

Extreme drought, host density, sex, and bullfrogs influence fungal pathogen infection in a declining lotic amphibian

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Abstract. Freshwater biodiversity is imperiled across the globe, and multiple stressors such as habitat alteration, non-native species invasion, disease, and climate change can act in concert to threaten vulnerable taxa. The amphibian chytrid fungus *Batrachochytrium dendrobatidis* (Bd), which causes the disease chytridiomycosis, is one of the causative factors of severe amphibian declines. The foothill yellow-legged frog (*Rana boylei*) is a stream-breeding anuran endemic to California and Oregon (USA) that has declined precipitously in recent decades, yet there is little information on its susceptibility to Bd. In the fall of 2013, we observed dead and dying juvenile *R. boylei* in a San Francisco Bay Area watershed where annual amphibian breeding censuses have been conducted since 1997 in a free-flowing reach and since 2003 in an anthropogenically modified stream reach. High pathogen loads on *R. boylei* and histologic lesions observed on a dead *R. boylei* metamorph collected from the site were consistent with lethal chytridiomycosis. The outbreak coincided with extremely low stream flows in autumn that concentrated frogs in drying pools and the absence of high peak flows in winter that allowed non-native American bullfrogs (*Rana catesbeiana*) to expand their spatial distribution in the stream network. Following the outbreak, we surveyed *R. boylei* and sympatric anurans at the site for the next two years to determine Bd trends within the population. Using mixed-effects models, we found that bullfrog presence was a positive predictor of both Bd prevalence and Bd load in *R. boylei*. Prevalence was also influenced by sex and life stage: Adult males were more likely to be infected than either females or juveniles. Moreover, we found that stream flow volume was negatively associated with Bd load. These results indicate that disease, drought, and flow regulation may interact synergistically to impact amphibians in ways not previously recognized, informing stream flow management strategies for native aquatic taxa.

Key words: amphibian declines; *Batrachochytrium dendrobatidis*; chytrid fungus; climate change; dams; disease; drought; multiple stressors; pathogen; *Rana boylei*; rivers; streams.

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INTRODUCTION

Fungal pathogens causing disease in wildlife are on the rise, with catastrophic consequences for

biodiversity (Fisher et al. 2012, Ercan et al. 2015). Anthropogenic disturbances, such as the transport and introduction of non-native species and habitat alteration, can facilitate the dispersal of fungal

pathogens and can cause shifts in their host-specific suitability, making disease outcomes difficult to predict (Fisher et al. 2012, Adlard et al. 2015). Shifts in environmental conditions can also alter host–pathogen relationships, changing disease risk (Dobson and Foufopoulos 2001). In some cases, climate change can increase the incidence and severity of animal pathogens by extending the suitable range of vectors and reservoir hosts, lengthening periods suitable for pathogen transmission, or directly affecting host susceptibility (Harvell et al. 2002, Greer et al. 2008, Eisenlord et al. 2016). In addition, climate change can combine with pre-existing stressors, resulting in cumulative effects to the host (Gallana et al. 2013).

The pathogenic chytrid fungus *Batrachochytrium dendrobatidis* (hereafter Bd) produces the amphibian disease chytridiomycosis in susceptible hosts and has caused declines and extinctions in over 200 species globally (Stuart et al. 2004, Wake and Vredenburg 2008). Bd has altered our understanding of the interaction between diseases and their hosts, in that Bd can cause host extinction, which is unlikely for most pathogens (MacPhee and Greenwood 2013). Bd's ability to infect multiple hosts allows it to maintain itself in less susceptible species while driving more susceptible species to extinction or near extinction (Catenazzi 2015). Therefore, it is crucial to understand the range of host species infected with Bd in ecosystems, and to identify potential reservoir hosts that could increase the threat of chytridiomycosis infection for susceptible species of conservation concern.

Both the biotic and abiotic contexts of Bd outcome must be considered because disease is also strongly shaped by the environment, which can influence the traits of the pathogen and hosts' responses to it (Blaustein et al. 2012). The prevalence and severity of Bd infection can be highly dependent upon local climatic conditions (Kriger and Hero 2007, Savage et al. 2011); however, these effects are not always observed (Knapp et al. 2011). Bd is an aquatic pathogen, requiring a minimum level of moisture to be viable in vitro (Johnson et al. 2003), and is often dependent upon moisture variables in the wild (Kriger 2009). Therefore, it is often suggested that warmer, drier climates may reduce Bd prevalence and loads (Becker and Zamudio 2011, Raffel et al. 2013); however, shifting climates may also

increase chytridiomycosis severity as warmer and drier conditions force amphibians to aggregate in reduced areas of moisture, increasing pathogen transmission rates (Burrowes et al. 2004, Lampo et al. 2006, Longo et al. 2010).

Here, we present the results of two years of Bd monitoring in the foothill yellow-legged frog (*Rana boylei*), a stream-dwelling species endemic to California and Oregon (USA) that has declined from over half of its former range (Davidson et al. 2002, Lind 2005). *Rana boylei* is a Species of Special Concern in the State of California (Thomson 2016) and is a candidate for federal Endangered Species Act listing, currently under review (U.S. Fish and Wildlife Service 1994, 2015). A primary driver of *R. boylei* declines is artificial stream flow and temperature regulation by dams (Lind et al. 1996, Kupferberg et al. 2012, Catenazzi and Kupferberg 2013), but the potential role of Bd in the precipitous decline of this species is not yet known. In a location where annual amphibian breeding censuses have been conducted since 2003, a highly anthropogenically modified watershed in California's East San Francisco Bay Area (Fig. 1), we observed dead and dying juvenile *R. boylei* in the fall of 2013. High pathogen loads at the site of dead and dying frogs suggested that the die-off was associated with an outbreak of chytridiomycosis, consistent with high susceptibility in early post-metamorphic individuals observed in other studies (Knapp et al. 2011, Abu Bakar et al. 2016).

The outbreak and two subsequent years of sampling approximately 16 km of stream coincided with the most severe drought event in California in the last 1200 yr (Griffin and Anchukaitis 2014), with 2012–2015 being the driest four consecutive water years since the record began in 1895 (Mann and Gleick 2015, California Department of Water Resources 2016). Extremely low stream flows concentrated frogs in shrinking pools throughout the dry season and the absence of peak flows in the rainy season allowed non-native American bullfrogs (*Rana catesbeiana*) to expand their spatial distribution. Bullfrogs occurred farther away from the lentic environs of a large water impoundment in the surrounding stream network's lotic habitats than had been observed since censuses began. Prior to the recent drought (2012–2015), *R. boylei* were consistently more abundant in the upstream unregulated portions of the study area (Kupferberg et al. 2012), but became relatively more abundant

