sporanox, Janssen Pharmaceuticals, 0.15% solution) for 5 min per day for 11 days.

To determine Bd status and load, we sampled frogs for Bd prior to each Bd exposure, at 7-day intervals during Bd infection, and three times, 2–4 days apart over the course of 7–10 days, after antifungal treatment. Frogs were also resampled for Bd 1–2 weeks prior to release into the wild to ensure all frogs were still Bd negative (Appendix S1:

TABLE 1 *Batrachochytrium dendrobatidis* (Bd) exposures and antifungal treatments by treatment group and day of experiment during Laboratory Experiment 1.

Exposure group	Days 1–3: Bd Exposure 1	Day 21: Antifungal Treatment 1	Days 51–53: Bd Exposure 2	Day 72: Antifungal Treatment 2
Infected 2×	Infected	Treated	Infected	Treated
Infected 1×	...	Treated	Infected	Treated
Control	...	Treated	...	Treated

Note: For all groups, $n = 20$.

TABLE 2 Individuals used in Laboratory Experiment 2 by treatment type, previous infection, and the year they were collected ("cohort").

Treatment group	Previous infection	Cohort	*n*
Infect (3×)	2× infected in Experiment 1	2016	10
Infect (2×)	1× infected in Experiment 1	2016	10
Infect (1×)	Naïve (sham control in Experiment 1)	2016	20
Infect (1×)	Naïve	2017	20
Sham control	Naïve	2017	20

Supplemental Methods). We swabbed frogs following a standardized protocol (Hyatt et al., 2007) using sterile rayon-tipped swabs (Medical Wire and Equipment Co., Wiltshire, England), extracted Bd DNA with PrepMan Ultra as described previously (Briggs et al., 2010), and quantified DNA with a TaqMan quantitative polymerase chain reaction (qPCR) assay (Boyle et al., 2004) (Appendix S1: Supplemental Methods). Standards were based on single internal transcribed spacer 1 (ITS1) PCR amplicons (Longo et al., 2013). To calculate zoospore equivalents (ZE; the quantification of Bd load), we multiplied the ITS copy number by 60 because Sierra Nevada strains have an average of 60 ITS1 copies per zoospore (Joseph & Knapp, 2018).

Releases and field sampling

Prior to release, we outfitted individuals with radio transmitters using aluminum beaded chains fitted around the waist following Tatarian (2008) and Rathbun and Murphey (1996). We released 45 frogs to Yosemite Valley in August 2019. In total, 15 frogs were released at each of three sites, five from each of the three exposure groups (1×, 2–3×, and sham control; Appendix S1: Table S1). Between August and December 2019, we used radiotelemetry to locate released frogs and attempted to recapture individual frogs once every 10–14 days. Upon capture, using a fresh pair of gloves for each frog, we recorded snout-vent length using dial calipers, weight using Pesola scales, and swabbed for Bd as described above. We placed swabs in individual screw-cap vials with approximately 200 µl of 95% ethanol, which was fully evaporated prior to DNA extraction. The DNA extraction and qPCR protocols followed the same procedure as described above.

Statistical analyses

Laboratory Experiments 1 and 2

In both laboratory experiments, we expected *R. draytonii* to exhibit infections ranging from resistance to infection or low-to-moderate Bd loads (~0–1000 ZE; a response consistent with innate immunity), based on previously published California field survey results (Adams, Kupferberg, et al., 2017; Adams, Pessier, & Briggs, 2017; Fellers et al., 2011). We also expected frogs infected for the first time to have higher peak Bd loads than previously infected frogs and for Bd loads to be lower for previously infected frogs than for frogs infected for the first time (i.e., exhibit a response consistent with adaptive immunity). We compared Bd load between exposure groups and performed all statistical analyses within exposure periods (on specific Bd sampling dates) so that comparisons were made among frogs exposed to the same Bd inoculate and concurrent laboratory conditions. We used *t* tests to compare Bd loads between exposure groups within experiment days, and paired *t* tests to compare Bd loads on the same individuals between two experiment days. The two dates selected for the paired *t* tests represent specific Bd load trajectory phases. For Laboratory Experiment 2, we expected Bd loads to decrease with the number of treatments; therefore, we compared Bd loads among all three exposure groups (1×, 2×, and 3× infected). We also expected frogs that had been inoculated one year earlier to continue to exhibit some level of pathogen resistance or tolerance; therefore, we combined the 2× and 3× infected groups into one "reinfected" category and compared it to the 1× group. We conducted ANOVA (with Tukey honestly significant difference post hoc pairwise comparison of means) to test if Bd loads differed across the three Bd exposure groups within each study day in Laboratory Experiment 2.

To compare Bd infection trajectories between exposure groups during the second infection phase of Laboratory Experiment 1, and during Laboratory Experiment 2, we fitted linear mixed effect (LME) models using the function "lme" from package "nlme" (Pinheiro et al., 2019), with exposure group and days post-exposure with orthogonal polynomial terms as fixed effects (Table 3), and Bd load as the response variable. We included polynomial terms in LME models of Bd load to account for the non-linear trajectory of Bd loads through time (Figure 2a). To account for repeated sampling of individuals in all LME models of the ex situ experimental data, we included a random effect of frog

TABLE 3 Variables used in mixed effects models of *Batrachochytrium dendrobatidis* (Bd) in experimentally infected *Rana draytonii*.

Variable	Range or levels	Description
Exposure group (predictor)	1×, 2×, 3×[a]	1×: previously uninfected frogs exposed to Bd once; 2×: frogs previously Bd-exposed once and re-exposed once; and 3×: frogs exposed to Bd three times
Exposure Group 2[a] (predictor)	Bd-naïve (previously uninfected; 1×), reinfected	Previously uninfected: 1× treatment group; reinfected: 2× and 3× individuals combined into one exposure group
Days post-exposure (predictor)	0–21	Number of days after Bd exposure
Bd load (response)	0–2932 ZE (Expt. 1) 0–17,492 ZE (Expt. 2)[a]	Bd infection burden, measured in ZE

Abbreviations: Expt., experiment; ZE, zoospore equivalents.
[a]Ranges or levels used only in models of Laboratory Experiment 2.

ID (i.e., PIT tag number). We excluded six frogs with equivocal Bd sample results from these statistical analyses—one Bd-negative frog that died of chytridiomycosis-unrelated causes and five frogs that each had one false Bd positive from qPCR well contamination (Appendix S1).

Post-release monitoring

We expected frogs to contract Bd in the wild post-release because Bd had previously been detected in Yosemite Valley (R. L. Grasso, unpublished data). Based on the results of our ex situ Bd exposure experiments, we expected frogs with prior Bd infections to have lower Bd loads and prevalence than Bd-naïve controls, but did not expect to observe a difference between 1× infected and 2–3× infected groups. We also expected a lower body condition in Bd-infected frogs. To test these hypotheses, we constructed four series of LME models, testing various predictors of Bd presence–absence, nonzero Bd loads, and body condition, with a fourth series testing various predictors of body condition in only Bd-positive samples (Table 4). We calculated body condition as $\log_{10}(\text{mass})/\log_{10}(\text{length})$ following Green (2001) and Brannelly et al. (2015). We used a paired *t* test to compare the body condition of frogs immediately before release and their mean body condition post-release. We used generalized linear mixed effect (GLME) models using the "glmer" function with a binomial response and logit-link function from the package lme4 (Bates, 2020) to determine the best predictors of Bd infection status (Bd presence or absence; Table 4); all other LME models were run with the "lme" function as described above. All models of post-release data included a nested random effect of frog ID within release site to account for repeated sampling of individuals at the same sites. This nested random effect was determined to be the best fit for the data compared to release site or frog ID alone. We calculated overall Bd prevalence (the proportion of released frogs infected with Bd) from individual swabs collected over the entire 4-month monitoring period. We considered swabs with ZE > 0 as positive and ZE = 0 as negative (Catenazzi & Kupferberg, 2017).

