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Decline and localized extirpation of the foothill yellow-legged frog (*Rana boylii*) in the presence of the fungal pathogen, *Batrachochytrium dendrobatidis*: Contemporary and historical perspectives

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in Ecology, Evolution, and Marine Biology

by

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March 2017

The dissertation of Andrea J. Adams is approved.

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I dedicate this work to the foothill yellow-legged frog and to my grandmother, Betty Adams, who always wanted me to be able to do what I wanted to do.

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Adams, A.J., A.P. Pessier, and C.J. Briggs. 2017. Historical rise in fungal pathogen prevalence coincides with rapid southern California extirpation of *Rana boylii.* California-Nevada Declining Amphibian Populations Task Force Meeting, Santa Barbara, California. (Presentation)

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ABSTRACT

Decline and localized extirpation of the foothill yellow-legged frog (*Rana boylii*) in the presence of the fungal pathogen, *Batrachochytrium dendrobatidis*: Contemporary and historical perspectives

by

Andrea J. Adams

The introduction and spread of novel pathogens is an increasingly important contributor to biodiversity loss, and *Batrachochytrium dendrobatidis* (Bd), the causative agent of the deadly amphibian disease chytridiomycosis, has led to the declines or extinctions of many amphibians globally. Repatriation programs are increasing in response to species extirpations; however, a prerequisite to these programs is an understanding of the causative factors in declines. The foothill yellow-legged frog (*Rana boylii*) was enigmatically extirpated from the southern California portion of its range between the late 1960s and the mid-1970s. Little is known about the cause of this rapid, localized extinction event, *R. boylii*'s Bd susceptibility, the impact of Bd on extant *R. boylii* populations, or the history and current status of Bd in amphibian populations in southern California. I tested the hypothesis that Bd could have played an important role in the rapid extirpation of *R. boylii* from southern California through field surveys, laboratory experiments, interviews, and museum specimen sampling. I found that where the species is extant in central California, it

is lethally susceptible to chytridiomycosis, and that infection is related to the presence of non-native American bullfrogs (*Rana catesbeiana*). I optimized methods to detect Bd DNA from formalin-fixed museum specimens using real-time quantitative polymerase chain reaction (qPCR), and found that both DNA extraction method and the pathogen load of frogs prior to formalin fixation are important predictors of successful Bd DNA detection. Finally, using an interdisciplinary approach that combined museum specimen sampling, interviews, and field notes, I examined the historical prevalence of Bd and abundance of *R. boylii* in southern California, and the timing of *R. boylii*'s extirpation. I found that Bd prevalence increased in the mid-to-late 20th century and coincided with *R. boylii*'s rapid decline from the region. This work reveals new insights into the current and historical role of chytridiomycosis in California amphibian declines and can serve to inform future *R. boylii* reintroduction efforts.

INTRODUCTION

The discipline of Conservation Biology arose in response to the need for a better understanding of increasing biodiversity loss, with the hope of ameliorating the crisis (Soulé 1986, Wilson and Peter 1988). As the "sixth mass extinction" (Novacek 2007) continues to whittle away at the diversity of life, the decline of amphibians has been characterized as the most dire, far exceeding that of birds and mammals (Stuart et al. 2004).

An influx of amphibian declines that were noted to have begun in the 1970s, mostly in the new world tropics and Australia, was the impetus for a worldwide effort among the herpetological community to better understand the magnitude and causes of declines in the late 1980s and early 1990s. The first World Congress of Herpetology in 1989 was a forum for herpetologists from around the world to convene and discuss their observations regarding amphibian declines and extinctions. In 1990, a National Research Council workshop on declining amphibians focused on next steps for research in ameliorating the situation and recommended more research on the responses of amphibians to certain pesticides, long-term monitoring of select amphibian populations, and a comparison of current species ranges with historical collections records (Barinaga 1990, Blaustein and Wake 1990). The Declining Amphibian Populations Task Force was subsequently formed to address the research methods and efforts necessary to begin addressing causes of decline. Although it was suggested that inexorable herpetologists were slow to voice their concerns about amphibian declines (Barinaga 1990), a more likely explanation is that herpetologists are familiar with the stochastic nature of most amphibian populations, and it did not become apparent to them that they were witnessing anything unusual until the accumulation of a sufficiently large number of independent observations of notable declines of amphibian

populations from around the world raised the possibility that the individual events might in fact be part of a global phenomenon. Debate ensued over whether amphibian declines were a real cause for concern or whether natural population fluctuations were being overstated (Wake 1991, Blaustein 1994, Pechmann and Wilbur 1994). Quantitative reviews of the evidence subsequently confirmed amphibian population declines were increasing dramatically throughout the 1950s and 1960s (Houlahan et al. 2000), and tests of null models suggested that it was highly unlikely that observed amphibian declines were due to natural fluctuations (Pounds and Crump 1994).

Once it became more widely accepted within the herpetological community that amphibian declines were indeed a real phenomenon, the next, and more difficult, obstacle to tackle was that of what was causing them. Anthropogenic causes of environmental degradation and their effects on faunal persistence were well-known by this time—habitat destruction, contaminants, and the thinning of the ozone layer had become household concepts. Amphibians began to be described as ecological indicators because of their permeable skin, often-shifting life stages between terrestrial and aquatic habitats, and relatively primitive immune systems (Wake and Vredenburg 2008). This "canary in the coal mine" idea gained popularity (Halliday 2000, Roy 2002), yet has been criticized for understating the role of declines in other taxa (Kerby et al. 2010).

The most puzzling aspect of amphibian declines was their occurrence in even relatively pristine environments subject to relatively little human influence. Global environmental change could be expected to affect all habitats regardless of the level of alteration, but would cause relatively slow, incremental changes through time, rather than abrupt die-off episodes. For example, the disappearance of the foothill yellow-legged frog

(*Rana boylii*) from the entire southern California portion of its range in a very short period of time suggests the action of one or more extreme events (Sweet 1983). Dramatic declines and extinctions such as this led some herpetologists to believe that there must be a "single, unitary cause" (Barinaga 1990). One of the few factors with enough influence to cause such widespread declines and extinctions in such a short period of time is that of epidemic disease.

The discovery of *Batrachochytrium dendrobatidis* (Bd), the fungal pathogen that causes the often-lethal disease chytridiomycosis in amphibians, shifted the focus of much of amphibian decline research. In the late 1990s, Joyce Longcore, a mycologist from the University of Maine, and her colleagues described an unusual fungus in an isolate from the skin of a blue poison dart frog that had died at the National Zoological Park in Washington, D.C. (Longcore et al. 1999). Previous work had identified similar fungi in arroyo toads (*Anaxyrus californicus*) from a captive population in southern California (Nichols et al. 1996), a captive White's tree frog (*Litoria caerulea*) (Longcore et al. 1999, Pessier et al. 1999), and other captive amphibians at zoos and research facilities in the United States (Nichols et al. 1998). Many amphibian declines have since been attributed to Bd throughout the world (Berger et al. 1998, Lips 1999, Bosch et al. 2001, Daszak et al. 2003, Lips et al. 2003, Muths et al. 2003), including in the Sierra Nevada of California (Rachowicz et al. 2006, Vredenburg et al. 2010), where all of the native anurans have experienced substantial declines (Drost and Fellers 1996).

In southern California, most native anuran species have also declined considerably (Jennings and Hayes 1994a, Jennings and Hayes 1994b, U.S. Fish and Wildlife Service 2002, 2012), and the most extreme case is the localized extirpation of *R. boylii*. Despite the

initial identification of Bd as a causative factor in California amphibian declines and extinctions (Rachowicz et al. 2006), the potential role of Bd in southern California amphibian declines has received little attention. Whether the declines in southern California are the result of global environmental change or local anthropogenic factors, the rapid extirpation of *R. boylii* warrants a closer examination of chytridiomycosis as a causative factor.

Before examining historical declines, it is important to know whether *R. boylii* is susceptible to Bd and to characterize Bd status in remaining populations. In Chapter 1, I investigated the Bd dynamics of an extant population of *R. boylii* in the San Francisco Bay Area of California, where a chytridiomycosis-induced die-off of *R. boylii* metamorphs occurred in the fall of 2013. I found that the presence of non-native American bullfrogs (*Rana catesbeiana*) was an important predictor of both the presence and intensity of Bd infection in *R. boylii*, and that a recent zone of contact between bullfrogs and *R. boylii* was caused by the extended drought between 2012 and 2016, which altered river hydrology and allowed bullfrogs to expand their spatial distribution in the stream network. This was further supported by the results of mixed effects models that indicated that stream flow volume was negatively associated with Bd load in *R. boylii*. Importantly, this study was the first published report to provide evidence of lethal chytridiomycosis in *R. boylii* in the field.