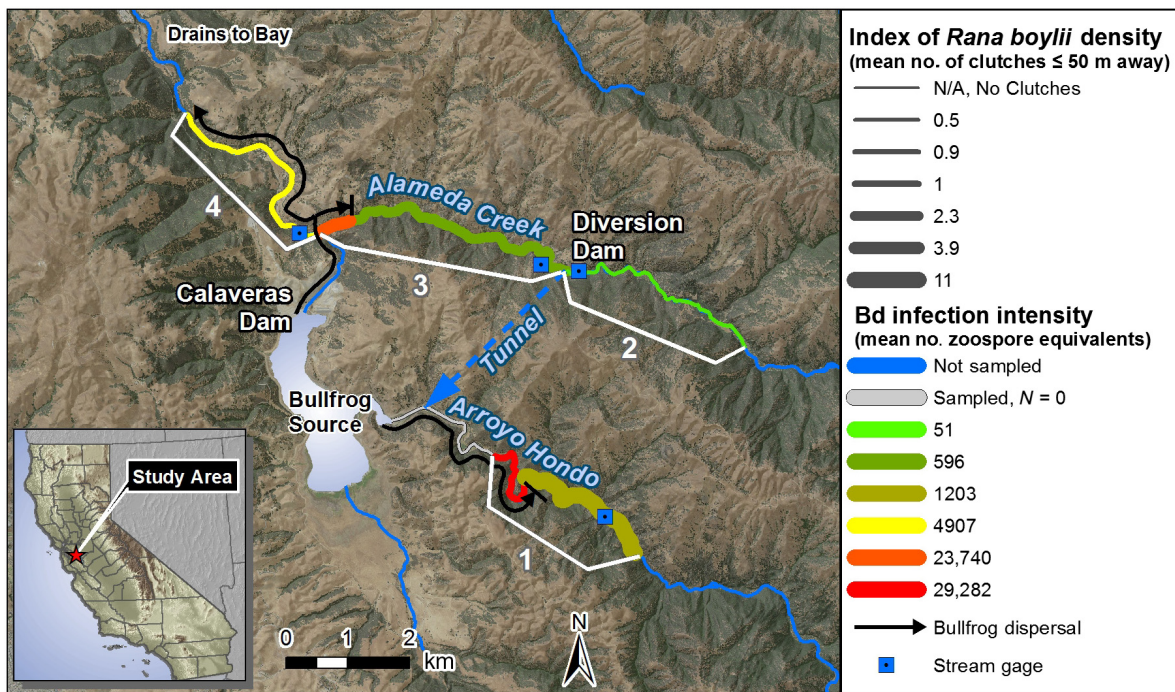


Fig. 1. The location of the study area in Alameda County, California (USA), and the four numbered hydrologically and geomorphologically distinct study reaches: (1) Arroyo Hondo upstream of the reservoir (ochre and red); (2) unregulated Alameda Creek upstream of the diversion dam which delivers water to the reservoir through a tunnel (bright green); (3) downstream of the diversion dam (dark green and orange); and (4) downstream of the confluence of the outflow of Calaveras Dam (bright yellow). Study reach colors correspond to the intensity of Bd infection on foothill yellow-legged frogs (*Rana boylei*) across the reach as well as Bd load of frogs within two infection hot spots (orange segment of Reach 3, and red segment of Reach 4). Line width indicates mean number of *R. boylei* clutches observed within 50 m of capture point of frogs sampled for Bd. Bullfrog (*Rana catesbeiana*) presence/absence status and direction of expansion are indicated by black arrows.

downstream in the regulated reaches (Fig. 2A) which remained wetted throughout the year, while the channels in the upper part of the watershed were completely without surface flow by midsummer (Fig. 2B, C). The 2013 chytridiomycosis outbreak also coincided with this period of shifting frog distribution, prompting us to hypothesize that drought could have played a synergistic role in the Bd outbreak we observed.

The goals of our study were to examine the potential causes of the Bd outbreak and die-off in juvenile *R. boylei*, assess the biotic and abiotic factors that may have influenced Bd prevalence and infection intensity in this population since the outbreak, and suggest which factors may have led to the die-off at this site. In addition, we wanted to better understand the potential for

synergistic effects of threats on this declining species and other species affected by Bd.

MATERIALS AND METHODS

Study site

Located in Alameda County, California, United States, the Alameda Creek watershed (Fig. 1) contains several large water impoundments, including Calaveras Reservoir, which provides a portion of the city of San Francisco's drinking water. Alameda Creek and Arroyo Hondo, the study streams, flow through a series of alluvial valleys and flood plains interspersed with narrow bedrock corridors. Elevations of the study stream reaches range from approximately 130 to 360 m above sea level. The Alameda Creek

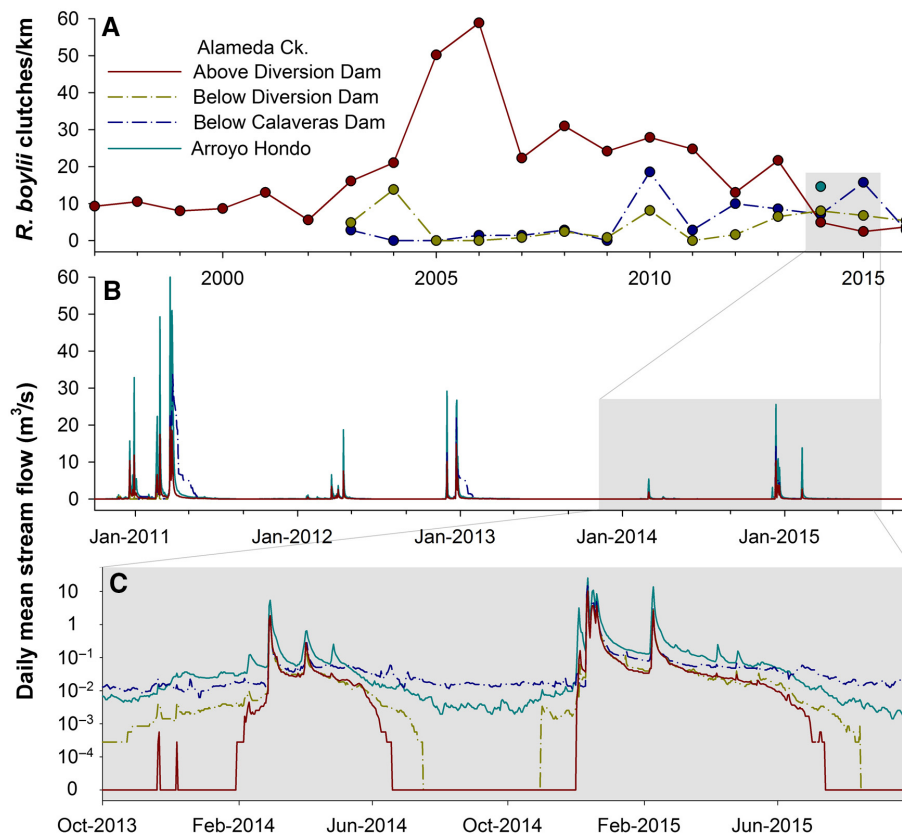


Fig. 2. (A) Breeding population size of *Rana boylei*; (B) daily mean stream flows in the study reaches of Arroyo Hondo and Alameda Creek prior to (i.e., 2011) and during a prolonged drought (2012–2015), showing reduced magnitude of winter flooding for all stream reaches in dry years and in regulated reaches in a normal rainfall year; and (C) differences in flow regime among reaches when sampling of amphibians for Bd occurred. Free-flowing reaches are indicated by solid lines, and regulated reaches by broken lines. A water year spans from 1 October to 30 September.

sampling area consists of three hydrologically distinct reaches: unregulated (i.e., no upstream dams or water diversions); below the Alameda Creek Diversion Dam (which delivers water to Calaveras Reservoir through a tunnel); and below the confluence with Calaveras Creek, which conveys releases from Calaveras Dam to Alameda Creek. The second stream sampled, Arroyo Hondo, is unregulated, but flows into the reservoir (Fig. 1). The four study reaches are also distinct from each other with respect to faunal composition of fish communities, land use (e.g., cattle grazing, recreation), and are different with respect to factors affecting water temperature such as composition of streamside vegetation (shrubs vs. trees), extent of shading by riparian canopy, and height of canyon walls. *Rana boylei*

can move upstream and downstream within both creeks, but migration distances in this system are unknown. A genetic analysis of frogs sampled in the various reaches indicates that *R. boylei* do not move around the reservoir and that it represents a barrier to gene flow (Peek 2012). The *R. boylei* population in the Alameda Creek watershed is one of the last populations of the species in the county, where it was formerly widespread.

Sampling methods

For two years following the fall 2013 Bd outbreak in which we observed dead and dying juveniles and recorded Bd loads that are lethally high in other ranid species (mean \log_{10} Bd load \pm standard error: 3.45 ± 0.36 ; Briggs et al.