For all mixed effect models of both the in situ and ex situ data, we $\log_{10}$-transformed Bd load and used likelihood-ratio tests to compare nested models when finding optimal residual autocorrelation structures (Zuur et al., 2009). To determine the relative importance of predictor variables within each model set, we ranked candidate models according to Akaike information criterion corrected for small sample sizes (AIC_c), where models with a $\Delta AIC_c > 2$ as compared to the model with the lowest AIC_c were considered not as well supported by the data (Burnham & Anderson, 2004). We used the "binom.test" function to calculate Bd prevalence for the in situ and ex situ experiments. All analyses were performed using R (R Core Team, 2019).

RESULTS

Laboratory Experiment 1

All post-metamorphic frogs were Bd-negative prior to the start of the experiment, and none of the unexposed controls tested positive for Bd for the duration of the experiment. No frogs exhibited outward symptoms of chytridiomycosis infection (skin sloughing or behavioral changes), and we observed high (>99%) survivorship (Appendix S1). One frog (from the 2× group) died, but did not test Bd positive after repeated Bd sampling before its death on Experiment Day 81. We do not attribute this mortality to chytridiomycosis due to the absence of characters typical of chytridiomycosis, such as epidermal lesions and zoosporangia (Pessier et al., 1999); see Appendix S1.

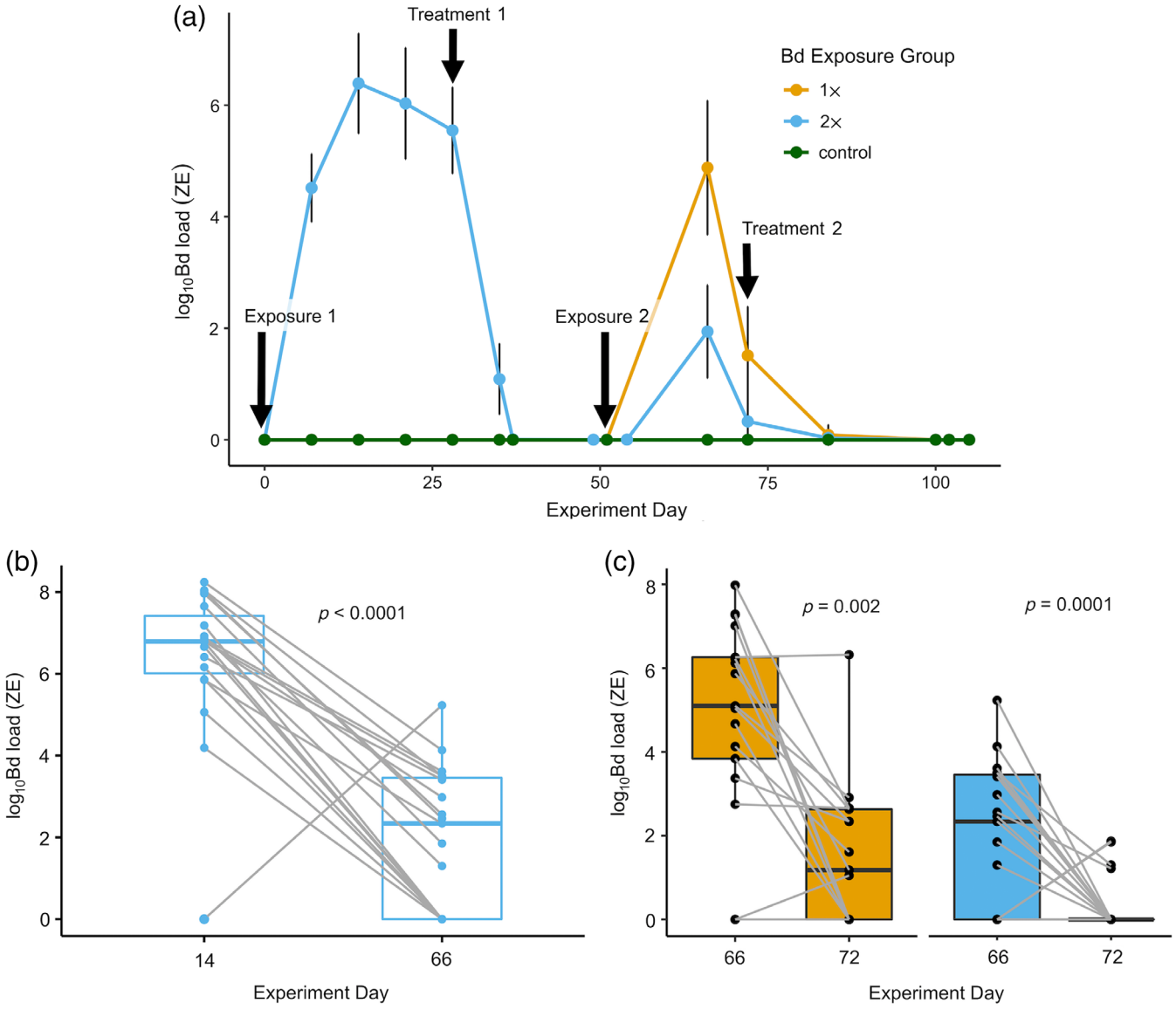

FIGURE 2 Pathogen burden of *Batrachochytrium dendrobatidis* (Bd load; in zoospore equivalents [ZE]) in California red-legged frogs (*Rana draytonii*) by exposure group during Laboratory Experiment 1. (a) Pathogen burden over Laboratory Experiment 1 timeline. Exposure groups: 1× = exposed to Bd once; 2× = Bd-exposed, cleared, and re-exposed; control = Bd-unexposed sham controls. "Exposure" refers to Bd exposure; "treatment" refers to the application of an antifungal drug to clear Bd infection. Error bars indicate 95% confidence intervals. (b) Paired data within the 2× Bd exposure group, showing the difference in peak pathogen burden (Experiment Days 14 and 66) after Bd exposure and prior to antifungal treatment. (c) Difference in peak Bd loads during the second exposure phase (between Experiment Days 66 and 72, prior to antifungal treatment) by exposure group (1× = gold; 2× = blue).

Exposure 1

All but one (95%) of the non-control frogs became infected during the first Bd exposure in Laboratory Experiment 1 (mean Bd load 905 ZE ± 1206 SD; Appendix S1: Table S2). Bd loads declined after the first Bd exposure prior to antifungal drug treatment, although not significantly (Figure 2a; Experiment Day 14 compared with Day 21; $t[17] = 1.506$ and $p = 0.15$).

Exposure 2

Following the second Bd exposure of Laboratory Experiment 1, mean Bd load (ZE ± SD, infected animals only) was 399 ± 686 for the 1× group and 28 ± 45 for the 2× group. Overall Bd prevalence during the second Bd exposure was 73% for the previously uninfected (1×) group and only 42% for the previously exposed (2×) group, as compared to the 95% infection rate of the same frogs during the first infection round (Appendix S1:

TABLE 4 Variables used in mixed effects models of *Batrachochytrium dendrobatidis* (Bd) load, Bd infection status, and body condition in post-release *Rana draytonii*.

Variable	Range or levels	Description
Exposure group	1×, 2–3×, and control	1×: frogs that were infected and cleared once in the laboratory prior to release; 2–3×: frogs that were infected and cleared two or three times prior to release; control: Bd-naïve (upon release) frogs that were enrolled in the laboratory exposure study as sham controls. Used as a predictor in all four models.
Exposure Group 2	Reinfected, Bd-naïve	"Reinfected" combines 1×, 2×, and 3× infected individuals; sham controls from the laboratory experiments are considered Bd-naïve.
Bd load	0–3458 zoospore equivalents	Used as a predictor in the model of body condition in only Bd-positive samples, and as a response in the Bd load model.
Bd presence–absence	Infected (1), uninfected (0)	Used as a predictor in the model of body condition using all (both Bd-positive and Bd-negative) samples; used as a response variable in models of Bd presence–absence.
Days post-release	8–100	Day after initial release that the frog was captured and sampled. Used in all models.
Sex	Female, male	Used as a predictor in models of Bd presence–absence and Bd load.
Body condition	0.82–0.97	Calculated as $\log_{10}(\text{mass})/\log_{10}(\text{length})$. Used as a predictor in Bd load and Bd presence–absence models, and as a response variable in body condition models.