Museum specimens provide indispensable repositories for information about historical ecological dynamics, including the presence of disease. Bd has been detected from formalin-fixed museum specimens using qPCR, but little is known about the efficacy of these techniques. In Chapter 2, I used bullfrogs of known Bd infection status to determine the effectiveness of different sampling and DNA extraction techniques in assessing Bd

status in formalin-fixed and ethanol-preserved specimens using qPCR. I found that a spin column kit DNA extraction method, which reduces the presence of qPCR inhibitors in the sample, provided for increased detection of Bd DNA in samples than did those with a simpler, more commonly used DNA extraction method. This resulted in significantly higher probabilities of qPCR detection of Bd DNA, and has important implications for the interpretation of results from studies that use different DNA extraction methods. I applied these methods to the museum specimen work I conducted in Chapter 3.

In Chapter 3, I used an interdisciplinary approach, combining interviews with herpetological experts, field notes, and museum specimen collections to examine the historical relative abundance of *R. boylii*, potential causes of *R. boylii* declines in the region, and historical prevalence of Bd. I found that an increase in Bd prevalence coincided with *R. boylii* declines and a time of rapid change in southern California, wherein fish stocking, backcountry recreation, urban development, and the amphibian pet trade were all on the rise. In addition, extreme flooding during the winter of 1969 was coincident with localized extirpations in *R. boylii* populations. I conclude that Bd likely played an important role in the rapid extirpation of this species from southern California, and that multiple natural and anthropogenic factors may have worked in concert to make this possible in such a relatively short period of time.

The research conducted as part of this dissertation aimed to advance understanding of the potential role of chytridiomycosis in the extirpation of *R. boylii* from southern California. The results show that *R. boylii* is susceptible to chytridiomycosis and other threats such as invasive species, drought, and flow regulation are likely acting synergistically to influence disease outcome. In addition, I demonstrate that it is important to

consider DNA extraction method when attempting to detect Bd DNA from formalin-fixed museum specimens. Furthermore, the coincidence of Bd's proliferation in southern California with *R. boylii*'s extirpation from the region suggests that chytridiomycosis could have played an important role in that extirpation. Taken together, the work presented here advances our understanding of the role of chytridiomycosis in shaping the occupancy of a declining California amphibian faced with numerous threats, and provides important information about Bd and *R. boylii* for managers that aim to reintroduce the species to southern California.

CHAPTER ONE: Extreme drought, host density, sex, and bullfrogs influence fungal pathogen infection in a declining lotic amphibian[1]

[1] At the time of this writing, Chapter One is in press in *Ecosphere* (DOI: 10.1002/ecs2.1740)

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 boylii*) 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. boylii* 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. boylii* and histologic lesions observed on a dead *R. boylii* 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. boylii* and sympatric anurans at the site for the next two years to determine Bd trends within the community. Using mixed effects models, we found that bullfrog presence was a positive predictor of both Bd prevalence and Bd load in *R. boylii*. 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.

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 boylii*), a stream dwelling species endemic to California and Oregon (USA) that has disappeared from over half of its former range (Davidson et al. 2002, Lind 2005). *R. boylii* 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. boylii* 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 (Figure 1.1), we observed dead and dying juvenile *R. boylii* in

the fall of 2013. High pathogen loads on 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 kilometers (km) of stream coincided with the most severe drought event in California in the last 1,200 years (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. boylii* were consistently more abundant in the upstream unregulated portions of the study area (Kupferberg et al. 2012), but became relatively more abundant downstream in the regulated reaches (Figure 1.2A) which remained wetted throughout the year while the channels in the upper part of the watershed were completely without surface flow by midsummer (Figure 1.2B,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. boylii,* 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, USA, the Alameda Creek watershed (Figure 1.1) 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 130-360 meters (m) above sea level. The Alameda Creek 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 (Figure 1.1). 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. *R. boylii* 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. boylii*

do not move around the reservoir and that it represents a barrier to gene flow (Peek 2012). The *R. boylii* 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 ± SE: 3.45 ± 0.36) (Briggs et al. 2010, Vredenburg et al. 2010, Kinney et al. 2011), we sampled *R. boylii* for Bd and sampled other frog species encountered during the course of our surveys. We also collected a dead *R. boylii* metamorph from the die-off on 7 November 2013 for histological analysis, which we conducted following Reeder et al. (2012). From November 2013 to September 2015 we surveyed 16 km of stream habitat (Figure 1.1) during the day, when *R. boylii* are most active. The cryptic nature of *R. boylii* and its low densities in this system make capturing a large number of individuals a challenge (Appendix 1: Figure 1). 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 to specifically to Bd sampling (Appendix 1: Table 1). 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 60Csx). 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 Co.)

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 hours).

Bd 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 uL of PrepMan Ultra (Applied Biosystems), 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. boylii* 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 ($km_{unregulated\ 1997\text{-}2016}$=1.64; $km_{below\ diversion\ dam\ 2003\text{-}2016}$=0.7; $km_{below\ Calaveras\ Dam\ 2003\text{-}2013}$=1.23; $km_{below\ Calaveras\ Dam\ 2015\text{-}2016}$=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). *R. boylii* clutches are readily visible on the rocks where they are attached, and are much more conspicuous than the frogs themselves (Appendix 1: Figure 1) 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. boylii* 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 days 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). 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. boylii* clutches", which is the number of *R. boylii* egg clutches observed 25 m upstream and 25 m downstream of point of capture (50 m total distance; Table 1.1).

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. boylii* and in bullfrogs (4 models 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 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.

Based on the literature, we expected Bd prevalence and load to vary according to different biotic and abiotic factors (Table 1.1). 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. 2015a). 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. boylii* egg clutches within 50 m of each frog location/Bd sampling locality (Table 1.1). 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 wave length of the riffle-pool cycle is 5-7 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 1.1) 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 were the best fit to the data. Predictor variables were sequentially tested for all four models in the order as presented in

Table 1.1. We log-transformed the ZE values for the model with *R. boylii* 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. boylii* were used in both *R. boylii* Bd prevalence and load models since all *R. boylii* 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 ΔAIC >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 less than 3 (Zuur et al. 2010). We conducted all analyses in the R computing environment (R Development Core Team 2012). GLMMs 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 *R. boylii* 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 1: Figure 2). 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. boylii* were the most frequently encountered species throughout the study reaches (Figure 1.3A). We captured and sampled 142 *R. boylii* individuals (127 post-metamorphic), along with 4 *Anaxyrus boreas halophilus* (California toad), 26 *Hyliola 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 (Figures 1.1 and 1.3A). All species tested positive for Bd, and infection prevalence (Figure 1.4A) 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. boylii* and bullfrogs (Figure 1.4B). Within *R. boylii*, males were more likely to be infected than either females or juveniles (Figure 1.5D).

The model analyses of post-metamorphic *R. boylii*, 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 1: Tables 2 and 3, Figures 1.5 and 1.6). While Bd prevalence was higher in water year 2015 than 2014 (Figure 1.5), none of the stream flow metrics included were important predictor variables based on the best-fit models for Bd presence-absence in *R. boylii* (Appendix 1: Table 2). 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 1.2, Appendix 1: Tables 2 and 3, Figure 1.6). Two of the best-fit models for Bd load in *R. boylii*

included an interaction of season and mean daily stream flow (Appendix 1: Table 3). Bd loads in *R. boylii* were generally lower in summer than in fall (Figure 1.6C), and stream flows were lowest in fall (Figure 1.2). The local density of conspecifics, as indicated by the number of *R. boylii* egg clutches within 50 m of each capture location, was also a significant positive predictor of Bd load in *R. boylii* (Table 1.2 and Appendix 1: Table 3, Figure 1.6E).

Because bullfrogs were among the most important predictors of Bd in both the *R. boylii* 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 1.2, Appendix 1: Table 4, Figure 1.7). In the bullfrog Bd load model, none of the predictors improved the model beyond the intercept-only model (Appendix 1: Table 5).