2010, Vredenburg et al. 2010, Kinney et al. 2011), we sampled *R. boylei* for Bd and sampled other frog species encountered during the course of our surveys. We also collected a dead *R. boylei* metamorph from the die-off on 7 November 2013 for histologic analysis, which we conducted following Reeder et al. (2012). From November 2013 to September 2015, we surveyed 16 km of stream habitat (Fig. 1) during the day, when *R. boylei* are most active. The cryptic nature of *R. boylei* and its low densities in this system make capturing a large number of individuals a challenge (Appendix S1: Fig. S1). Effort was made to equalize sample size among seasons—we made several more visits during cold weather in the winter when frogs are extremely difficult to locate in order to not disproportionately weight the number of summer samples. There were a total of 30 field days dedicated specifically to Bd sampling (Appendix S1: Table S1). We walked along the banks, waded in the channel, and captured amphibians with gloved hands. We recorded water temperature with a quick-read thermometer and recorded latitude and longitude of capture locations with a hand-held GPS device (Garmin GPSmap 60C_{sx}, Olathe, Kansas, USA). Locations of all encountered bullfrogs and signal crayfish (*Pacifastacus leniusculus*), even if not captured, were also noted. Upon capture, we recorded sex, length (snout-urostyle length for post-metamorphic individuals; body length for tadpoles, using dial calipers), and swabbed for Bd using sterile, rayon-tipped swabs (Medical Wire and Equipment, Corsham, Wiltshire, England) following a standardized protocol (Hyatt et al. 2007). Post-metamorphic individuals were swabbed five times each on the bottoms of the feet, on the ventral thighs, and both sides of the drink patch. Tadpoles were sampled by swabbing across the beak and tooth rows 30 times. A fresh pair of gloves was used to handle each animal to prevent cross-contamination. Swabs were individually placed in sterile screw-cap vials and then frozen upon return from the field (within 6–8 h).

Batrachochytrium dendrobatidis dynamics in a population are often characterized by a positive relationship between Bd prevalence (the proportion of infected individuals) and Bd load (a measure of infection intensity) during an epidemic (Briggs et al. 2010). To test for the quantity of Bd in each sample (Bd load), we used a real-time PCR assay (qPCR). Using qPCR analysis, infection

intensity is determined in terms of zoospore equivalents (ZE), the number of zoospores on the swab sample as compared to a standard curve of serial dilutions of standard Bd DNA. After extracting DNA from swabs using 40 μ L of PrepMan Ultra (Applied Biosystems, Foster City, California, USA), qPCR analysis followed a standardized protocol (Boyle et al. 2004), and samples were run on a StepOnePlus real-time PCR system (Applied Biosystems). Positive controls in quantities of 0.1, 1, 10, and 100 ZE were run in addition to negative controls (PCR water only). Negative controls indicated that there was no false-positive amplification on any of the qPCR plates.

We assessed the local density of *R. boylei* by conducting a breeding census of the full study reach (16 km) in the spring of 2014 following the same protocol (Kupferberg et al. 2012) used for long-term monitoring of sub-sections of Alameda Creek ($\text{km}_{\text{unregulated 1997–2016}} = 1.64$; $\text{km}_{\text{below diversion dam 2003–2016}} = 0.7$; $\text{km}_{\text{below Calaveras Dam 2003–2013}} = 1.23$; $\text{km}_{\text{below Calaveras Dam 2015–2016}} = 3.69$). For ranid frogs that oviposit a discrete mass of eggs (clutch) per year, clutch counts are a commonly used index (Petranka et al. 2007). *Rana boylei* clutches are readily visible on the rocks where they are attached, and are much more conspicuous than the frogs themselves (Appendix S1: Fig. S1) which spend more than half their time below water and under substrates (Gonsolin 2010). Clutch counts closely correspond to the number of adult females (Van Wagner 1996). Spatial clustering in 2015 mirrored that of 2014, so the 2014 density estimates were applied to both study years. This mirroring is consistent with the pattern that *R. boylei* congregate and breed at the same lek sites from year to year (Kupferberg 1996, Wheeler and Welsh 2008). Males begin arriving at the breeding sites in early March and remain in the vicinity of the leks for several weeks after the last female oviposits, and tadpoles and juveniles generally remain within the natal riffle–pool sequence until fall rains trigger dispersal. Every 10–14 d we searched for clutches and marked them by placing a bamboo skewer with flagging in the stream bed. We repeated surveys until no new clutches were found and noted any previously overlooked clutches. For each swab or egg mass location, we took a GPS reading and converted that latitude and longitude to a stream station. Stream stations

are given in river kilometers, defined as a measure of distance tracing the line of steepest ascent in the river channel from its outlet. San Francisco Bay is designated as zero, and distances increase as one moves upstream (U.S. Geological Survey 2015). We calculated stream distances as the difference between the stream station values for each swab location using ArcGIS 10.1 (ESRI, Redlands, California, USA). We calculated two variables from stream station: (1) “Bullfrog Distance,” which is the distance from point of capture to the nearest bullfrog observation, and (2) “*R. boylei* clutches,” which is the number of *R. boylei* egg clutches observed 25 m upstream and 25 m downstream of point of capture (50 m total distance; Table 1).

Analyses and hypothesis evaluation

We used generalized linear mixed-effects models (GLMMs) and an information-theoretic approach to test various hypotheses for which factors best predict Bd prevalence and load in *R. boylei* and in bullfrogs (four models in total). Using mixed-effects models enabled us to account for non-independence of samples that were collected at the same locality on the same day, as well as to evaluate predictors at the individual or site-specific level. To accomplish this, in every model we included “survey event,” a variable created to group frogs sampled on the same date and within the same one of the four study reaches, as a random effect; there were 24 levels of this random effect. To ensure that our results were not driven by this choice of random effect, we also explored a number of alternative random effects in the model, including a continuous random effect accounting for spatial autocorrelation between samples (Appendix S2). These more complex random effects (i.e., error structures) did not affect our inference, and thus, we present the simpler “survey event” random effect in this paper.

Based on the literature, we expected Bd prevalence and load to vary according to different biotic and abiotic factors (Table 1). We hypothesized that drought conditions would positively affect Bd prevalence and load if amphibians become highly concentrated in some reaches as the stream flow becomes intermittent—we expected Bd transmission rates and Bd susceptibility (due to stress) to increase as a function of host density (Rachowicz and Briggs 2007, Peterson and McKenzie 2014, Brannelly et al. 2015). We therefore included as

predictor variables in the model several metrics of hydrologic conditions derived from stream gauge data (collected by the U.S. Geological Survey) that could be indicative of the current drought (including water temperature) and the number of *R. boylei* egg clutches within 50 m of each frog location/Bd sampling locality (Table 1). This 50-m distance scales with the morphology of the channel and the boundaries of a given riffle–pool habitat unit. The typical wetted width of our sampling sites was 8–9 m, and the wavelength of the riffle–pool cycle is five to seven times the channel width (Langbein and Leopold 1964).

In addition to the temperature-dependent rate of both Bd growth (Piotrowski et al. 2004) and responses of amphibians to chytridiomycosis (Raffel et al. 2010, Becker et al. 2012), seasonal changes in climate can affect Bd outcome through host factors such as behavior, transmission opportunities, and immune function (Kriger and Hero 2007, Rowley and Alford 2007, Ribas et al. 2009, Kinney et al. 2011). Therefore, we hypothesized that there could be variation in seasonal effects on Bd. We used a water year variable to determine whether there was an effect of time as the multi-year drought continued. Water year 2014 is 1 October 2013 through 30 September 2014, and water year 2015 is 1 October 2014 through 30 September 2015.