Table S2). Peak Bd loads were significantly lower for the second Bd exposure round of the 2× infected group ($t[18] = 7.26$ and $p < 0.0001$), and the single frog that did not get infected in the first exposure had the highest Bd load in the second exposure (Figure 2b). Peak Bd loads (Experiment Day 66; Figure 2a) were significantly higher for the 1× infected (previously uninfected) group than the 2× (previously infected) group ($t[29] = 4.23$ and $p = 0.0002$; Figure 2a).

Bd load decreased significantly after peak infection, even before antifungal treatment (Figure 2a; Experiment Days 66–72) for both exposure groups (2× group: $t[18] = 3.538$ and $p = 0.002$; 1× group: $t[16] = 5.039$ and $p = 0.0001$; Figure 2c). During the same period, 55% of the 2× infected group cleared their infections, and 28% did not get infected, while 35% of the 1× exposure group cleared their infections, and one frog did not get infected. The best-fit LME model comparing the trajectory of Bd infection through time by exposure group during the second Bd exposure phase of Laboratory Experiment 1 included significant terms for days post-exposure, days post-exposure^2, and the interaction exposure group × days post-exposure (Appendix S1: Tables S3 and S4). Across all post-infection sampling events prior to antifungal treatment, nonzero Bd loads were significantly lower for reinfected (2×) frogs than previously unexposed (1×) frogs ($\beta_{\text{reinfected}} = -2.06$, 95% CI −2.89 to −1.23; Appendix S1: Figure S1).

Laboratory Experiment 2

No frogs died during the course of Laboratory Experiment 2, and none of the sham controls became infected. Mean Bd load (ZE ± SD, infected animals only) was 1595 ± 2463 for the 1× (previously uninfected) group, 417 ± 382 for the 2× group, and 362 ± 828 for the 3× group. Overall Bd prevalence during Laboratory Experiment 2 was 72% for 1× group, 72% for 2× group, and 69% for the 3× group (Appendix S1: Table S2). Across all exposure groups, Bd loads significantly declined after initially peaking (Days 5–13; $t[53] = 6.07$ and $p < 0.0001$; Figure 3a,b), and four individuals (7%) lost their infections during this experimental phase (Figure 3b). Peak Bd load (Experiment Day 5; Figure 3a) differed among the three infected exposure groups (ANOVA, $F_{2,51} = 7.84$ and $p = 0.001$), but only the 1× and 3× exposure groups were significantly different from one another (Tukey post hoc test, $p = 0.001$; Figure 3c). Reinfected frogs (2× and 3× exposure groups combined) had significantly lower peak Bd loads (Experiment Day 5) than frogs infected for the first time ($t[39] = 3.71$ and $p = 0.0006$; Appendix S1: Figure S2).

The best-fit LME model of Bd infection trajectory within Laboratory Experiment 2 included significant terms for the orthogonal polynomial of days post-exposure^3, Exposure Group 2 (i.e., first-time infected [1×] or reinfected [2–3×]; Table 3), and the interaction of days post-exposure^3 and Exposure Group 2 (Appendix S1: Table S5). Bd load was significantly lower for the reinfected group than for the 1× group ($\beta_{\text{reinfected}} = -0.99$, 95% CI −1.66 to −0.31; Appendix S1: Table S6 and Figure S1). One model with exposure group (1×, 2×, and 3×; Table 3) was equivalent to the best-fit model as evidenced by $\Delta AIC_c < 2$ (Appendix S1: Table S5).

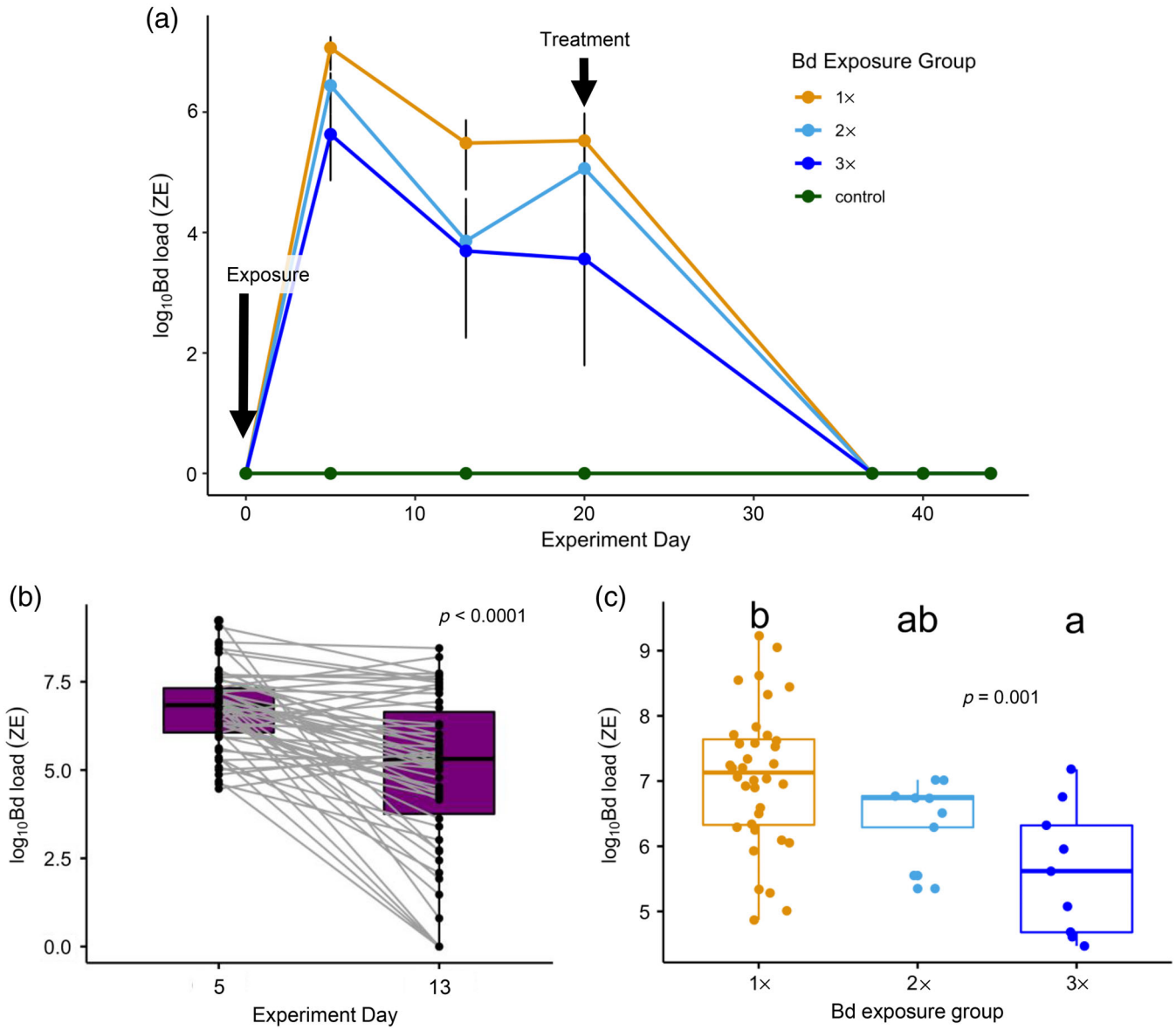

FIGURE 3 Pathogen burden of *Batrachochytrium dendrobatidis* (Bd load; in zoospore equivalents [ZE]) in California red-legged frogs (*Rana draytonii*) by exposure group during Laboratory Experiment 2. (a) Pathogen burden over Laboratory Experiment 2 timeline. Exposure groups: 1× = exposed to Bd once; 2× = Bd-exposed, cleared, and re-exposed; 3× = 2× frogs that were cleared and re-exposed a third time; and control = Bd-unexposed sham controls. "Exposure" refers to Bd exposure; "treatment" refers to the application of an antifungal drug to clear Bd infection. (b) Paired data across all Bd-exposed groups, showing a decrease in peak pathogen burden after Bd exposure and prior to antifungal treatment (Experiment Days 5 and 13). (c) Difference in peak Bd loads (Experiment Day 5) by exposure group.