DISCUSSION

Bd susceptibility in R. boylii

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. boylii* 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 (for example, 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. boylii* can be susceptible to the lethal consequences of chytridiomycosis in the field are in contrast to laboratory experiments (Davidson et al. 2003, Davidson et al. 2007) and a field study (Lowe 2009) that found reduced growth or body condition in Bd-positive juveniles, but which were inconclusive with respect to chytridiomycosis-induced mortality. Significant within-species variation in Bd outcome are not uncommon (Briggs et al. 2010, Bradley et al. 2015). Indeed, when the experiment of Davidson et al. (2007) was repeated, and *R. boylii* 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. boylii* 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. boylii* decline suggested that climate change may be influencing the species' northward range 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 years of annual monitoring (Kupferberg et al. 2012 and Figure 1.2A). This, coupled with our observation that Bd loads in *R. boylii* increase at lower stream flows, indicates that climate change, water extraction for human use, and disease may be acting synergistically to threaten *R. boylii* 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 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 (Figure 1.7B), 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, Figure 1.1) by as much as 5°C (McBain Associates 2014). This is below the realized thermal niche for *R. boylii* 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 influence Bd infection in bullfrogs in this system, we observed lower Bd loads in *R. boylii* in summer (Figure 1.6C). However, temperature was not an important predictor of either Bd load or prevalence in the *R. boylii* models.

Bullfrogs

Our findings that both the probability of Bd infection and Bd load are higher in *R. boylii* 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. boylii* 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. boylii* 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 *H. regilla* than in the bullfrogs observed in this study (Figure 1.4). Moreover, *R. boylii* and *H. regilla* (which is terrestrial for part of its life history) share the same stream channel habitat less frequently compared to

R. boylii and bullfrogs, so transmission opportunities between *R. boylii* and *H. regilla* are fewer at this site.

In addition to their role as Bd vectors (Greenspan et al. 2012, Schloegel et al. 2012), bullfrogs may also increase native ranids' susceptibility to Bd by decreasing their survivorship in other ways. In mesocosm experiments, both *R. draytonii* tadpoles (Kiesecker and Blaustein 1998) and *R. boylii* 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. boylii* males than either females or juveniles could be caused by behavioral or physiological factors. Several behaviors observed in *R. boylii* males may increase opportunities for Bd transmission, therefore increasing the likelihood that they will be infected with Bd. For example, adult male *R. boylii* frequently engage in aggressive wrestling behavior, likely induced by calling activity (Wheeler and Welsh 2008, Murphy et al. 2011). *R. boylii* 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. boylii* 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, USA 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 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. boylii* may be multiplicative, although we did not find strong statistical evidence for this interaction in either of the *R. boylii* models (Appendix 1: Tables 2 and 3). *R. boylii* frequently amplex bullfrogs in an attempt to breed where the species are sympatric (SJK and SB personal observations, Figure 1.8; Lind et al. 2003), suggesting that *R. boylii* 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).

Bd 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 years (SJK, personal observation),

suggesting that Bd infection status may influence this behavior if such a causative mechanism exists.

Density

Our finding that Bd loads in *R. boylii* increase with increasing density of *R. boylii* 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, Briggs et al. 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. 2015a). *R. boylii* 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 (Figure 1.1), 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.

R. boylii 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. boylii* 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 Farrer 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 Figure 1.2B) promotes 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).

CONCLUSION

R. boylii 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. boylii* populations in this system. During extreme drought, when the reach downstream of the dam remained wet while other reaches went dry, a 20-year pattern of higher *R. boylii* densities in unregulated reaches was reversed (Figure 1.2A). 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. boylii* 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.

TABLES

Table 1.1. 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: December 1 to May 31; Summer: June 1 to August 31; Fall: September 1 to November 30.
Season 2	Environmental/Temporal	Wet, Dry	Wet season: December 1 to May 31; Dry season: June 31-November 30.
Hydrologic Unit/Flow Regime	Environmental/Geographic (anthropogenic)	Alameda Creek: Unregulated; Diversion; Dam Release & 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 days	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

Covariate	Type	Range or levels	Description
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
R. boylii Clutches	Biological	0 - 19	Number of *R. boylii* egg clutches within 50 m (25 m upstream & 25 m downstream) of sample collection site

† 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, CA; 11172945 - Alameda Creek above Diversion Dam, near Sunol, CA; 11172955 - Alameda Creek below Diversion Dam, near Sunol, CA; 11173510 - Alameda Creek below Calaveras Creek, near Sunol, CA

§ Variables used in *R. boylii* models only

¶ Variables used in bullfrog models only

Table 1.2. Parameter estimates for best-fit models (see Appendix 1: Tables 2-4) used to determine the best predictors of: (A) Bd presence-absence in *Rana boylii*; (B) Bd presence-absence in bullfrogs; and (C) Bd load in *R. boylii*. 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

Model	**Parameter**	**Estimate**	**SE**	**z**	**p**
(A) Bd presence-absence (*R. boylii*) (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 (*R. boylii*) (R^2 = 0.53)	(Intercept) †	4.42	(2.09, 6.76)		
	Season3 (Summer) †	-4.60	(-6.24, -2.95)		
	Season3 (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)		
	R. boylii Clutches†	0.82	(0.14, 1.53)		

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

FIGURES

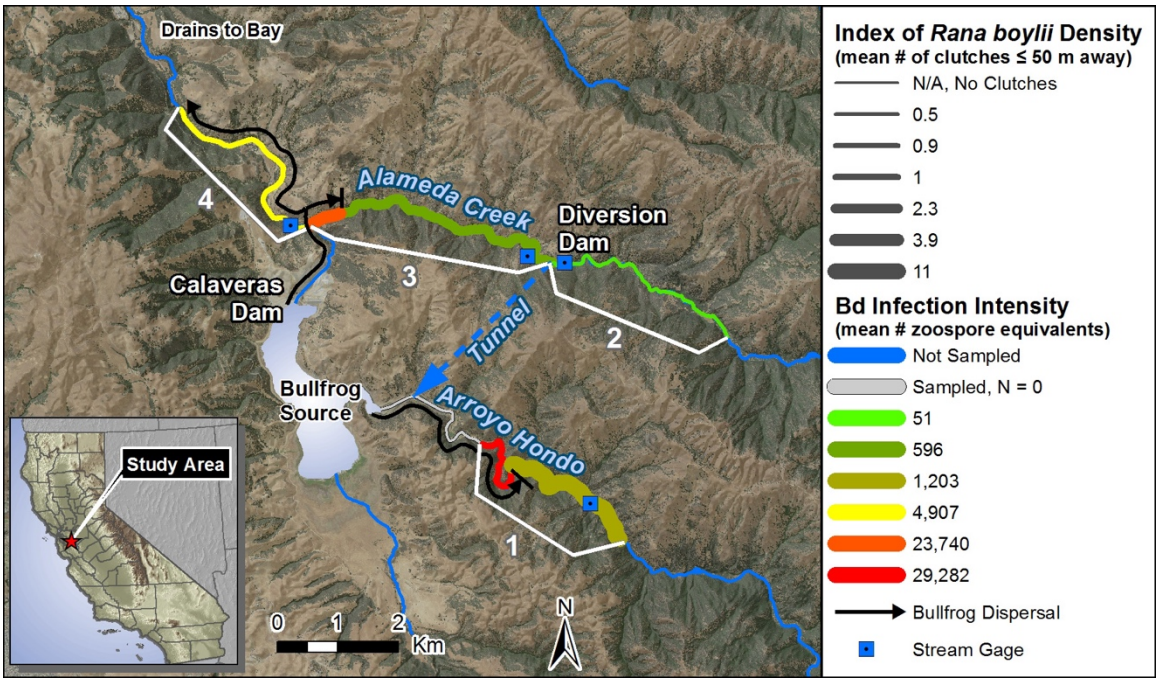

Figure 1.1. The location of the study area in Alameda County, California (USA) and the 4 numbered hydrologically and geomorphologically distinct study reaches: 1) Arroyo Hondo upstream of the reservoir (ochre and red); 2) unregulated Alameda Creek upstream of the diversion dam which delivers water to 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 boylii*) 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. boylii* clutches observed within 50 m of capture point of frogs sampled for Bd. Bullfrog (*Rana catesbeiana*) presence/absence status and direction of expansion is indicated by black arrows.