Additional biotic variables of interest in this system include the presence of non-native species, bullfrogs, and crayfish. Bullfrogs are capable of becoming infected with Bd, but do not appear to succumb to chytridiomycosis when infected with most strains, making them a potential disease vector and reservoir, both in the live amphibian trade and in the wild (Daszak et al. 2004, Garner et al. 2006, Schloegel et al. 2012, Gervasi et al. 2013). Since both bullfrogs and crayfish can harbor Bd and are capable of transferring infection to amphibian hosts (Greenspan et al. 2012, McMahon et al. 2013, but see Betancourt-Roman et al. 2016), we expected the presence of these invasive species to positively influence Bd in the system. We also tested for effects of different biometric variables (i.e., length, stage, and sex; Table 1) as these have been associated with Bd prevalence and load in amphibian populations (Kriger et al. 2007, Garner et al. 2009, Imasuen et al. 2011).

Driven by the aforementioned hypotheses, we used a forward selection procedure with the GLMMs to determine the predictor variables that

Table 1. Variables used in mixed-effects models to predict Bd load and probability of Bd infection.

Covariate	Type	Range or levels	Description
Season 3	Environmental/ Temporal	Winter/Spring; summer; fall	Winter/Spring: 1 December to 31 May; Summer: 1 June to 31 August; Fall: 1 September to 30 November
Season 2	Environmental/ Temporal	Wet, dry	Wet season: 1 December to 31 May; Dry season: 31 June–30 November
Hydrologic unit/ Flow regime	Environmental/ Geographic (anthropogenic)	Alameda Creek: unregulated; diversion; dam release and diversion Arroyo Hondo: unregulated	Stream and flow regime where sampling occurred
Water year	Environmental (drought)	2014, 2015	1 October–30 September
Water temperature	Environmental/ Temporal (drought)	9.9–23.0°C	Temperature of stream†
Days since peak stream flow	Environmental (drought)	6–429 d	Number of days since peak stream flow for the respective water year
Preceding peak stream flow	Environmental (drought)	4.9–121.8 m ³ /s	Peak flow of respective water year that preceded survey date‡
Mean daily stream flow	Environmental (drought)	0–0.12 m ³ /s	Mean daily flow on the survey date‡
Drought index	Environmental (drought)	1.0–54.4 d/m ³ s ⁻¹	Days since the peak flow of the respective water year divided by the magnitude of that peak flow
Sex-stage§	Biological	Tadpole, juvenile, female, male	Combination of sex (if stage is adult) and stage (larval or juvenile) if not adult
Stage¶	Biological	Tadpole, post- metamorphic	Indicates whether pre- (i.e., tadpoles) or post-metamorphic (juveniles and adults)
Length	Biological	21.2–67 mm	Snout-vent length (for adults); body length (for tadpoles and juveniles); measured with dial calipers
Crayfish§	Biological (invasive species)	Present, absent	Binary indication of whether crayfish are present at the site
Bullfrogs§	Biological (invasive species)	Present, absent	Binary indication of whether bullfrogs observed at the site
Bullfrog time§	Biological (invasive species)	Absent, recent, established	History of bullfrog observations at site (absent = no observations 1997–2015; recent = observed during drought 2012–2015; established = observed pre-drought)
Bullfrog distance§	Biological (invasive species)	0.0–8027.8 m	Distance to the nearest bullfrog along the stream
<i>Rana boylei</i> clutches	Biological	0–19	Number of <i>R. boylei</i> egg clutches within 50 m (25 m upstream and 25 m downstream) of sample collection site

Note: Bd, *Batrachochytrium dendrobatidis*.

† Water temperature was measured with a thermometer at the site of capture. If thermometer temperature was not available, then median daily temperature from the nearest USGS gage station was used, via National Water Information System: Web Interface (<https://waterdata.usgs.gov/nwis>).

‡ Measured at USGS stream gage for respective reach, accessed using National Water Information System: Web Interface. Gages used: 11173200—Arroyo Hondo near San Jose, California; 11172945—Alameda Creek above diversion dam, near Sunol, California; 11172955—Alameda Creek below diversion dam, near Sunol, California; 11173510—Alameda Creek below Calaveras Creek, near Sunol, California.

§ Variables used in *R. boylei* models only.

¶ Variables used in bullfrog models only.

were the best fit to the data. Predictor variables were sequentially tested for all four models in the order as presented in Table 1. We log-transformed the ZE values for the model with *R. boylei* Bd load as a response variable. We z-transformed all

continuous predictor variables so that effect sizes of different predictors were comparable. Only post-metamorphic *R. boylei* were used in both *R. boylei* Bd prevalence and load models since all *R. boylei* tadpoles were Bd negative. Interactions

were included in the models whenever biologically appropriate. We ranked candidate models according to Akaike's information criterion (AIC) to determine the relative importance of predictor variables within each model set. The models with the lowest AIC were considered the best-supported models by the data, and models with a $\Delta\text{AIC} > 2$ as compared to the model with the lowest AIC were considered not as well supported by the data (Burnham and Anderson 2004). We complemented this information-theoretic approach by computing likelihood ratio tests for nested models. Variance inflation factors (VIFs) were used to determine that none of the fixed effects in the best-fit models were collinear, as indicated by VIF values < 3 (Zuur et al. 2010). We conducted all analyses in the R computing environment (R Development Core Team 2012). Generalized linear mixed-effects models were fit using the "glmer" (for Bd presence/absence models) and "lmer" (for the Bd load models) functions in the "lme4" package (Bates 2010). If a model failed to converge using these functions, we refit the identical model using a Bayesian approach with slightly regularizing prior distributions on the model coefficients. This allowed for convergence of the model while guarding against overfitting (McElreath 2016).

RESULTS

Histologic examination of serial transverse sections of a dead *Rana boylei* metamorph collected from the 2013 die-off revealed lesions of moderate to severe epidermal hyperplasia and hyperkeratosis and myriad intralesional chytrid-type fungal organisms consistent with lethal chytridiomycosis (Appendix S1: Fig. S2). No visceral lesions of other infectious diseases known to cause mortality events of metamorphs (e.g., Ranavirus) were observed. Between 7 November 2013 and 11 September 2015, *R. boylei* were the most frequently encountered species throughout the study reaches (Fig. 3A). We captured and sampled 142 *R. boylei* individuals (127 post-metamorphic), along with four *Anaxyrus boreas halophilus* (California toad), 26 *Hyla regilla* (Pacific treefrog), 10 *Rana draytonii* (California red-legged frog), and 33 *Rana catesbeiana* (American bullfrog). Bullfrog observations were restricted to sites downstream of 170 m elevation in Alameda Creek and

downstream of 228 m elevation in Arroyo Hondo (Figs. 1, 3A). All species tested positive for Bd, and infection prevalence (Fig. 4A) for all species combined was 40% (87 of 216 samples). Bd-positive individuals were found across all reaches, from the most downstream to the most upstream extent of the surveys. Among species, Bd loads were highest in *R. boylei* and bullfrogs (Fig. 4B). Within *R. boylei*, males were more likely to be infected than either females or juveniles (Fig. 5D).

The model analyses of post-metamorphic *R. boylei*, for both Bd presence/absence and Bd load, indicated that spatial and temporal environmental factors were important. The presence of bullfrogs had a positive influence on Bd infection (Appendix S1: Tables S2, S3; Figs. 5, 6). While Bd prevalence was higher in water year 2015 than in 2014 (Fig. 5), none of the stream flow metrics included were important predictor variables based on the best-fit models for Bd presence/absence in *R. boylei* (Appendix S1: Table S2). For Bd load however, a marginally significant negative association of mean daily stream flow was included among the best-fit models (i.e., significant at $\alpha = 0.1$, but not at $\alpha = 0.05$; Table 2, Appendix S1: Tables S2, S3; Fig. 6). Two of the best-fit models for Bd load in *R. boylei* included an interaction between season and mean daily stream flow (Appendix S1: Table S3). Bd loads in *R. boylei* were generally lower in summer than in fall (Fig. 6C), and stream flows were lowest in fall (Fig. 2). The local density of conspecifics, as indicated by the number of *R. boylei* egg clutches within 50 m of each capture location, was also a significant positive predictor of Bd load in *R. boylei* (Table 2 and Appendix S1: Table S3; Fig. 6E).