Field monitoring

During the 14-week post-release monitoring period, mean Bd load was 296 ZE ± 619 SD, and overall Bd prevalence was 20%. Neither Bd load nor Bd prevalence were significantly different among exposure groups (Appendix S1: Figure S3). None of the candidate mixed effect models of nonzero Bd load outperformed the null model, indicating that none of the predictors tested (Table 4) significantly influenced Bd infection intensity (Appendix S1: Table S7). The best-fit model of Bd presence–absence included the orthogonal polynomial of days post-release^2, indicating a nonlinear effect of time on Bd infection (Appendix S1: Tables S8 and S9).

In LME models that included all (i.e., Bd-positive and Bd-negative) frogs, exposure group was a significant predictor of body condition ($\beta_{2-3\times} = 0.02$, 95% CI 0.01–0.04; Appendix S1: Tables S10 and S11), which was higher in the 2–3× group compared to the 1× and control groups (Appendix S1: Figure S4). The best-fit model of body condition in Bd-positive frogs included exposure group, but it was equivalent ($\Delta < 2$ AIC$_c$) to the null model

(Appendix S1: Table S12), so we did not consider exposure group to be a significant predictor of body condition in Bd-positive frogs. There was no significant difference between the body condition of individual frogs just before release and their mean body condition post-release ($t[46] = -1.54$ and $p = 0.13$). The overall frog recapture rate was 85%. At the conclusion of monitoring on 10 December 2019, seven individuals had died post-release: three from predation, two from drowning after being trapped in underwater debris by the transmitter belt, and two of unknown causes that could not be investigated further due to decomposition.

DISCUSSION

The Amphibian Conservation Action Plan (Wren et al., 2015) calls for efforts to ensure that ex situ conservation programs are more intentionally coupled with field recovery efforts. Our study links these important components and combines the benefits of ex situ experimental manipulation with a field translocation and subsequent monitoring. In addition, our study begins to fill an important gap in the literature of Bd susceptibility in *R. draytonii*.

Experimental Bd outcomes in *R. draytonii*

In both ex situ experiments, infection burdens were lower for previously exposed frogs than for frogs exposed to Bd for the first time. Post-release, we did not observe an effect of previous Bd exposure on Bd prevalence or load, but frogs that had been previously exposed and cleared of Bd 2–3 times ex situ had slightly higher body condition than the frogs infected once ex situ or previously uninfected controls. Future studies should examine additional parameters that can influence body condition, including microbiome diversity (Hernández-Gómez et al., 2018), which has a complex relationship with Bd infection (Jani & Briggs, 2014).

Our approach was to use four different Bd isolates, but little is known about competition among isolates, and we did not evaluate the relative proportions of each strain's infection on multiple frogs. It is unlikely that individual strain presence could have affected our measurements of infection burden because previous experiments with the same four Bd isolates we used showed similar Bd load trajectories when frogs were exposed to the isolates either together or one at a time (A. Jani, unpublished data). We also selected this mix of four Bd strains because their performance is known from previous Bd studies (Jani et al., 2021; Jani & Briggs, 2018). Any variations in ITS1 copy number between strains do not alter the significance of our laboratory experiment findings because we analyzed and observed differences in pathogen load on the logarithmic scale, and the ITS1 copy number of North American wild strains is on the same order of magnitude (Longo et al., 2013; Rebollar et al., 2017); therefore, even in the unlikely event that a high-copy strain dominated different treatments, the bias would only result in minimal differences in qPCR-measured loads and would not have affected our results. Finally, all of the frogs in our study were exposed to the same mix of four strains under the same laboratory conditions. If there are any strain differences, either in ITS copy number or load, we would expect the effect to be the same across treatments and therefore should not affect our results.

R. draytonii in California: In a Bd-enzootic state?

The initial arrival of Bd to *R. draytonii*'s range may have initiated a selective sweep against alleles that did not confer immune defenses against the pathogen, leaving extant populations in an enzootic state. This transition from epizootic to enzootic conditions has been described for other amphibian species, with population-level outcomes ranging from slow, continued declines to recruitment-mediated stability (Briggs et al., 2010; Catenazzi et al., 2017; Kupferberg et al., 2021; Retallick et al., 2004; Scheele et al., 2015). Multiple southern California *R. draytonii* populations exhibit signals of recent genetic bottlenecks, possibly the result of a chytridiomycosis-induced selective sweep (Richmond et al., 2011). In addition, the rate and pattern of the *R. draytonii* southern range contraction is consistent with pathogenic disease (Richmond et al., 2013). The earliest Bd record in California is from Los Angeles County in 1915 (Adams, Pessier, & Briggs, 2017), and Bd likely arrived in the Yosemite Region in the 1970s (Green & Kagarise Sherman, 2001). We sourced the egg masses in this study from a *R. draytonii* population where Bd had previously been detected; therefore, although individual frogs were Bd-naïve at the beginning of our study (because they were collected as eggs and reared in Bd-free enclosures), they were obtained from a likely enzootic population.

Our observations are in accord with the hypothesis that Sierra Nevada *R. draytonii* is enzootic. Our experimental observations of *R. draytonii* responses to Bd are consistent with innate and adaptive immunity, though because we did not directly measure immunological markers, the immunological mechanisms for our observations are unclear. Bd is ubiquitous in *R. draytonii* across the range (Adams, 2022; Adams, Kupferberg, et al., 2017; Adams, Peralta-García, et al., 2022; Adams, Pessier, & Briggs, 2017; Fellers

et al., 2011; Padgett-Flohr & Hopkins, 2009), suggesting that these populations persist with some level of pathogen resistance, though multi-year capture–mark–recapture studies are needed in conjunction with disease monitoring to determine the long-term effects of the pathogen on the species in the wild.

When Bd is enzootic, populations can still experience persistent, slow, time-lagged declines (Catenazzi et al., 2017). This can happen even if disease prevalence is very low, owing to environmental or amphibian host reservoirs, high pathogenicity, or poor immune defenses (Valenzuela-Sánchez et al., 2017; Voyles et al., 2018). In some *R. draytonii* populations, higher Bd prevalence is associated with lower survivorship (Russell et al., 2019), possibly a signature of this slow-decline phenomenon.

Life stage considerations

In this study, we reared frogs from eggs through metamorphosis in a Bd-free facility and exposed frogs to Bd one or two years after hatching. Like some other frog species of western North America, *R. draytonii* may be most susceptible in the earliest post-metamorphic life stages (Adams et al., 2020; Adams, Kupferberg, et al., 2017; Waddle et al., 2019). Adequate immune systems to effectively fight Bd infection are usually lacking during metamorphosis (Rollins-Smith et al., 2011). Repeating the present study with Bd exposures during metamorphosis may capture what could be the most immunocompromised stage of the *R. draytonii* life cycle. Further, the post-metamorphic, captive-reared frogs we used were observed under benign conditions with adequate food and no predators; therefore, the ex situ results of this study should not be extrapolated to all *R. draytonii* in the wild.

Management implications

Based on our results, ex situ Bd treatments may not confer additional chytridiomycosis defense for translocations from this source population to Yosemite Valley. However, differential susceptibility among populations suggests that if this study was repeated with frogs collected from a different source population, with a potentially different evolutionary history with Bd, the outcome may differ. For example, endangered alpine tree frogs (*Litoria verrauxii alpina*) collected from two Bd-endemic populations demonstrated more robust immune responses to Bd exposure compared to frogs from a Bd-naïve population (Grogan et al., 2018).