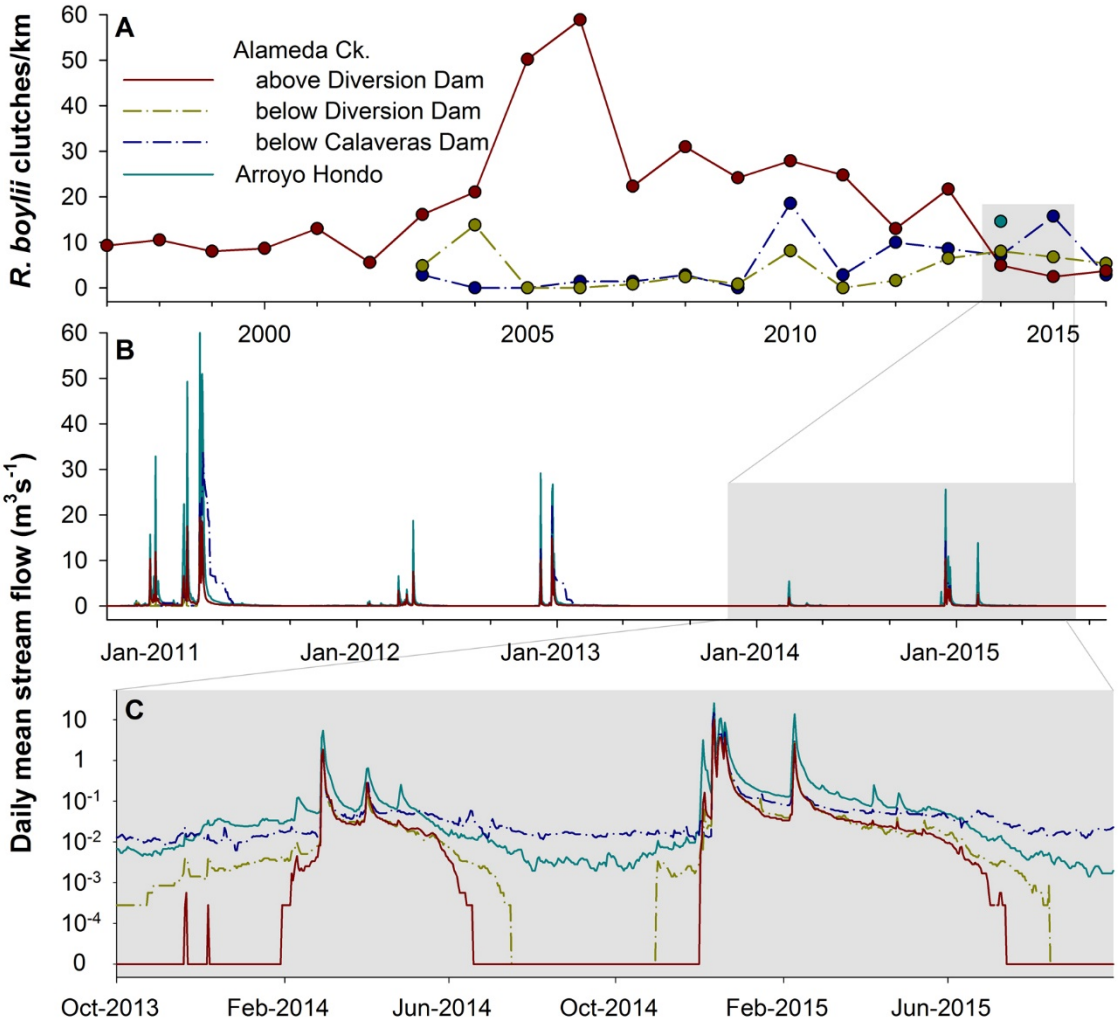

Figure 1.2. (A) Breeding population size of *Rana boylii*; (B) daily mean stream flows in the study reaches of Arroyo Hondo and Alameda Creek prior to (i.e., 2011) and during a prolonged drought (2012-2015), showing reduced magnitude of winter flooding for all stream reaches in dry years and in regulated 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, regulated reaches by broken lines. A water year spans from 1 October to 30 September.

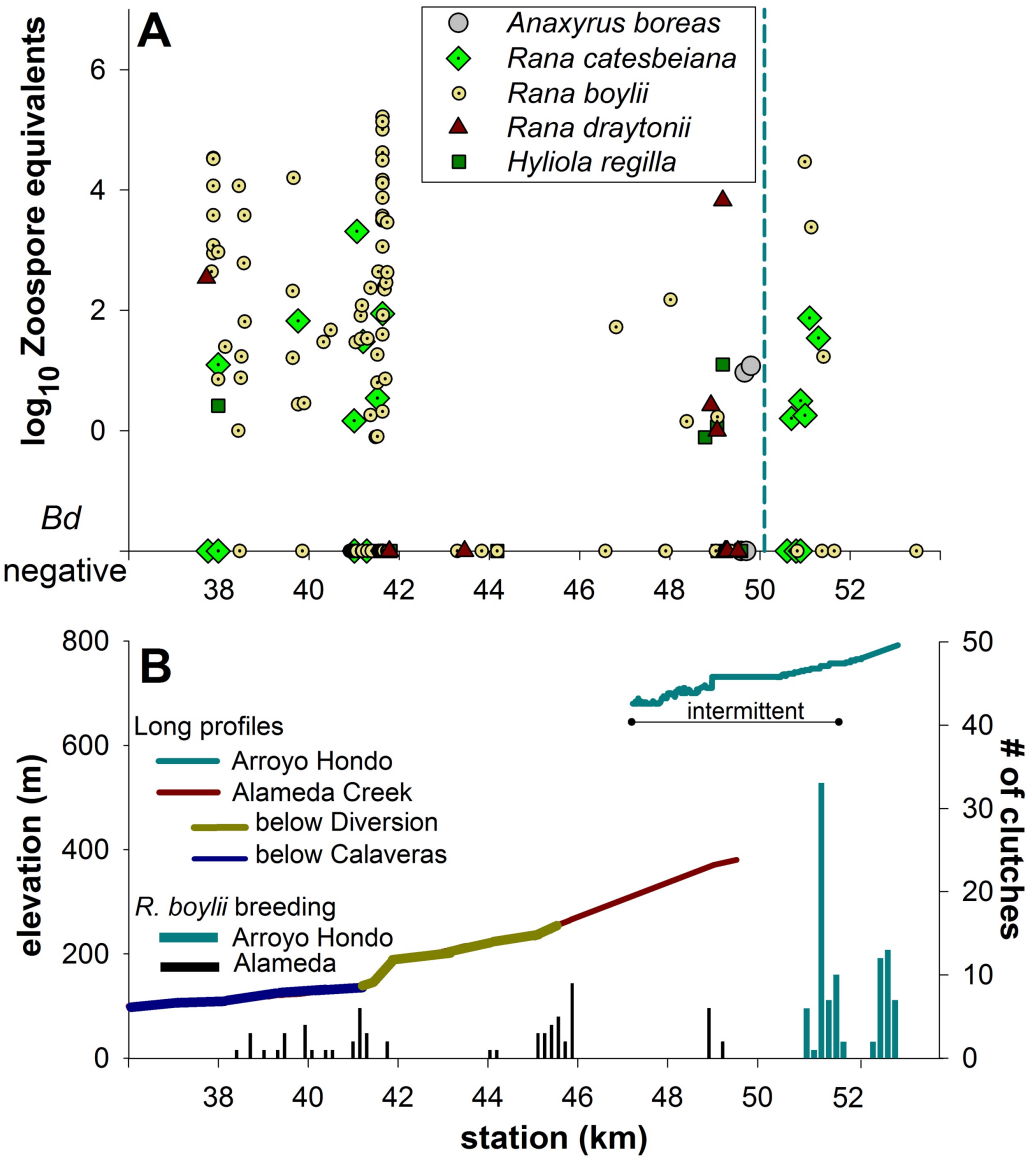

Figure 1.3. (A) Pathogen load by species at stream station locations. Left of the vertical dashed line is Alameda Creek; right of the dashed line is Arroyo Hondo. (B) Stream profiles (lines) and number of *Rana boylii* clutches (bars) observed at stream station locations along Alameda Creek and Arroyo Hondo in 2014. Breeding sites were visited an average of 4 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.