Because bullfrogs were among the most important predictors of Bd in both the *R. boylei* load and prevalence models, and bullfrogs are potentially a Bd reservoir in the systems they inhabit, we also included models of Bd in bullfrogs to see which factors best predict Bd infection in that species. The best predictors of Bd presence/absence in bullfrogs included a positive effect of water temperature and an effect of life stage, in which post-metamorphic individuals were more likely to be Bd positive than tadpoles (Table 2; Appendix S1: Table S4; Fig. 7). In the bullfrog Bd load model, none of the predictors improved the model beyond the intercept-only model (Appendix S1: Table S5).

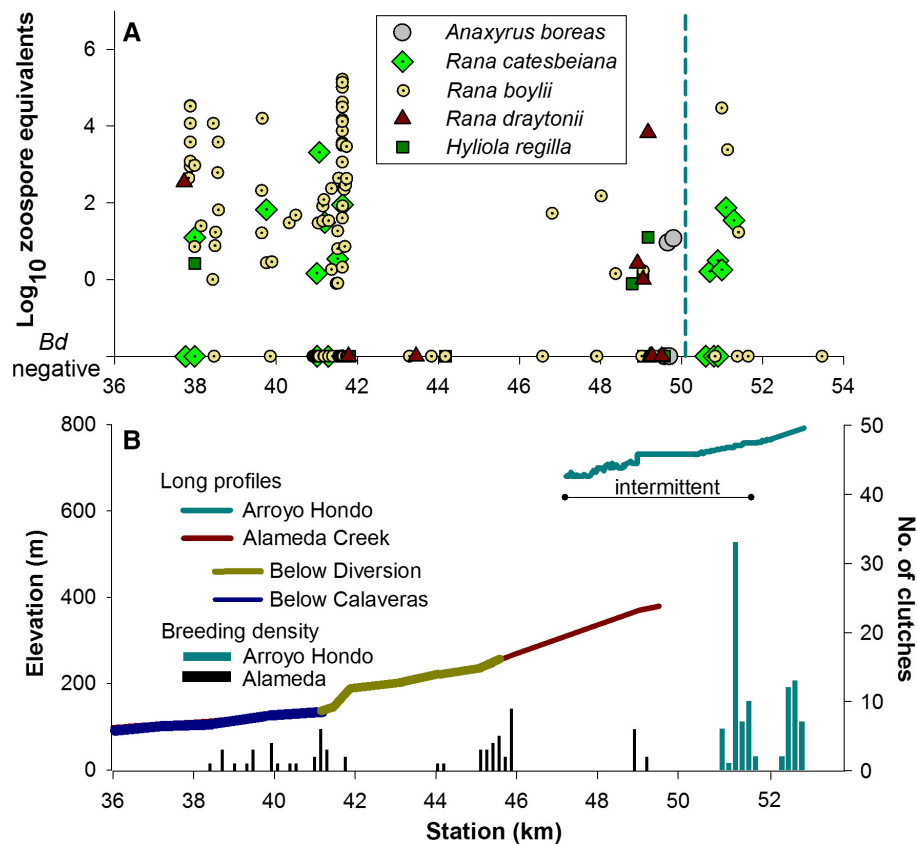


Fig. 3. (A) Pathogen load by species at stream station locations. Left of the vertical dashed line is Alameda Creek; right of the dashed line is Arroyo Hondo. (B) Stream profiles (lines) and number of *Rana boylei* clutches (bars) observed at stream station locations along Alameda Creek and Arroyo Hondo in 2014. Breeding sites were visited an average of four times between 13 March and 14 May (during the oviposition season). In Alameda Creek, only the reach depicted in dark blue remained continuously wetted throughout the drought.

DISCUSSION

Bd susceptibility in *Rana boylei*

Our observations of relatively high *Bd* loads and lesions consistent with severe chytridiomycosis coinciding with a mass mortality event make this the first published report of lethal chytridiomycosis in *R. boylei* in the field. Although *Bd* has been detected many miles upstream of the current study site in a tributary of Arroyo Hondo over the last decade (Padgett-Flohr and Hopkins 2010), these were the first indications of negative effects of *Bd* infection among lotic-breeding frogs in the watershed. *Bd* has been documented in the watershed from museum specimens collected in 1966, and in live animals as recently as 2007 (Padgett-Flohr and Hopkins 2009, 2010), approximately

5 miles upstream of the closest sampling location used in this study, but it is possible that the 2013 outbreak may have been the result of an introduction of a novel genotype of *Bd* to the watershed. Even when genotypes are the same (e.g., belonging to the widespread, deleterious Global Panzootic Lineage of *Bd*), local variation in phenotype can lead to differential *Bd* outcome in the host (Lambertini et al. 2016), so a novel variation in genotype is not essential for a shift from enzootic to epizootic conditions.

Our observations that *R. boylei* can be susceptible to the lethal consequences of chytridiomycosis in the field are in contrast to laboratory experiments (Davidson et al. 2003, 2007) and a field study (Lowe 2009) that found reduced growth or body condition in *Bd*-positive juveniles, but which

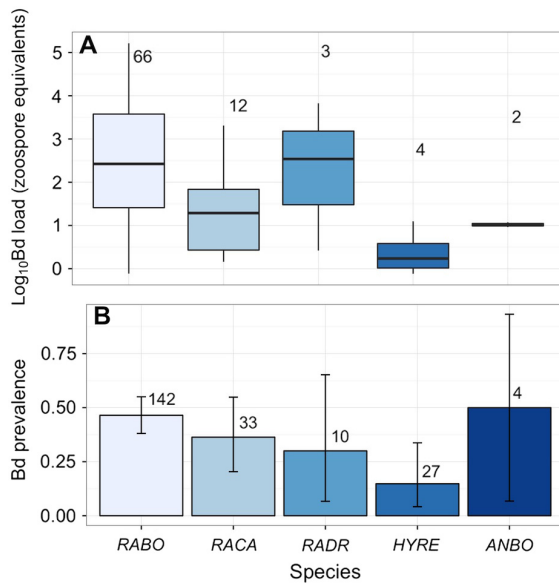


Fig. 4. (A) Bd load and (B) Bd prevalence for all anuran species sampled at the study site. Bold horizontal lines within each boxplot in (A) indicate the median, boxes show the interquartile (IQ) range, and whiskers show the range within 1.5 times the IQ range. Error bars in (B) represent the 95% Clopper–Pearson binomial confidence intervals. Numbers above the bars indicate (A) the number of Bd-positive individuals or (B) total sample size for each species. Species codes: RABO, *Rana boylei* (foothill yellow-legged frog); RACA, *Rana catesbeiana* (American bullfrog); RADR, *Rana draytonii* (California red-legged frog); HYRE, *Hyllola regilla* (Pacific treefrog); ANBO, *Anaxyrus boreas halophilus* (California toad).

were inconclusive with respect to chytridiomycosis-induced mortality. Significant within-species variation in Bd outcome is not uncommon (Briggs et al. 2010, Bradley et al. 2015). Indeed, when the experiment of Davidson et al. (2007) was repeated, and *R. boylei* from the same location were exposed to the same Bd strain, the result was 100% mortality (C. Davidson, unpublished data). Different disease outcomes could result from variation in a variety of biotic or abiotic factors, including immunity-related factors, such as composition of the skin microbiome (Krynak et al. 2016), differences in antimicrobial peptides (AMPs), behavior, or major histocompatibility complex genotype (Rollins-Smith and Conlon 2005, Savage and Zamudio 2011). The AMPs in *R. boylei* skin have been found to be highly active against Bd in culture (Davidson

et al. 2007); however, species with peptides active in vitro such as the mountain yellow-legged frog (*Rana muscosa*) can still be highly susceptible to Bd infection in nature (Rachowicz et al. 2006, Rollins-Smith et al. 2006).