We ensured that Bd-exposed frogs were cleared before release, minimizing the risk of pathogen transport from the ex situ to the in situ portion of the study. Managers conducting translocations with *R. draytonii* and other species should continue to be vigilant with rigorous biosecurity protocols whenever working with or transporting amphibians between sites. The apparent enzootic status of Bd in much of *R. draytonii*'s range does not necessarily indicate that such precautions are no longer necessary. On the contrary, the Bd genome is much more diverse than previously imagined, and distinct lineages of highly differential virulence continue to spread globally (Byrne et al., 2019), perpetually threatening amphibian populations.

CONCLUSION

In response to the global emergence of Bd, some species are showing the effects of natural selection on immunity (Brannelly et al., 2016; Savage & Zamudio, 2016), so inoculation against disease may not confer additional benefit. Based on our ex situ and in situ results, *R. draytonii* may be one of these species, potentially holding keys to the mechanisms behind innate and adaptive immunity for future research. We caution, however, that the evidence for innate and adaptive immunity we observed under laboratory conditions in this study may not mirror *R. draytonii* Bd susceptibility across the species' life cycle in the wild. This study demonstrates how innovative collaborations between ex situ and in situ conservation partners can contribute to a more comprehensive understanding of the threats facing imperiled species in reintroduction programs as well as their solutions.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data (Adams, Bushell, & Grasso, 2022) are available from Dryad: https://doi.org/10.25349/D9Z020.

ORCID

Andrea J. Adams https://orcid.org/0000-0001-5609-6150
Robert L. Grasso https://orcid.org/0000-0002-3027-071X

REFERENCES

Adams, A., J. Bushell, and R. Grasso. 2022. "Data from: To Treat or Not to Treat? Experimental Pathogen Exposure, Treatment, and Release of a Threatened Amphibian." Dryad. Dataset. https://doi.org/10.25349/D9Z020.

Adams, A. J., K. C. Brown, M. Jennings, and R. L. Grasso. n.d. "Homecoming or New Pad: Historical Evidence for California Red-Legged Frogs in the Yosemite Region." (in press).

Adams, A. J. 2022. *Surveys for Amphibian Chytrid Fungus (Batrachochytrium dendrobatidis) in the Santa Monica Mountains National Recreation Area at California Red-Legged Frog (Rana draytonii) Source and Translocation Sites, 2020–2021*. Thousand Oaks, CA: U.S. National Park Service.

Adams, A. J., S. J. Kupferberg, M. Q. Wilber, A. P. Pessier, M. Grefsrud, S. Bobzien, V. T. Vredenburg, and C. J. Briggs. 2017. "Extreme Drought, Host Density, Sex, and Bullfrogs Influence Fungal Pathogen Infection in a Declining Lotic Amphibian." *Ecosphere* 8(3): e01740. https://doi.org/10.1002/ecs2.1740.

Adams, A. J., A. Peralta-García, C. A. Flores-López, J. H. Valdez-Villavicencio, and C. J. Briggs. 2022. "High Fungal Pathogen Loads and Prevalence in Baja California Amphibian Communities: The Importance of Species, Elevation, and Historical Context." *Global Ecology and Conservation* 33: e01968. https://doi.org/10.1016/j.gecco.2021.e01968.

Adams, A. J., A. P. Pessier, P. Cranston, and R. L. Grasso. 2020. "Chytridiomycosis-Induced Mortality in a Threatened Anuran." *PLoS One* 15(11): e0241119. https://doi.org/10.1371/journal.pone.0241119.

Adams, A. J., A. P. Pessier, and C. J. Briggs. 2017. "Rapid Extirpation of a North American Frog Coincides with an Increase in Fungal Pathogen Prevalence: Historical Analysis and Implications for Reintroduction." *Ecology and Evolution* 7(23): 10216–32. https://doi.org/10.1002/ece3.3468.

Bates, D. M. 2020. *lme4: Mixed-Effects Modeling with R*. New York: Springer.

Becker, C. G., S. E. Greenspan, K. E. Tracy, J. A. Dash, C. Lambertini, T. S. Jenkinson, D. S. Leite, et al. 2017. "Variation in Phenotype and Virulence among Enzootic and Panzootic Amphibian Chytrid Lineages." *Fungal Ecology* 26: 45–50. https://doi.org/10.1016/j.funeco.2016.11.007.

Berger, L., G. Marantelli, L. F. Skerratt, and R. Speare. 2005. "Virulence of the Amphibian Chytrid Fungus *Batrachochytium dendrobatidis* Varies with the Strain." *Diseases of Aquatic Organisms* 68(1): 47–50. https://doi.org/10.3354/dao068047.

Berger, L., R. Speare, D. Peter Daszak, E. Green, A. A. Cunningham, C. Louise Goggin, R. Slocombe, et al. 1998. "Chytridiomycosis Causes Amphibian Mortality Associated with Population Declines in the Rain Forests of Australia and Central America." *Proceedings of the National Academy of Sciences* 95(15): 9031–6.

Boyle, D. G., D. B. Boyle, V. Olsen, J. A. T. Morgan, and A. D. Hyatt. 2004. "Rapid Quantitative Detection of Chytridiomycosis (*Batrachochytrium dendrobatidis*) in Amphibian Samples Using Real-Time Taqman PCR Assay." *Diseases of Aquatic Organisms* 60(2): 141–8. https://doi.org/10.3354/dao060141.

Brannelly, L. A., D. A. Hunter, L. F. Skerratt, B. C. Scheele, D. Lenger, M. S. McFadden, P. S. Harlow, and L. Berger. 2016. "Chytrid Infection and Post-Release Fitness in the Reintroduction of an Endangered Alpine Tree Frog." *Animal Conservation* 19(2): 153–62. https://doi.org/10.1111/acv.12230.

Brannelly, L. A., D. A. Hunter, D. Lenger, B. C. Scheele, L. F. Skerratt, and L. Berger. 2015. "Dynamics of Chytridiomycosis during the Breeding Season in an Australian Alpine Amphibian." *PLoS One* 10(12): e0143629. https://doi.org/10.1371/journal.pone.0143629.

Briggs, C. J., R. A. Knapp, and V. T. Vredenburg. 2010. "Enzootic and Epizootic Dynamics of the Chytrid Fungal Pathogen of Amphibians." *Proceedings of the National Academy of Sciences, USA* 107(21): 9695–700. https://doi.org/10.1073/pnas.0912886107.

Briggs, C. J., V. T. Vredenburg, R. A. Knapp, and L. J. Rachowicz. 2005. "Investigating the Population-Level Effects of Chytridiomycosis: An Emerging Infectious Disease of Amphibians." *Ecology* 86(12): 3149–59.

Burnham, K. P., and D. R. Anderson. 2004. "Multimodel Inference: Understanding AIC and BIC in Model Selection." *Sociological Methods & Research* 33(2): 261–304.

Byrne, A. Q., V. T. Vredenburg, A. Martel, F. Pasmans, R. C. Bell, D. C. Blackburn, M. C. Bletz, J. Bosch, C. J. Briggs, and R. M. Brown. 2019. "Cryptic Diversity of a Widespread Global Pathogen Reveals Expanded Threats to Amphibian Conservation." *Proceedings of the National Academy of Sciences* 116(41): 20382–7.

Canessa, S., A. S.-v. der Sluijs, A. Martel, and F. Pasmans. 2019. "Mitigation of Amphibian Disease Requires a Stronger Connection between Research and Management." *Biological Conservation* 236: 236–42. https://doi.org/10.1016/j.biocon.2019.05.030.

Cashins, S. D., L. F. Grogan, M. McFadden, D. Hunter, P. S. Harlow, L. Berger, and L. F. Skerratt. 2013. "Prior Infection Does Not Improve Survival against the Amphibian Disease Chytridiomycosis." *PLoS One* 8(2): e56747. https://doi.org/10.1371/journal.pone.0056747.

Catenazzi, A., and S. J. Kupferberg. 2017. "Variation in Thermal Niche of a Declining River-Breeding Frog: From Counter-Gradient Responses to Population Distribution Patterns." *Freshwater Biology* 62(7): 1255–65.