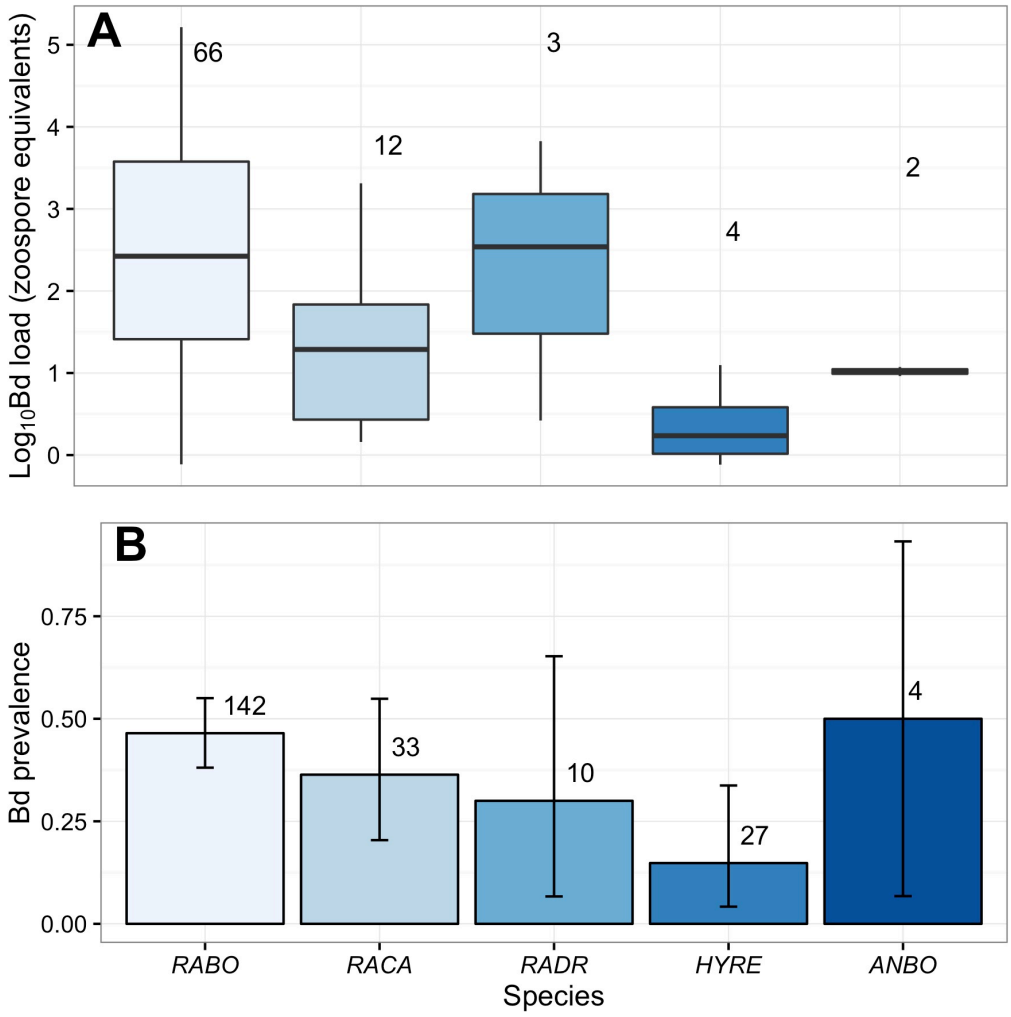

Figure 1.4. (A) Bd load and (B) Bd prevalence for all anuran species sampled at the study site**.** Bold horizontal lines within each boxplot in (A) indicate the median, boxes show the interquartile (IQ) range, and whiskers show the range within 1.5 times the IQ range. Error bars in (B) represent the 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 boylii* (foothill yellow-legged frog); *RACA* = *Rana catesbeiana* (American bullfrog); *RADR* = *Rana draytonii* (California red-legged frog)*; HYRE* = *Hyliola regilla* (Pacific treefrog); *ANBO* = *Anaxyrus boreas halophilus* (California toad).

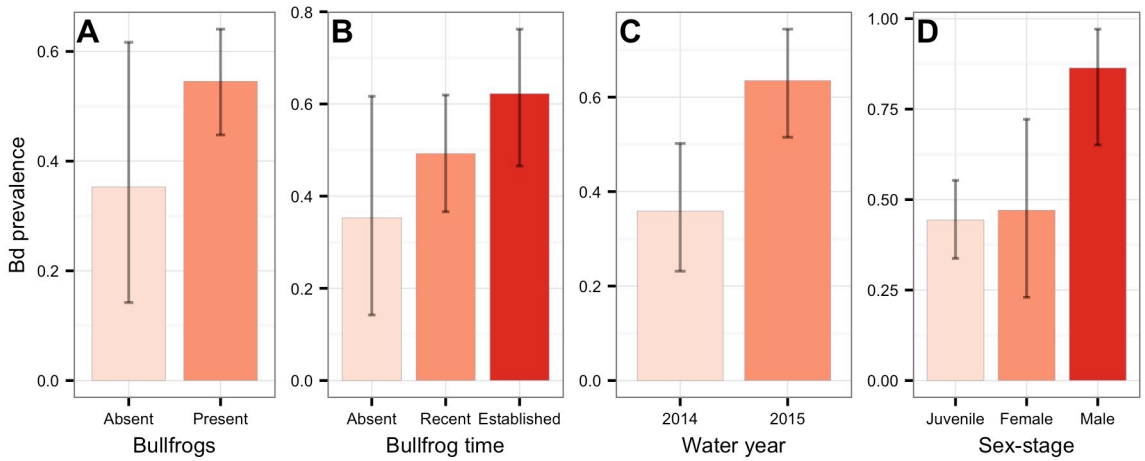

Figure 1.5. Relationship between Bd prevalence in *Rana boylii* and the most important explanatory variables as determined by the best-fit mixed effects models (Tables 1.2 and 1.6), including (A) Bullfrog presence-absence; (B) Length of time of bullfrog presence; (C) Water Year; (D) Sex/life stage. Error bars indicate 95% Clopper-Pearson binomial confidence intervals. Descriptions of explanatory variables are in Table 1.1.

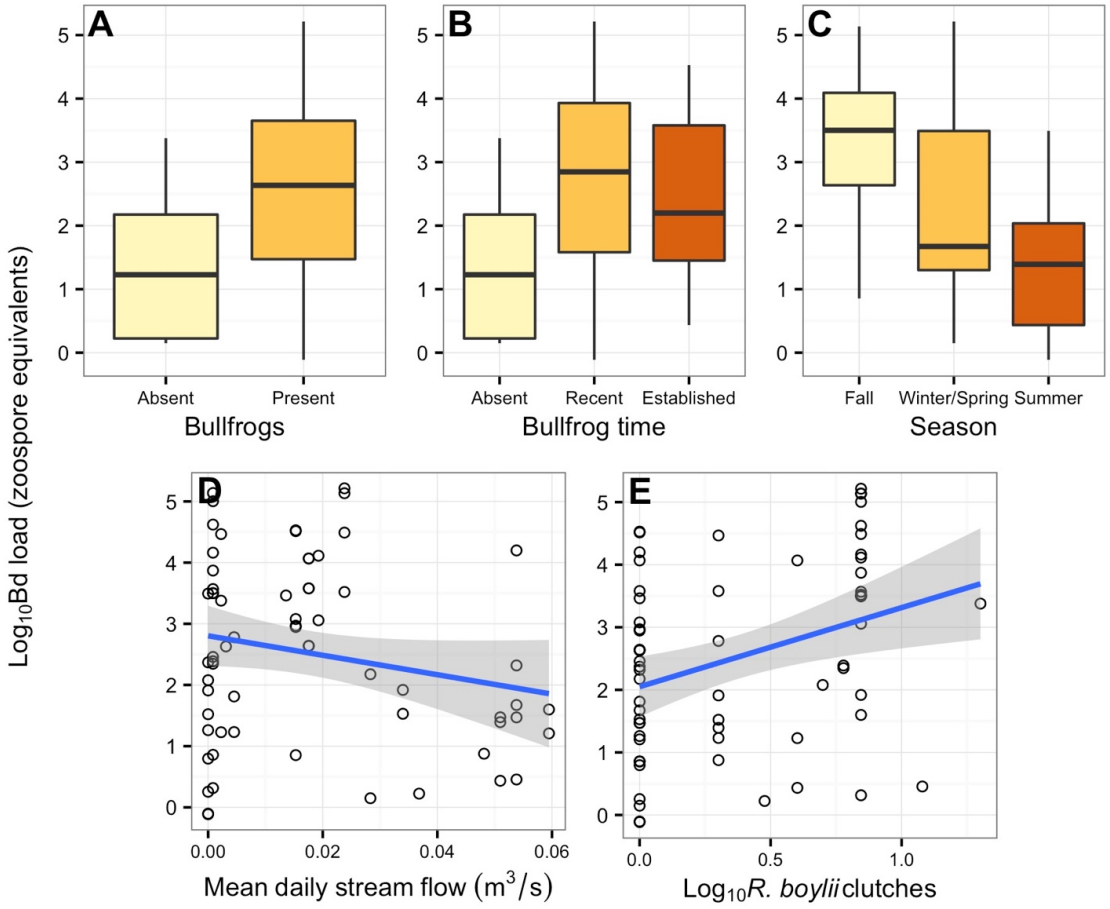

Figure 1.6. Relationship between Bd loads of infected *Rana boylii* and the most important explanatory variables as determined by the best-fit mixed effects models (Table 1.2 and Appendix 1: Table 3), including (A) Bullfrog presence-absence; (B) Length of time of bullfrog presence; (C) Season; (D) Mean daily stream flow; and (E) Number of *R. boylii* 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 1.1.