Climate

Increasing volatility and variability in predicted precipitation is expected to have considerable conservation consequences for amphibians, which can have highly specific flow and moisture requirements (Walls et al. 2013). A spatial analysis of *R. boylei* decline suggested that climate change may be influencing the species' northward range

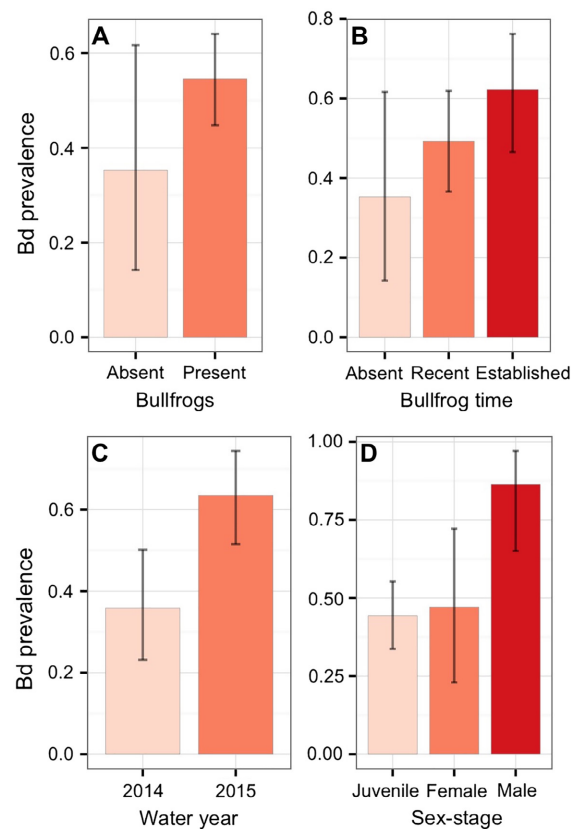


Fig. 5. Relationship between Bd prevalence in *Rana boylei* and the most important explanatory variables as determined by the best-fit mixed-effects models (Table 2 and Appendix S1: Table S2), including (A) Bullfrog presence/absence; (B) length of time of bullfrog presence; (C) water year; (D) sex/life stage. Error bars indicate 95% Clopper–Pearson binomial confidence intervals. Descriptions of explanatory variables are in Table 1.

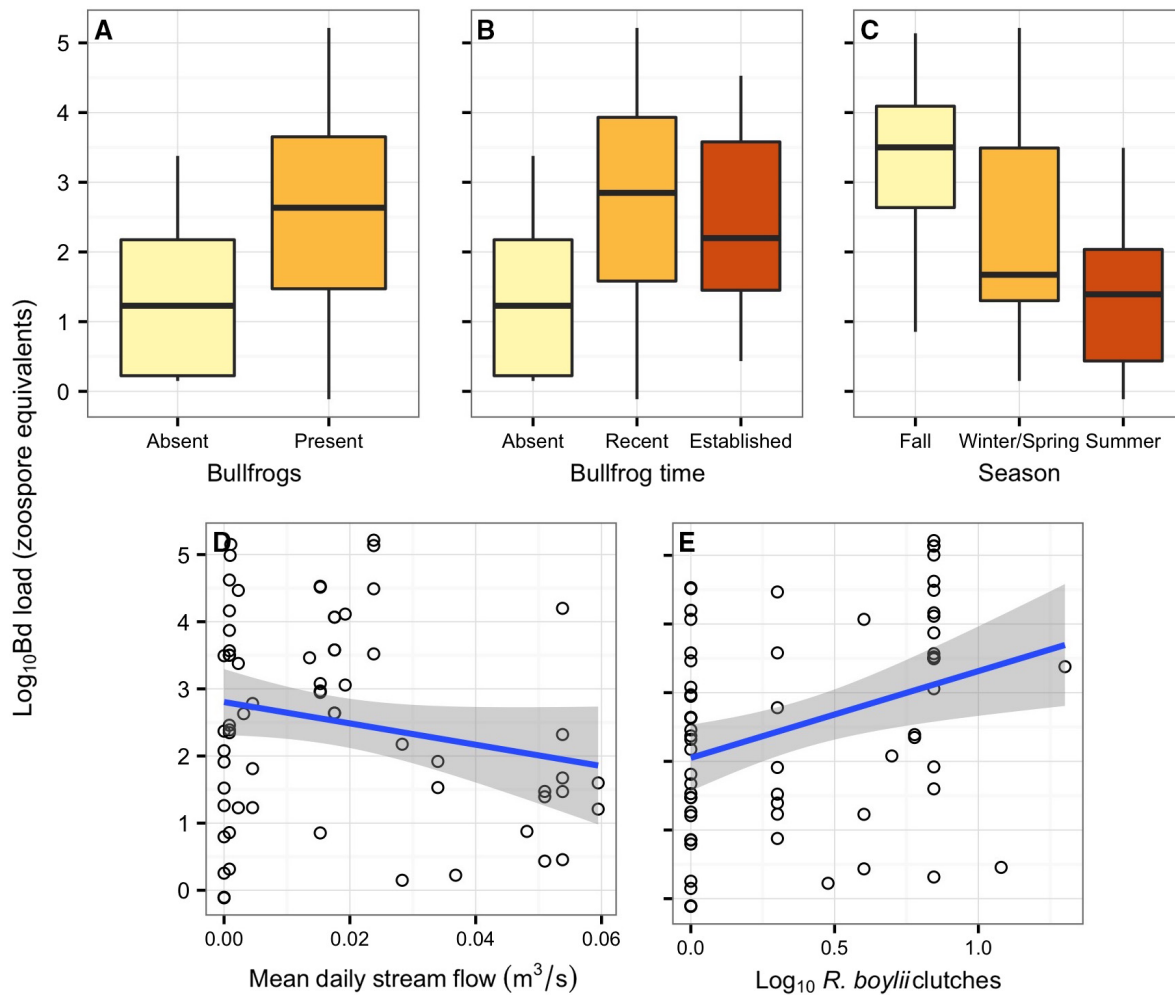


Fig. 6. Relationship between Bd loads of infected *Rana boylei* and the most important explanatory variables as determined by the best-fit mixed-effects models (Table 2 and Appendix S1: Table S3), including (A) bullfrog presence/absence; (B) length of time of bullfrog presence; (C) season; (D) mean daily stream flow; and (E) number of *R. boylei* clutches within 50 m of each Bd sampling point. Both (D) and (E) depict the best-fit line for a regression of the two continuous variables against \log_{10} Bd load. Descriptions of explanatory variables are in Table 1.

contraction (Davidson et al. 2002). In addition, during the drought, the previously robust population in the upstream unregulated reach of Alameda Creek declined steadily to the lowest number observed during 20 yr of annual monitoring (Kupferberg et al. 2012, Fig. 2A). This, coupled with our observation that Bd loads in *R. boylei* increase at lower stream flows, indicates that climate change, water extraction for human use, and disease may be acting synergistically to threaten *R. boylei* populations in central California and amphibians globally. Bd zoospores, the infective stage of the pathogen, are flagellated and

actively swim in the water column (Piotrowski et al. 2004), so could be concentrated at lower flows. This has been observed in laboratory experiments, in which Bd naïve frogs had significantly decreased time to mortality and Bd growth rate at higher flow rates, presumably because of the increased availability of zoospores at lower current velocities (Tunstall 2012).