Catenazzi, A., A. Swei, J. Finkle, E. Foreyt, L. Wyman, and V. T. Vredenburg. 2017. "Epizootic to Enzootic Transition of a Fungal Disease in Tropical Andean Frogs: Are Surviving

Species Still Susceptible?" *PLoS One* 12(10): e0186478. https://doi.org/10.1371/journal.pone.0186478.

Collins, J. P., M. L. Crump, and T. E. Lovejoy, III. 2009. *Extinction in Our Times: Global Amphibian Decline*. Oxford, England: Oxford University Press.

Daszak, P., L. Berger, A. A. Cunningham, A. D. Hyatt, D. Earl Green, and R. Speare. 1999. "Emerging Infectious Diseases and Amphibian Population Declines." *Emerging Infectious Diseases* 5(6): 735–48.

DiRenzo, G. V., P. F. Langhammer, K. R. Zamudio, and K. R. Lips. 2014. "Fungal Infection Intensity and Zoospore Output of *Atelopus zeteki*, a Potential Acute Chytrid Supershedder." *PLoS One* 9(3): e93356. https://doi.org/10.1371/journal.pone.0093356.

Doddington, B. J., J. Bosch, J. A. Oliver, N. C. Grassly, G. Garcia, B. R. Schmidt, T. W. J. Garner, and M. C. Fisher. 2013. "Context-Dependent Amphibian Host Population Response to an Invading Pathogen." *Ecology* 94(8): 1795–804. https://doi.org/10.1890/12-1270.1.

Farrer, R. A., L. A. Weinert, J. Bielby, T. W. Garner, F. Balloux, F. Clare, J. Bosch, et al. 2011. "Multiple Emergences of Genetically Diverse Amphibian-Infecting Chytrids Include a Globalized Hypervirulent Recombinant Lineage." *Proceedings of the National Academy of Sciences, USA* 108(46): 18732–6. https://doi.org/10.1073/pnas.1111915108.

Fellers, G. M., R. A. Cole, D. M. Reinitz, and P. M. Kleeman. 2011. "Amphibian Chytrid Fungus (*Batrachochytrium dendrobatidis*) in Coastal and Montane California, USA Anurans." *Herpetological Conservation and Biology* 6(3): 383–94.

Fellers, G. M., D. F. Bradford, D. Pratt, and L. L. Wood. 2007. "Demise of Repatriated Populations of Mountain Yellow-Legged Frogs (*Rana muscosa*) in the Sierra Nevada of California." *Herpetological Conservation and Biology* 2(1): 5–21.

Fisher, M. C., T. W. Garner, and S. F. Walker. 2009. "Global Emergence of *Batrachochytrium dendrobatidis* and Amphibian Chytridiomycosis in Space, Time, and Host." *Annual Review of Microbiology* 63: 291–310. https://doi.org/10.1146/annurev.micro.091208.073435.

Garmyn, A., P. Van Rooij, F. Pasmans, T. Hellebuyck, W. Van Den Broeck, F. Haesebrouck, and A. Martel. 2012. "Waterfowl: Potential Environmental Reservoirs of the Chytrid Fungus *Batrachochytrium dendrobatidis*." *PLoS One* 7(4): e35038. https://doi.org/10.1371/journal.pone.0035038.

González-del-Pliego, P., R. P. Freckleton, D. P. Edwards, M. S. Koo, B. R. Scheffers, R. Alexander Pyron, and W. Jetz. 2019. "Phylogenetic and Trait-Based Prediction of Extinction Risk for Data-Deficient Amphibians." *Current Biology* 29(9): 1557–1563.e3. https://doi.org/10.1016/j.cub.2019.04.005.

Gosner, K. L. 1960. "A Simplified Table for Staging Anuran Embryos and Larvae with Notes on Identification." *Herpetologica* 16(3): 183–90. https://doi.org/10.2307/3890061.

Green, A. J. 2001. "Mass/Length Residuals: Measures of Body Condition or Generators of Spurious Results?" *Ecology* 82(5): 1473–83. https://doi.org/10.1890/0012-9658(2001)082[1473:mlrmob]2.0.co;2.

Green, D. E., and C. Kagarise Sherman. 2001. "Diagnostic Histological Findings in Yosemite Toads (*Bufo canorus*) from a Die-Off in the 1970s." *Journal of Herpetology* 35(1): 92–103.

Grogan, L. F., S. D. Cashins, L. F. Skerratt, L. Berger, M. S. McFadden, P. Harlow, D. A. Hunter, B. C. Scheele, and J. Mulvenna. 2018. "Evolution of Resistance to Chytridiomycosis Is Associated with a Robust Early Immune Response." *Molecular Ecology* 27(4): 919–34. https://doi.org/10.1111/mec.14493.

Harding, G., R. A. Griffiths, and L. Pavajeau. 2016. "Developments in Amphibian Captive Breeding and Reintroduction Programs." *Conservation Biology* 30(2): 340–9. https://doi.org/10.1111/cobi.12612.

Hernández-Gómez, O., J. T. Briggler, and R. N. Williams. 2018. "Influence of Immunogenetics, Sex and Body Condition on the Cutaneous Microbial Communities of Two Giant Salamanders." *Molecular Ecology* 27(8): 1915–29. https://doi.org/10.1111/mec.14500.

Hudson, M. A., R. A. Griffiths, L. Martin, C. Fenton, S.-L. Adams, A. Blackman, M. Sulton, et al. 2019. "Reservoir Frogs: Seasonality of *Batrachochytrium dendrobatidis* Infection in Robber Frogs in Dominica and Montserrat." *PeerJ* 7: e7021. https://doi.org/10.7717/peerj.7021.

Hyatt, A. D., D. G. Boyle, V. Olsen, D. B. Boyle, L. Berger, D. Obendorf, A. Dalton, et al. 2007. "Diagnostic Assays and Sampling Protocols for the Detection of *Batrachochytrium dendrobatidis*." *Diseases of Aquatic Organisms* 73(3): 175–92. https://doi.org/10.3354/dao073175.

Jani, A. J., and C. J. Briggs. 2014. "The Pathogen *Batrachochytrium dendrobatidis* Disturbs the Frog Skin Microbiome during a Natural Epidemic and Experimental Infection." *Proceedings of the National Academy of Sciences* 111(47): E5049–58. https://doi.org/10.1073/pnas.1412752111.

Jani, A. J., and C. J. Briggs. 2018. "Host and Aquatic Environment Shape the Amphibian Skin Microbiome but Effects on Downstream Resistance to the Pathogen *Batrachochytrium dendrobatidis* Are Variable." *Frontiers in Microbiology* 9: 487.

Jani, A. J., J. Bushell, C. G. Arisdakessian, M. Belcaid, D. M. Boiano, C. Brown, and R. A. Knapp. 2021. "The Amphibian Microbiome Exhibits Poor Resilience Following Pathogen-Induced Disturbance." *The ISME Journal* 15(6): 1628–40. https://doi.org/10.1038/s41396-020-00875-w.

Jennings, M. R., and M. P. Hayes. 1994. *Amphibian and Reptile Species of Special Concern in California*. Rancho Cordova, CA: California Department of Fish and Game.

Joseph, M. B., and R. A. Knapp. 2018. "Disease and Climate Effects on Individuals Drive Post-Reintroduction Population Dynamics of an Endangered Amphibian." *Ecosphere* 9(11): e02499. https://doi.org/10.1002/ecs2.2499.

Kamoroff, C., N. Daniele, R. L. Grasso, R. Rising, T. Espinoza, and C. S. Goldberg. 2020. "Effective Removal of the American Bullfrog (*Lithobates catesbeianus*) on a Landscape Level: Long Term Monitoring and Removal Efforts in Yosemite Valley, Yosemite National Park." *Biological Invasions* 22(2): 617–26. https://doi.org/10.1007/s10530-019-02116-4.

Karlsdóttir, B., A. T. Knight, K. Johnson, and J. Dawson. 2021. "Lessons from Practitioners for Designing and Implementing Effective Amphibian Captive Breeding Programmes." *Oryx* 55(3): 382–92. https://doi.org/10.1017/S0030605320000332.