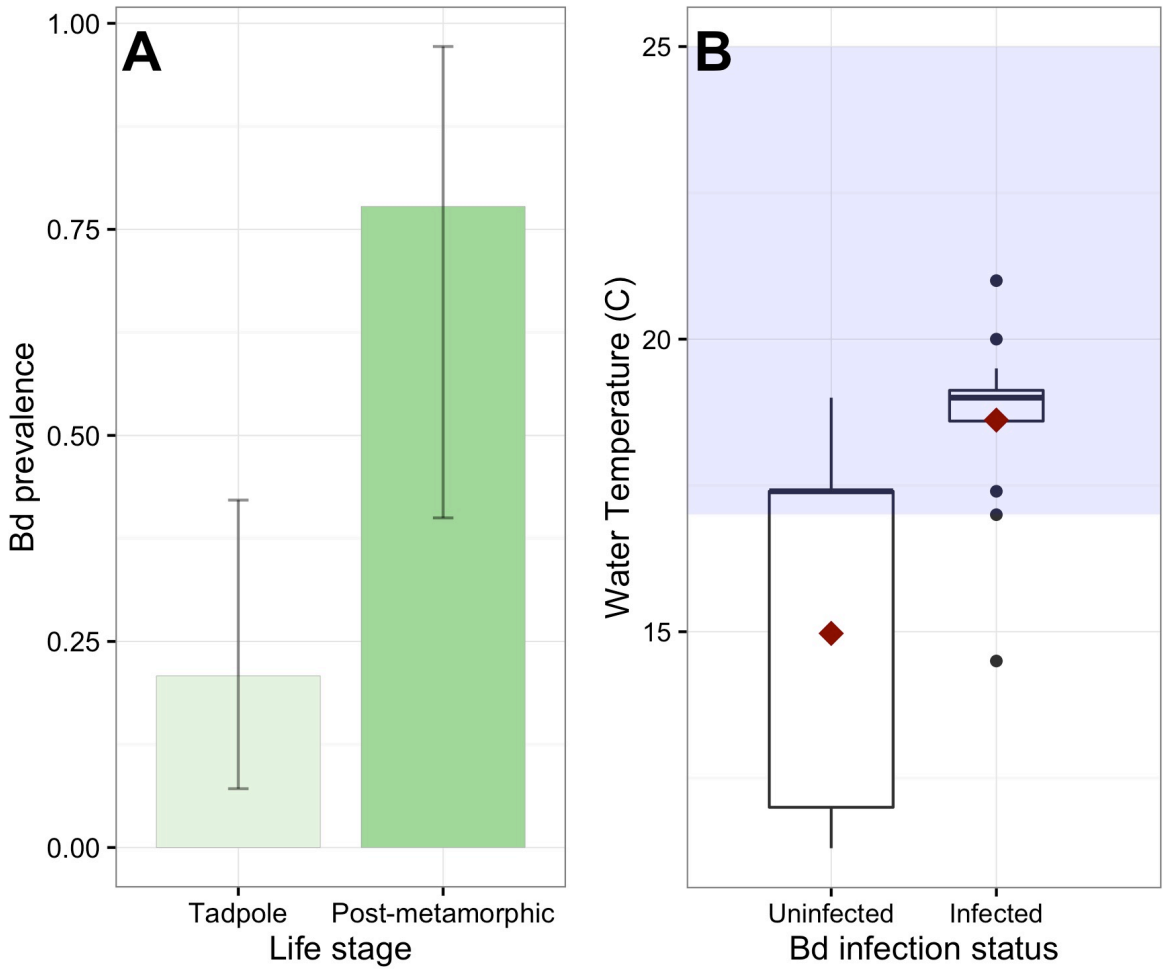

Figure 1.7. Bd infection in non-native American bullfrogs (*Rana catesbeiana*), with (A) Bd prevalence by life stage and (B) Bd infection status at the range of water temperatures observed. Error bars in (A) indicate 95% Clopper-Pearson binomial confidence intervals. Bold bars in (B) indicate the median, 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 * 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).

Figure 1.8. Male foothill yellow legged frog (*Rana boylii*; 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.

CHAPTER TWO: DNA extraction method affects the detection of a fungal pathogen in formalin-fixed specimens using qPCR[2]

[2] Chapter Two was first published on 20 August 2015 in *PLoS ONE* (DOI: 10.1371/journal.pone.0135389)

ABSTRACT

Museum collections provide indispensable repositories for obtaining information about the historical presence of disease in wildlife populations. The pathogenic amphibian chytrid fungus *Batrachochytrium dendrobatidis* (Bd) has played a significant role in global amphibian declines, and examining preserved specimens for Bd can improve our understanding of its emergence and spread. Quantitative PCR (qPCR) enables Bd detection with minimal disturbance to amphibian skin and is significantly more sensitive to detecting Bd than histology; therefore, developing effective qPCR methodologies for detecting Bd DNA in formalin-fixed specimens can provide an efficient and effective approach to examining historical Bd emergence and prevalence. Techniques for detecting Bd in museum specimens have not been evaluated for their effectiveness in control specimens that mimic the conditions of animals most likely to be encountered in museums, including those with low pathogen loads. We used American bullfrogs (*Rana catesbeiana*) of known infection status to evaluate the success of qPCR to detect Bd in formalin-fixed specimens after three years of ethanol storage. Our objectives were to compare the most commonly used DNA extraction method for Bd (PrepMan, PM) to Macherey-Nagel DNA FFPE (MN), test optimizations for Bd detection with PM, and provide recommendations for maximizing Bd detection. We found that successful detection is relatively high (80-90%) when Bd loads before formalin fixation are high, regardless of the extraction method used; however, at lower infection levels, detection probabilities were significantly reduced. The MN DNA extraction method increased Bd detection by as much as 50% at moderate infection levels. Our results indicate that, for animals characterized by lower pathogen loads (i.e., those most commonly encountered in museum collections), current methods may underestimate the

proportion of Bd-infected amphibians. Those extracting DNA from archived museum specimens should ensure that the techniques they are using are known to provide high-quality throughput DNA for later analysis.

INTRODUCTION

Natural history collections are becoming increasingly important for ecological and conservation research (Lips 2011, Rocha et al. 2014), facilitating studies as diverse as those documenting the effects of environmental contaminants (Porter and Wiemeyer 1969) and morphological responses to anthropogenic climate change (Gardner et al. 2011). With the advancement of molecular techniques, museum specimens have considerable value for inquiries about infectious diseases and their conservation consequences (Weldon et al. 2004, Wyatt et al. 2008, Avila-Arcos et al. 2013, Hewson et al. 2014). The discovery and description of the fungal pathogen *Batrachochytrium dendrobatidis* (hereafter Bd) in the late 1990s (Berger et al. 1998, Pessier et al. 1999) provided an explanation for many amphibian declines that had previously been enigmatic (e.g., (Berger et al. 1998, Muths et al. 2003, Rachowicz et al. 2006)); however, most threatened species have inadequate data tying their declines to Bd as a primary factor (Heard et al. 2011). The comprehensive extent of the global distribution of Bd (Olson et al. 2013) remains unclear, and understanding the spatiotemporal dynamics of Bd emergence is important in evaluating the possible role of this pathogen in the declines of amphibian species.

The "spreading pathogen" hypothesis (Skerratt et al. 2007) posits that Bd is a novel pathogen that has recently dispersed around the world. This hypothesis has been the focus of much recent research (as reviewed in (Kilpatrick et al. 2010)) and has been supported by both genetic (Morehouse et al. 2003, Morgan et al. 2007, James et al. 2009, Farrer et al. 2011) and spatiotemporal data (Lips et al. 2008, Vredenburg et al. 2010). Museum specimen collections have an increasingly important function in enabling ecologists to address specific questions about the potential role of Bd in historical amphibian populations

(Lips 2011). Patterns of Bd emergence and spread have been deduced from work with archived amphibian specimens (Weldon et al. 2004, Ouellet et al. 2005, de Queiroz Carnaval et al. 2006, Weinstein 2009, Soto-Azat et al. 2010, Cheng et al. 2011, Huss et al. 2013, Soto-Azat et al. 2013, Zhu et al. 2014), using both histological and polymerase chain reaction (PCR) techniques.