Our observation that water temperature has a positive relationship with Bd infection in bullfrogs is consistent with the optimum range of temperatures for Bd growth in amphibian species of temperate regions (Raffel et al. 2010, Becker

Table 2. Parameter estimates for best-fit models (see Appendix S1: Tables S2–S4) used to determine the best predictors of (A) Bd presence/absence in *Rana boylei*; (B) Bd presence/absence in bullfrogs; and (C) Bd load in *R. boylei*.

Model	Parameter	Estimate	SE	z	P
(A) Bd presence/absence (<i>R. boylei</i> ; $R^2 = 0.35$)	(Intercept)	−5.32	2.99	−1.78	0.08
	Water year 2015	4.29	2.73	1.57	0.12
	Sex-stage (Juveniles)	−0.47	1.49	−0.32	0.75
	Sex-stage (Males)	4.90	2.56	1.92	0.06
	Bullfrog time (Established)	3.25	2.48	1.31	0.19
	Bullfrog time (Recent)	5.10	2.62	1.94	0.05
(B) Bd presence/absence (bullfrogs; $R^2 = 0.95$)	(Intercept)	−4.54	1.84	−2.47	0.01*
	Water temperature	5.88	2.49	2.36	0.02*
	Stage (Post-metamorphic)	9.78	4.53	2.16	0.03*
		Estimate		95% CI (lower, upper)	
(C) Bd load (<i>R. boylei</i> ; $R^2 = 0.53$)	(Intercept)†	4.42		2.09, 6.76	
	Season 3 (Summer)†	−4.60		−6.24, −2.95	
	Season 3 (Winter/Spring)	−0.43		−2.97, 1.89	
	Mean daily stream flow	−1.13		−2.40, 0.22	
	Bullfrog time (Established)†	3.06		0.80, 5.47	
	Bullfrog time (Recent)†	2.48		0.18, 4.84	
	<i>R. boylei</i> clutches†	0.82		0.14, 1.53	

Notes: SE, standard error. Confidence intervals (CI) were calculated using a parametric bootstrap. R^2 values presented for each model were calculated for the fixed effects.

* $P < 0.05$.

† Parameter estimates with 95% CI that do not overlap zero.

et al. 2012), although temperature variability and not just absolute temperature can also affect host responses to Bd (Raffel et al. 2013). The majority of Bd positives in bullfrogs in this study occurred when water was warmer than 17°C (Fig. 7B), which is the lower end of the thermal optimum growth range of Bd (Piotrowski et al. 2004, Woodhams et al. 2008). California climate change projections under a range of emissions scenarios predict a 1.5–4.5°C increase in air temperatures within the next century (Cayan et al. 2008), consistent with historical observations and projections of future river temperatures in the United States (Kaushal et al. 2010, van Vliet et al. 2013). Therefore, temperatures could rise into Bd's thermal optimum growth range in portions of the Alameda Creek watershed, potentially increasing the prevalence of Bd in bullfrogs in this system. Although stream temperatures largely follow air temperatures, they are spatially heterogeneous as a result of microgeographic factors such as tributary plumes, influx of groundwater, and canopy shading, creating locally cooler conditions (Webb et al. 2008, Fullerton et al. 2015, Wawrzyniak et al. 2016). For example, planned hypolimnetic releases from Calaveras Reservoir after the completion of the Calaveras Dam Replacement Project

(now under construction) will cool Alameda Creek downstream of the confluence with Calaveras Creek (Study Reach 4, Fig. 1) by as much as 5°C (McBain Associates 2014). This is below the realized thermal niche for *R. boylei* tadpoles (Catenazzi and Kupferberg 2013, Wheeler et al. 2015), but may limit Bd in bullfrogs.

In vitro, Bd has the ability to rapidly adapt to a broad spectrum of thermal conditions by optimizing its growth rate, which may affect the severity of chytridiomycosis in the host (Voyles et al. 2012). Therefore, while present temperatures may be in the optimum range for growth in the bullfrog reservoir host, Bd may be able to adapt to local temperature shifts. Our observation that Bd prevalence is higher in bullfrogs at temperatures that are optimum for the fungus in vitro is supported by the thermal optimum hypothesis, but is speculative given that Bd's response to temperature in the host is complex (Fisher et al. 2009, Raffel et al. 2013). In laboratory experiments, hosts infected with Bd have shown different responses to increased temperatures, ranging from no response to increased survival (Berger et al. 2004, Carey et al. 2006, Andre et al. 2008). In contrast to our observations that warmer temperatures appear to positively

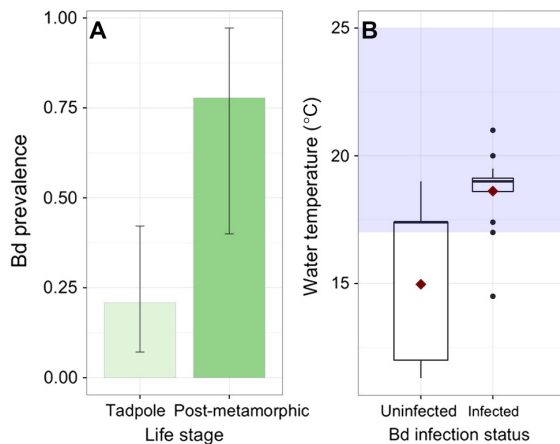


Fig. 7. Bd infection in non-native American bullfrogs (*Rana catesbeiana*), with (A) Bd prevalence by life stage and (B) Bd infection status at the range of water temperatures observed. Error bars in (A) indicate 95% Clopper-Pearson binomial confidence intervals. Bold bars in (B) indicate the median; lower and upper hinges indicate the 25% and 75% quantiles, respectively; and lower and upper whiskers indicate the smallest and largest observations greater than or equal to the upper and lower hinges—1.5 times the interquartile range, respectively. Red diamonds represent the means and the blue shaded area represents the Bd thermal optimum growth range from Piotrowski et al. (2004).

influence Bd infection in bullfrogs in this system, we observed lower Bd loads in *R. boylei* in summer (Fig. 6C). However, temperature was not an important predictor of either Bd load or prevalence in the *R. boylei* models.

Bullfrogs

Our findings that both the probability of Bd infection and the probability of Bd load are higher in *R. boylei* when bullfrogs are present are supported by a prior field study that showed a positive relationship between Bd prevalence and load and bullfrog density in native amphibian populations sympatric with non-native bullfrogs (Peterson and McKenzie 2014). Because they prefer pools with little or no flow, bullfrog densities in rivers can increase during drought years in California's Mediterranean climate (i.e., cool, wet winters and warm, dry summers), particularly after years with low winter peak discharges (Kupferberg 1997, Doubledee et al. 2003). We attribute the influence of water year on Bd

prevalence in *R. boylei* to the continued expansion of bullfrogs into the study area through water year 2015. In addition, the site of the 2013 die-off is the zone of most recent contact with bullfrogs in the stream, so Bd naïve *R. boylei* juveniles were located in the area where the density of alternate hosts was increasing as the result of the drought. In recent drought years, bullfrogs expanded their range at the Alameda Creek site, providing a Bd reservoir host species where previously there had been none. Although it has been suggested that *Hyliola (Pseudacris)* species may act as a Bd vector and reservoir in California (Padgett-Flohr and Hopkins 2009, Reeder et al. 2012), Bd prevalence and load were lower in *Hyliola regilla* than in the bullfrogs observed in this study (Fig. 4). Moreover, *H. regilla* (which is terrestrial for part of its life history) and *R. boylei* share the same stream channel habitat less frequently compared to *R. boylei* and bullfrogs, so transmission opportunities between *R. boylei* and *H. regilla* are fewer at this site.