Knapp, R. A., G. M. Fellers, P. M. Kleeman, D. A. W. Miller, V. T. Vredenburg, E. B. Rosenblum, and C. J. Briggs. 2016. "Large-Scale Recovery of an Endangered Amphibian despite Ongoing Exposure to Multiple Stressors." *Proceedings of the National Academy of Sciences* 113(42): 11889–94. https://doi.org/10.1073/pnas.1600983113.

Knapp, R. A., M. B. Joseph, T. C. Smith, E. E. Hegeman, V. T. Vredenburg, J. E. Erdman, D. M. Boiano, A. J. Jani, and C. J. Briggs. 2021. "Effectiveness of Antifungal Treatments during Chytridiomycosis Epizootics in Populations of an Endangered Frog." *bioRxiv* 10: e12712.

Kupferberg, S. J., H. Moidu, A. J. Adams, A. Catenazzi, M. Grefsrud, S. Bobzien, R. Leidy, and S. M. Carlson. 2021. "Seasonal Drought and Its Effects on Frog Population Dynamics and Amphibian Disease in Intermittent Streams." *Ecohydrology* 15: e2395. https://doi.org/10.1002/eco.2395.

Longo, A. V., D. Rodriguez, D. da Silva Leite, L. F. Toledo, C. M. Almeralla, P. A. Burrowes, and K. R. Zamudio. 2013. "ITS1 Copy Number Varies among *Batrachochytrium dendrobatidis* Strains: Implications for qPCR Estimates of Infection Intensity from Field-Collected Amphibian Skin Swabs." *PLoS One* 8(3): e59499. https://doi.org/10.1371/journal.pone.0059499.

Mazzoni, R., A. A. Cunningham, P. Daszak, A. Apolo, E. Perdomo, and G. Speranza. 2003. "Emerging Pathogen of Wild Amphibians in Frogs (*Rana catesbeiana*) Farmed for International Trade." *Emerging Infectious Diseases* 9(8): 995–8.

McFadden, M., D. Hunter, P. Harlow, R. Pietsch, and B. Scheele. 2010. "Captive Management and Experimental Reintroduction of the Booroolong Frog on the South Western Slopes Region, New South Wales, Australia." In *Global Re-Introduction Perspectives: Additional Case Studies from around the Globe*, edited by P. S. Soorae, 77–80. Abu Dhabi: IUCN/SSC Re-introduction Specialist Group.

McMahon, T. A., B. F. Sears, M. D. Venesky, S. M. Bessler, J. M. Brown, K. Deutsch, N. T. Halstead, et al. 2014. "Amphibians Acquire Resistance to Live and Dead Fungus Overcoming Fungal Immunosuppression." *Nature* 511(7508): 224–7. https://doi.org/10.1038/nature13491.

Mendelson, J. R., S. M. Whitfield, and M. J. Sredl. 2019. "A Recovery Engine Strategy for Amphibian Conservation in the Context of Disease." *Biological Conservation* 236: 188–91. https://doi.org/10.1016/j.biocon.2019.05.025.

Mitchell, K. M., T. S. Churcher, T. W. J. Garner, and M. C. Fisher. 2008. "Persistence of the Emerging Pathogen *Batrachochytrium dendrobatidis* outside the Amphibian Host Greatly Increases the Probability of Host Extinction." *Proceedings of the Royal Society B: Biological Sciences* 275(1632): 329–34. https://doi.org/10.1098/rspb.2007.1356.

Murphy, P. J., S. St-Hilaire, and P. S. Corn. 2011. "Temperature, Hydric Environment, and Prior Pathogen Exposure Alter the Experimental Severity of Chytridiomycosis in Boreal Toads." *Diseases of Aquatic Organisms* 95(1): 31–42. https://doi.org/10.3354/dao02336.

Nichols, D. K., A. P. Pessier, and J. E. Longcore. 1998. "Cutaneous Chytridiomycosis in Amphibians: An Emerging Disease?" American Association of Zoo Veterinarians and American Association of Wildlife Veterinarians Joint Conference.

Nichols, D. K., E. W. Lamirande, A. P. Pessier, and J. E. Longcore. 2001. "Experimental Transmission of Cutaneous Chytridiomycosis in Dendrobatid Frogs." *Journal of Wildlife Diseases* 37(1): 1–11.

Padgett-Flohr, G. E. 2008. "Pathogenicity of *Batrachochytrium dendrobatidis* in Two Threatened California Amphibians: *Rana draytonii* and *Ambystoma californiense*." *Herpetological Conservation and Biology* 3(2): 182–91.

Padgett-Flohr, G. E., and R. L. Hopkins, II. 2009. "*Batrachochytrium dendrobatidis*, a Novel Pathogen Approaching Endemism in Central California." *Diseases of Aquatic Organisms* 83(1): 1–9. https://doi.org/10.3354/dao02003.

Peralta-Garcia, A., B. D. Hollingsworth, J. Q. Richmond, J. H. Valdez-Villavicencio, G. Ruiz-Campos, R. N. Fisher, P. Cruz-Hernandez, and P. Galina-Tessaro. 2016. "Status of the California Red-Legged Frog (*Rana draytonii*) in the State of Baja California, Mexico." *Herpetological Conservation and Biology* 11(1): 168–80.

Pessier, A. P., D. K. Nichols, J. E. Longcore, and M. S. Fuller. 1999. "Cutaneous Chytridiomycosis in Poison Dart Frogs (*Dendrobates* spp.) and White's Tree Frogs (*Litoria caerulea*)." *Journal of Veterinary Diagnostic Investigation* 11(2): 194–9. https://doi.org/10.1177/104063879901100219.

Pinheiro, J., D. Bates, S. Debroy, D. Sarkar, and R Core Team. 2019. "nlme: Linear and Nonlinear Mixed Effects Models." R Package Version 3.1-141.

Piovia-Scott, J., S. Karen Pope, J. Worth, E. B. Rosenblum, T. Poorten, J. Refsnider, L. A. Rollins-Smith, et al. 2015. "Correlates of Virulence in a Frog-Killing Fungal Pathogen: Evidence from a California Amphibian Decline." *The ISME Journal* 9(7): 1570–8. https://doi.org/10.1038/ismej.2014.241.

R Core Team. 2019. "R: A Language and Environment for Statistical Computing." https://www.r-project.org/.

Rathbun, G. B., and T. G. Murphey. 1996. "Evaluation of a Radio-Belt for Ranid Frogs." *Herpetological Review* 27(4): 187–8.

Rebollar, E. A., D. C. Woodhams, B. LaBumbard, J. F. Kielgast, and R. N. Harris. 2017. "Prevalence and Pathogen Load Estimates for the Fungus *Batrachochytrium dendrobatidis* Are Impacted by ITS DNA Copy Number Variation." *Diseases of Aquatic Organisms* 123(3): 213–26.

Retallick, R. W. R., H. McCallum, and R. Speare. 2004. "Endemic Infection of the Amphibian Chytrid Fungus in a Frog Community Post-Decline." *PLoS Biology* 2(11): 1965–71. https://doi.org/10.1371/journal.pbio.0020351.g001.

Richmond, J. Q., K. R. Barr, A. R. Backlin, R. N. Fisher, and A. G. Vandergast. 2011. *Population Genetics of the California Red-Legged Frog (Rana draytonii): Effects of Wildfires on Genetic Diversity and Strategies for Selecting Source Populations for Translocation in Southern California*. Sacramento, CA: U.S. Department of the Interior, U.S. Geological Survey, Western Ecological Research Center.

Richmond, J. Q., K. R. Barr, A. R. Backlin, A. G. Vandergast, and R. N. Fisher. 2013. "Evolutionary Dynamics of a Rapidly Receding Southern Range Boundary in the Threatened California Red-Legged Frog (*Rana draytonii*)." *Evolutionary Applications* 6: 808–22. https://doi.org/10.1111/eva.12067.