Histopathology has been used successfully to identify chytridiomycosis in live and fresh-dead amphibians (Berger et al. 1998, Berger et al. 1999b, Pessier et al. 1999) and in formalin-fixed specimens (Weldon et al. 2004, Ouellet et al. 2005, Lips et al. 2006, Padgett-Flohr and Hopkins 2009, Weinstein 2009), made possible by the characteristic thickening of metamorphosed amphibian skin, accompanied by reproductive zoosporangia structures presented by cutaneous chytridiomycosis (Berger et al. 1998, Longcore et al. 1999, Pessier et al. 1999, Berger et al. 2005). Histological methods for large-scale sampling, however, are labor intensive and time-consuming, causing issues of feasibility when large numbers of specimens are to be sampled to determine landscape-level effects and emergence of Bd. This can result in small sample sizes, increasing the risk of sampling bias (Ouellet et al. 2005, Padgett-Flohr 2009). Infection with Bd typically has a patchy distribution on amphibian skin, and histology can produce false negatives even when Bd's reproductive zoosporangia are present, if zoosporangia density is low, or if distribution is uneven, which is possible even in highly infected individuals (Pessier et al. 1999, Boyle et al. 2004, Daszak et al. 2004, Reeder et al. 2012). In addition, histological methods require that a portion of the skin be removed for analysis, which damages specimens—a primary concern for museum curators.

The development of PCR (Annis et al. 2004) and quantitative PCR (qPCR) techniques for detecting Bd (Boyle et al. 2004) has enabled Bd detection with minimal disturbance to amphibian skin and substantially increased sensitivity: qPCR is significantly more sensitive than histology (Kriger et al. 2006, Hyatt et al. 2007). Because of the limitations posed by histology and the advantages of qPCR, developing effective qPCR-based methods for detecting Bd DNA in formalin-fixed specimens can provide an efficient and effective approach to examining historical Bd emergence and prevalence.

Before 2011, attempts to detect Bd DNA from formalin-fixed specimens were relatively unsuccessful (reviewed in Richards-Hrdlicka (Richards-Hrdlicka 2012)). Cheng et al. (2011) were able to successfully recover Bd in 83% of histologically-confirmed, infected specimens using PrepMan Ultra DNA extraction reagent (Life Technologies, Grand Island, NY, USA; hereafter PM) and qPCR, following the qPCR protocol of Boyle et al. (Boyle et al. 2004). This was the first study to effectively use qPCR to detect Bd in infected specimens that had been fixed in formalin, and created a hopeful prospect for applying the same method to other collections. Richards-Hrdlicka (Richards-Hrdlicka 2012) also successfully used qPCR to detect Bd from formalin-fixed amphibians using two extraction kits specifically designed to extract DNA from formalin-fixed tissue: DNA IQ (Promega, Madison, WI, USA), and Macherey-Nagel DNA FFPE (Bethlehem, PA, USA; hereafter MN). In other taxa, the MN extraction method has been shown to increase DNA yield and quality as compared to other DNA extraction kits (Popa et al. 2007). Since Cheng et al.'s pioneering success with the PM DNA extraction for detecting Bd from formalin-fixed specimens (Boyle et al. 2004, Cheng et al. 2011), the PM DNA extraction has become the most commonly used method to detect Bd in formalin-fixed specimens (Soto-Azat et al.

2013, Vredenburg et al. 2013, Mendelson III et al. 2014, Muletz et al. 2014, Rodriguez et al. 2014, Fong et al. 2015, Talley et al. 2015), (although some have used other methods, such as Qiagen spin column-based extractions (Cheng et al. 2011, Muletz et al. 2014, Talley et al. 2015)).

The ability to detect Bd DNA using qPCR can be moderated by several factors, including individual pathogen load. The infection intensity, or Bd load, on an amphibian is commonly measured by taking a skin swab following a standard swabbing protocol (Hyatt et al. 2007). 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. Bd loads higher than 10,000 ZE are within the lethal range of some species (Briggs et al. 2010, Vredenburg et al. 2010, Kinney et al. 2011), and specimens with similarly high infection levels have been used to calibrate detection in formalin-fixed tissues (Cheng et al. 2011). Although it is important to understand detection success in formalin-fixed specimens under high Bd DNA conditions, the most common conditions of museum specimens are likely to be those characterized by low to moderate Bd infection intensities. Low Bd loads are usually exhibited by individuals that are not considered susceptible, are subclinical, are potentially in a post-die-off, enzootic state (e.g., a median of ≈ 20 ZE was measured in one study of Bd in the enzootic state (Briggs et al. 2010)), or are exhibiting nascent lethal infections. The probability of a collection event coinciding with a die-off event is small (unless individuals were being collected intentionally because of a die-off event). Even in multi-year field studies with evidence of Bd-related die-off events, moribund individuals are rarely encountered after thousands of visual survey hours (Piovia-Scott et al. 2015). In Bd-susceptible species, when

individuals reach lethally high Bd loads, they die promptly and morbid amphibians are typically removed quickly by predators or scavengers (Green et al. 2002), so those individuals are less likely to be encountered nor desired by collectors. Thus, high pathogen loads are likely rare in most herpetological museum collections.

In addition to the importance of understanding Bd detection success under a variety of infection loads, both length of time in formalin and the pH of the solution can affect the ability to detect DNA after fixation of a specimen in formalin (Bucklin and Allen 2004). Despite this, the length of time amphibians are exposed to formalin fixative is highly variable. A standard manual for amphibian preservation recommends that amphibians be left in a formalin bath "for a minimum of 4 days, or preferably for the remainder of the field season"(McDiarmid 1994); another widely-used source recommends "1 week to 10 days" for formalin fixation (Simmons 2002). Collectors throughout history have practiced a range of time courses for formalin fixation of amphibian tissues, and many specimens have likely been fixed and/or stored in formalin for longer periods than recommended.

Here, we used qPCR to characterize Bd loads on live frogs then fixed them in formalin, preserved them in ethanol, and re-swabbed them three years later to assess the ability to successfully detect Bd on frogs of known infection status using qPCR. We also compared the efficacy of the PM (Boyle et al. 2004, Cheng et al. 2011) and MN (Richards-Hrdlicka 2012) extraction protocols across a range of zoospore loads. Our specific goals in this study were to: 1) test various optimizations for improving detection of Bd DNA from the commonly-used PM DNA extraction; 2) determine the robustness of qPCR detection of Bd from formalin-fixed museum specimens in conditions that most closely mimic those of formalin-fixed, archived museum specimens; 3) within the aforementioned conditions,

examine the effectiveness of PM vs. MN; and 4) determine the potential for false positives to occur in specimen sampling after mixing individuals of known infection status in common containers for formalin fixation and ethanol storage. The results will serve to improve methods used in qPCR detection of Bd DNA from formalin-fixed and ethanol-archived natural history collections.

MATERIALS AND METHODS

Field Sampling and Preservation

We used the Bd infection status of live frogs to assess our ability to detect Bd DNA after formalin fixation and ethanol preservation. We sampled American bullfrogs (n=62) for Bd in the wild (Vandenberg Air Force Base, Santa Barbara County, California, USA) using sterile dacron swabs (MW100 fine-tip; Medical Wire & Equipment, Corsham, Wiltshire, England) following Hyatt et al. (Hyatt et al. 2007), with five strokes of the swab in each of the six body regions (inner thighs, feet and drink patch; 30 strokes total). Following Boyle et al. (Boyle et al. 2004) to determine Bd loads, we used PM to extract DNA from those swabs (Boyle et al. 2004), and diluted extracts 1:10 in DNase-free 0.25X TE (Tris-EDTA) (hereafter TE). We used triplicate positive controls in quantities of 0.1, 1, 10, and 100 ZE, and triplicate negative controls to detect any false positives. DNA standards were provided by the Australian Animal Health Laboratory, CSIRO Livestock Industries, Victoria, Australia (isolate AAHL 98 1810/3, from Australia), or developed by MHT (isolate CJB7, from California). Standards developed by MHT were quality controlled for equivalent standard quantification to isolate AAHL 98 1810/3 prior to use in qPCR using a protocol provided by the Hyatt laboratory. The Hyatt standard Bd DNA protocol was followed precisely, and new standards were run concurrently with Hyatt standards. Care was taken to

ensure a dilution of new standard Bd DNA to 100 ZE (the highest standard DNA amount in the standard curve) matched an average of multi-year Hyatt 100 ZE standard Bd DNA qPCR data, averaged across all plates set to a threshold of 0.1. qPCR amplification parameters followed Boyle et al. (Boyle et al. 2004) and were performed on an Applied Biosystems StepOnePlus Real-Time qPCR System. To calculate a ZE score for each swab, we multiplied raw genomic qPCR output for each sample that was diluted 1:10 by 80 to account for dilution of the sample during the extraction process. Of the 62 bullfrogs sampled in the wild, 94% (n=58) were Bd-positive at the time of capture, with Bd loads ranging from <1 to over 70,000 ZE (median 17 ZE). The remaining four individuals served as negative controls to test for cross-contamination from storage in common containers.