In addition to their role as Bd vectors (Green-span et al. 2012, Schloegel et al. 2012), bullfrogs may also increase native ranids' susceptibility to Bd by decreasing their fitness in other ways. In mesocosm experiments, both *Rana draytonii* tadpoles (Kiesecker and Blaustein 1998) and *R. boylei* tadpoles (Kupferberg 1997) had increased time to metamorphosis and decreased mass when housed with bullfrog tadpoles and/or adults, presumably because of shifts in behavior, habitat use, and resource availability. Such stresses can act synergistically to increase Bd susceptibility in sympatric species.

The male effect

Our observation that Bd prevalence is higher in *R. boylei* males than in either females or juveniles could be caused by behavioral or physiological factors. Several behaviors observed in *R. boylei* males may increase opportunities for Bd transmission, therefore increasing the likelihood that they will be infected with Bd. For example, adult male *R. boylei* frequently engage in aggressive wrestling behavior, likely induced by calling activity (Wheeler and Welsh 2008, Murphy et al. 2011). *Rana boylei* is a prolonged breeder (i.e., breeding occurs over a period of greater than one month), as indicated by their male-biased daily operational sex ratio (Wheeler and Welsh 2008). At breeding sites, *R. boylei* males will congregate

and stay in the water for extended periods, while females arrive at different times throughout the breeding season (Wheeler and Welsh 2008), so the higher incidence of Bd infection observed in males in this study could be due to higher rates of contact with each other and with the water, which Bd needs to survive (Johnson et al. 2003). A similar trend has been observed in Boreal toad (*Anaxyrus boreas boreas*) populations in Colorado, United States, wherein males in chytridiomycosis-infected populations have much lower survival rates than adult females (Carey et al. 2006).

In addition to behavior, physiological factors such as testosterone and other sex hormones can lead to higher parasite loads in male amphibians. For example, the prevalence and intensity of macroparasite infections are generally higher in males than in females, owing to the relationship between sex hormones and immune function (Klein 2004). In addition, testosterone may play an immunosuppressive role in amphibians as it does in mammals and birds; in one study, higher Ranavirus titers were associated with higher testosterone levels in males (Crespi et al. 2015).

Our observations that both bullfrog presence and sex influence Bd presence in *R. boylei* may be multiplicative, although we did not find strong statistical evidence for this interaction in either of the *R. boylei* models (Appendix S1: Tables S2, S3). *Rana boylei* frequently amplex bullfrogs in an attempt to breed where the species are sympatric (S. J. Kupferberg and S. Bobzien, *personal observations*, Fig. 8, Lind et al. 2003), suggesting that *R. boylei* males may experience direct Bd transmission from contact with bullfrog reservoir hosts. In addition, Bd-infected bullfrogs have been observed shedding more infective zoospores than other native western species (Peterson and McKenzie 2014).

Batrachochytrium dendrobatidis itself may alter male host behavior to increase opportunities for transmission or increase reproductive investment in infected males that have a shorter lifespan due to chytridiomycosis infection (Chatfield et al. 2013, An and Waldman 2016). In Alameda Creek after the Bd outbreak, young-of-last-year males were observed amplexing other males during the day, behaviors not seen in the prior 19 yr (S. J. Kupferberg, *personal observation*), suggesting that Bd infection status may influence this behavior if such a causative mechanism exists.



Fig. 8. Male foothill yellow-legged frog (*Rana boylei*; 51 mm snout-urostyle length) in amplexus with a non-native American bullfrog (*Rana catesbeiana*) at the site of the 2013 chytridiomycosis die-off in Alameda Creek. Photo credit: Steve Bobzien.

Density

Our finding that Bd loads in *R. boylei* increase with increasing density of *R. boylei* clutches within 50 m of a Bd sampling site (i.e., individual frog location) is consistent with the hypothesis that Bd transmission is density dependent (Briggs et al. 2005, 2010). Large increases in Bd prevalence have been observed during the breeding season of aggregate breeding species (Kinney et al. 2011), likely related to this density dependence phenomenon (Brannelly et al. 2015). *Rana boylei* density at the 2013 die-off site may have increased as a result of the drought on two spatial and temporal scales. First, over the course of the drought, the number of frogs breeding and laying eggs increased in the vicinity. Second, within a given breeding season, the drought caused individual pools to become isolated with little surface flow, likely allowing Bd's infective zoospore stage to increase in the shrinking pools. The bedrock lithology of the steepest part of Study Reach 3 (Fig. 1), where we observed very high Bd loads, forces subsurface flows above ground, so the area remains wetted when the channel dries out upstream. Therefore, by creating a refuge for frogs during the drought, the canyon morphology of this reach may have also created a refuge for Bd.

Rana boylei population trajectories through 2010 indicate that historically the more dense populations occurred upstream in the unregulated

reaches (Kupferberg et al. 2012), but since the drought began, trends have reversed. The perennial reach of Alameda Creek, which remained wet because of discharge from Calaveras Reservoir, and the perennial reach of Arroyo Hondo, which drains a large watershed and flows into Calaveras Reservoir, provide refugia for *R. boylei* but also expose them to increased risk because bullfrogs can thrive there. The potential indirect negative effects of bullfrogs as Bd reservoir hosts, which our results suggest can be added to their well-documented direct effects on native amphibians as predators (Kats and Ferrer 2003).

Flow regulation

Globally, flow regulation can cause a plethora of environmental problems, and the influence of dams and diversions on invasive species and pathogens is not unique to the system in this study. When stream or river flow is manipulated, it can create complex cascades of indirect effects on disease outcomes (Ong et al. 2016). Fish can be more susceptible to parasites in regulated systems, especially when dams increase abundance of an alternate reservoir host (Bartholomew et al. 2007), but ours is the first study that we are aware of to recognize the potential for indirect effects of flow regulation on Bd outcome for native amphibians. Non-native species proliferate when flow regulation creates habitat similar to their native ranges (Rahel 2002, Lobos and Jaksic 2005), especially when ephemeral lotic systems become permanent lentic ones. In California's rivers, habitat conversion and diminution of winter flooding (due to dams and inter-annual variation in precipitation as shown in Fig. 2B) promote persistence and expansion of bullfrog populations (Kupferberg 1997, Doubledee et al. 2003, Fuller et al. 2011). In addition, the pattern we observed of low flows assisting an advancement of the bullfrog invasion upstream is similar to a study of California fish, wherein non-native fish assemblages were favored in drought years and natives in non-drought years (Marchetti and Moyle 2001).

CONCLUSIONS

Rana boylei appears to be susceptible to the lethal consequences of chytridiomycosis in the field, and flow regulation, drought, invasive bullfrogs, and Bd may be acting synergistically to impact

R. boylei populations in this system. During extreme drought, when the reach downstream of the dam remained wet, while other reaches went dry, a 20-yr pattern of higher *R. boylei* densities in unregulated reaches was reversed (Fig. 2A). Because loss of the young-of-the-year cohort (e.g., scouring of eggs after ill-timed dam releases) has been associated with subsequent declines of *R. boylei* in this and other rivers (Kupferberg et al. 2012), we anticipate that the effects of chytridiomycosis-induced mortality on recent metamorphs may have a time-lagged impact on the population that survived the drought. Furthermore, shifts to epizootic states among populations in space and time can cause mortality with population-level consequences even after Bd has reached a state of endemism (Briggs et al. 2010, Pilliod et al. 2010, Piovia-Scott et al. 2015), so a greater understanding of the biotic and abiotic factors that affect Bd outcome is critical. Our findings highlight the importance of implementing management actions (e.g., eradicating bullfrogs, mimicking the natural disturbance regime) that increase resilience in declining wildlife populations that are threatened by flow regulation, climate change, invasive species, and disease.

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