Richmond, J. Q., A. E. Savage, K. R. Zamudio, and E. B. Rosenblum. 2009. "Toward Immunogenetic Studies of Amphibian Chytridiomycosis: Linking Innate and Acquired Immunity." *Bioscience* 59(4): 311–20.

Rollins-Smith, L. A. 2017. "Amphibian Immunity–Stress, Disease, and Climate Change." *Developmental & Comparative Immunology* 66: 111–9. https://doi.org/10.1016/j.dci.2016.07.002.

Rollins-Smith, L. A., J. P. Ramsey, J. D. Pask, L. K. Reinert, and D. C. Woodhams. 2011. "Amphibian Immune Defenses against Chytridiomycosis: Impacts of Changing Environments." *Integrative and Comparative Biology* 51(4): 552–62. https://doi.org/10.1093/icb/icr095.

Rosenblum, E. B., J. Voyles, T. J. Poorten, and J. E. Stajich. 2010. "The Deadly Chytrid Fungus: A Story of an Emerging

Pathogen." *PLoS Pathogens* 6(1): e1000550. https://doi.org/10.1371/journal.ppat.1000550.

Russell, R. E., B. J. Halstead, B. A. Mosher, E. Muths, M. J. Adams, E. H. C. Grant, R. N. Fisher, et al. 2019. "Effect of Amphibian Chytrid Fungus (*Batrachochytrium dendrobatidis*) on Apparent Survival of Frogs and Toads in the Western USA." *Biological Conservation* 236: 296–304.

Savage, A. E., and K. R. Zamudio. 2011. "MHC Genotypes Associate with Resistance to a Frog-Killing Fungus." *Proceedings of the National Academy of Sciences, USA* 108(40): 16705–10. https://doi.org/10.1073/pnas.1106893108.

Savage, A. E., and K. R. Zamudio. 2016. "Adaptive Tolerance to a Pathogenic Fungus Drives Major Histocompatibility Complex Evolution in Natural Amphibian Populations." *Proceedings of the Royal Society of London B: Biological Sciences* 283(1827): 20153115. https://doi.org/10.1098/rspb.2015.3115.

Scheele, B. C., D. A. Hunter, L. F. Grogan, L. E. E. Berger, J. E. Kolby, M. S. McFadden, G. Marantelli, L. F. Skerratt, and D. A. Driscoll. 2014. "Interventions for Reducing Extinction Risk in Chytridiomycosis-Threatened Amphibians." *Conservation Biology* 28(5): 1195–205. https://doi.org/10.1111/cobi.12322.

Scheele, B. C., D. A. Hunter, L. F. Skerratt, L. A. Brannelly, and D. A. Driscoll. 2015. "Low Impact of Chytridiomycosis on Frog Recruitment Enables Persistence in Refuges despite High Adult Mortality." *Biological Conservation* 182: 36–43. https://doi.org/10.1016/j.biocon.2014.11.032.

Shaffer, H. B., G. M. Fellers, S. R. Voss, J. C. Oliver, and G. B. Pauly. 2004. "Species Boundaries, Phylogeography and Conservation Genetics of the Red-Legged Frog (*Rana aurora/draytonii*) Complex." *Molecular Ecology* 13(9): 2667–77. https://doi.org/10.1111/j.1365-294X.2004.02285.x.

Stice, M. J., and C. J. Briggs. 2010. "Immunization Is Ineffective at Preventing Infection and Mortality Due to the Amphibian Chytrid Fungus *Batrachochytrium dendrobatidis*." *Journal of Wildlife Diseases* 46(1): 70–7. https://doi.org/10.7589/0090-3558-46.1.70.

Stockwell, M., S. Clulow, J. Clulow, and M. Mahony. 2008. "The Impact of the Amphibian Chytrid Fungus *Batrachochytrium dendrobatidis* on a Green and Golden Bell Frog Litoria Aurea Reintroduction Program at the Hunter Wetlands Centre Australia in the Hunter Region of NSW." *Australian Zoologist* 34(3): 379–86. https://doi.org/10.7882/AZ.2008.015.

Tatarian, P. 2008. "Movement Patterns of California Red-Legged Frogs (*Rana draytonii*) in an Inland California Environment." *Herpetological Conservation and Biology* 3(2): 155–69.

U.S. Fish and Wildlife Service. 1996. "Endangered and Threatened Wildlife and Plants; Determination of Threatened Status for the California Red-Legged Frog." *Federal Register* 61: 25813–33.

U.S. Fish and Wildlife Service. 2002. *Recovery Plan for the California Red-Legged Frog (Rana aurora draytonii)*. Portland, OR: U.S. Fish and Wildlife Service.

Valenzuela-Sánchez, A., B. R. Schmidt, D. E. Uribe-Rivera, F. Costas, A. A. Cunningham, and C. Soto-Azat. 2017. "Cryptic Disease-Induced Mortality May Cause Host Extinction in an Apparently Stable Host-Parasite System." *Proceedings of the Royal Society B: Biological Sciences* 284(1863): 20171176. https://doi.org/10.1098/rspb.2017.1176.

Venesky, M. D., T. R. Raffel, T. A. McMahon, and J. R. Rohr. 2014. "Confronting Inconsistencies in the Amphibian-Chytridiomycosis System: Implications for Disease Management." *Biological Reviews* 89(2): 477–83. https://doi.org/10.1111/brv.12064.

Voyles, J., D. C. Woodhams, V. Saenz, A. Q. Byrne, R. Perez, G. Rios-Sotelo, M. J. Ryan, et al. 2018. "Shifts in Disease Dynamics in a Tropical Amphibian Assemblage Are Not Due to Pathogen Attenuation." *Science* 359(6383): 1517–9. https://doi.org/10.1126/science.aao4806.

Waddle, A. W., J. E. Levy, R. Rivera, F. van Breukelen, M. Nash, and J. R. Jaeger. 2019. "Population-Level Resistance to Chytridiomycosis Is Life-Stage Dependent in an Imperiled Anuran." *EcoHealth* 16(4): 701–11. https://doi.org/10.1007/s10393-019-01446-y.

Waddle, A. W., R. Rivera, H. Rice, E. C. Keenan, G. Rezaei, J. E. Levy, Y. S. Vasquez, et al. 2021. "Amphibian Resistance to Chytridiomycosis Increases Following Low-Virulence Chytrid Fungal Infection or Drug-Mediated Clearance." *Journal of Applied Ecology* 58(10): 2053–64. https://doi.org/10.1111/1365-2664.13974.

Wilber, M. Q., R. A. Knapp, M. Toothman, and C. J. Briggs. 2017. "Resistance, Tolerance and Environmental Transmission Dynamics Determine Host Extinction Risk in a Load-Dependent Amphibian Disease." *Ecology Letters* 20(9): 1169–81. https://doi.org/10.1111/ele.12814.

Woodhams, D. C., K. Ardipradja, R. A. Alford, G. Marantelli, L. K. Reinert, and L. A. Rollins-Smith. 2007. "Resistance to Chytridiomycosis Varies among Amphibian Species and Is Correlated with Skin Peptide Defenses." *Animal Conservation* 10(4): 409–17. https://doi.org/10.1111/j.1469-1795.2007.00130.x.

Woodhams, D. C., J. Bosch, C. J. Briggs, S. Cashins, L. R. Davis, A. Lauer, E. Muths, et al. 2011. "Mitigating Amphibian Disease: Strategies to Maintain Wild Populations and Control Chytridiomycosis." *Frontiers in Zoology* 8(1): 8. https://doi.org/10.1186/1742-9994-8-8.

Wren, S., A. Angulo, H. Meredith, J. Kielgast, M. Dos Santos, and P. Bishop. 2015. "Amphibian Conservation Action Plan." April 2015. IUCN SSC Amphibian Specialist Group.

Zuur, A., E. N. Ieno, N. Walker, A. A. Saveliev, and G. M. Smith. 2009. *Mixed Effects Models and Extensions in Ecology with R*. New York: Springer Science & Business Media.

SUPPORTING INFORMATION

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

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