Because Bd loads detectable on moribund frogs are the most accurate within 24 hours of death (Savage et al. 2011), bullfrogs were immediately euthanized after swabbing and then fixed in a 10% buffered formalin solution (pH 7) for four days. Since formalin fixation was historically conducted in the field, as trips often lasted for multiple days, immediate formalin fixation is also consistent with methods most likely encountered by those using animals preserved under historical museum specimen methods. After four days of formalin fixation, frogs were placed in a water bath for 20 minutes to rinse them, placed in 70% ethanol for long-term storage, and were assigned randomly to three separate 19 L buckets so that individuals of varying Bd loads were intermixed in the ethanol storage solution, mimicking natural history collections. Specimens were resampled after three years of storage in the ethanol solution.

Specimen Sampling

To assess Bd detection after formalin fixation and ethanol storage, frogs were re-sampled multiple times for qPCR. We used five separate swabbing events to test the

following optimizations, as outlined in Table 2.1: 1) number of times swabbed (swab events A-C); 2) extract dilution (swab event D); 3) Genereleaser treatment (swab events C & D); 4) TE rehydration (swab events A-C) and 5) DNA extraction method (swab events D & E). Prior to sampling, individual frogs were thoroughly rinsed with clean 70% ethanol to minimize the chances of Bd cross-contamination from other frogs in the same container. Fresh nitrile gloves were used to handle each specimen. Specimens were swabbed in each of the same six areas as live swabbed frogs (Hyatt et al. 2007) (bottoms of the feet, inner thighs, drink patch) in five separate events, using a new swab for each individual: first, five times in each of the six areas (5x6; Swab Event "A" in Table 2.1), followed by 15 strokes in each of the six areas (15x6; "B", Table 2.1) and finally 25x6, which was repeated three times ("C", "D", "E", Table 2.1) to test different extraction techniques. Swab events were discrete and consecutive, so Swab Event B always followed Swab Event A, and so on. We limited the swabs to 25x6 strokes because after 25 strokes in each area, the rayon swab tip began to disintegrate. Even with this many strokes, the specimens remained undamaged. Because no differences have been observed in the probability of Bd detection between the use of swabs and brushes (Cheng et al. 2011), swabs, rather than interdental brushes, were used. These swabs are also less expensive than brushes and identical to those used for field sampling. All specimen swabs were air dried under a laminar flow hood in the laboratory for 48 hours to evaporate residual ethanol from samples prior to DNA extraction.

Specimen DNA extractions

To compare the efficacy of subsequent Bd DNA detection using qPCR, DNA from individual swabs were extracted by two separate extraction methods, PM and MN. Although phenol:chloroform may be the extraction method of choice in museum specimens when full animal tissues are being sampled, phenol:chloroform is not demonstrated to be

superior when extracting low copy number DNA (e.g., Bd DNA) in samples with a high DNA background (in this case, preserved amphibian DNA) (Barta et al. 2014), so it was not tested in this study. Multiple optimizations (TE rehydration, reduced dilution, and Genereleaser) were used on PM-extracted swabs in an attempt to improve Bd DNA detection from swabs that were extracted using that method. We performed our pre-PCR sample manipulations (drying, extraction, qPCR plate setup) in a dedicated room separate from our qPCR facilities. This standard work flow for PCR work ensures that unamplified samples cannot be contaminated by post-PCR amplicons. In addition, we followed time-tested decontamination procedures for all benches and equipment (treatment with bleach followed by a 70% ethanol rinse) and employed frequent glove changes to ensure no incidental contamination between samples occurred during pre-PCR processing.

PM extraction and optimizations. In the first three PM extractions (Events A-C, Table 2.1), 20μL TE was added in addition to the PM, to rehydrate the swab in the extraction process and to putatively increase DNA yield from the swab, as TE solubilizes DNA. Swabs from Events A-D (Table 2.1) were extracted using PM and following Boyle et al. (Boyle et al. 2004), in the same manner described above for the field-collected samples. All PM-extracted swabs (Events A-D) were initially diluted 1:10 with TE (Boyle et al. 2004). To test if an increase in the amount of DNA template in each reaction would increase recovery rates, additional extract from swabs C and D were diluted only 1:5. To calculate a ZE score for each swab, raw genomic qPCR output for each sample that was diluted 1:10 was multiplied by 80 to account for dilution of the sample during the extraction process. PM-extracted samples that were diluted 1:5 were multiplied by 40. A subset of remaining extracts from swabs C and D were treated with 45 μL of Genereleaser

(BioVentures, Murfreesboro, TN, USA), a proprietary formula that quickly releases genetic material from cells and separates inhibitors from the sample, in order to test whether the treatment would increase DNA amplification. All PM-extracted samples were run in singlicate, with the exception of the 1:10 dilution of the D swabs, which were run in duplicate (Table 2.1).

MN Extraction and optimizations. Swabs (Event E) were extracted with MN following Richards-Hrdlicka (Richards-Hrdlicka 2012), with minor modifications for the use of swabs rather than interdental brushes. Briefly, 100 µL of FL buffer and 10µL of proteinase K (10mg/mL) were added to the swab and incubated at 37°C overnight for lysis, then 100 µL of D-link crosslink buffer was added and swabs were incubated for 30 minutes at 90°C. After cooling, conditions were adjusted by adding 200 µL of 98% ethanol. To bind the DNA, swab extract was aliquotted into spin columns (TissueSpin XS, Macherey-Nagel) and centrifuged for 1.5 minutes at 2000 rpm, twice, and the flow-through discarded. In some instances, rayon from the swab prevented filter flow-through, so after the first centrifugation, if all of the liquid did not pass through, the filter would be gently agitated with a clean 10µL pipette tip and centrifugation would be repeated. To wash the membrane, 200 µL of B5 buffer was added to the spin column for 5 minutes, then spin columns were centrifuged for 11,000 rpm for 30 seconds, and flow through was discarded. Columns were then placed in 1.5 ml microcentrifuge tubes and left open for 30 minutes to allow ethanol evaporation. To elute the DNA from the filter, BE buffer was warmed to 70°C and 45µL was pipetted directly onto the column's filter. After 1 minute, columns were centrifuged at 11,000 rpm for 30 seconds. Extracts (5 µL per reaction) were run in duplicate using qPCR as described in Boyle et al. (Boyle et al. 2004). Bd standards used were the same as

described above for field-collected swabs. For all MN extractions, raw genomic output was multiplied by 9 to calculate the total number of ZE per swab (since the total extract volume was 45 μL).

Data Analysis

A sample was considered positive when any replicates in the sample exhibited a logarithmic curve in the amplification profile that crossed the ΔRn threshold (set at 0.1 (Boyle et al. 2004)). When sample size varied between treatments (refer to superscripts in Table 2.1), individual swabs were excluded so that those used for paired analyses were from the same frog. Also for paired analyses, when replicate number varied between treatments (singlicate versus duplicate), the first replicate of duplicate runs was used. When comparing PM to MN, the results of duplicate runs were averaged to determine a mean ZE for each sample. We conducted paired t tests (on square root-transformed ZE data to achieve normality) to look for treatment effects on all nonzero ZE values (samples that returned a positive result) and McNemar's tests to look for treatment effects on recovery rates (number of positives detected/total true positives) within each swab type (C, D, and E).

We used generalized linear mixed models (GLMMs) with a binomial response and a logit-link function to test whether pre-preservation Bd load, DNA extraction method (PM or MN), and individual live frog mass were significant predictors of Bd detection (success or failure) after formalin fixation using duplicate results from swab events D and E. For this analysis, we used only data for individual frogs that we had individual mass recorded (n = 52). We square root-transformed both the live ZE and post-preservation ZE data to facilitate data analysis. We treated initial Bd load, individual mass, and extraction protocol as fixed effects and the identity of each individual frog as a random effect. Treating individual frogs as a random effect allows us to test for differences among extraction protocol and initial load

(the main predictors of interest), while accounting for the fact that individual frogs were necessarily swabbed multiple times to collect samples for separate DNA extractions. We used the "MuMIn" package in R (R Development Core Team 2012) to average the best-fit (within 2 AIC, Akaike Information Criterion) models and then used coefficients from the model averaging in a linear regression to predict the probability of Bd detection at varying levels of Bd load to compare PM and MN.

To determine if there was a relationship between live Bd load and post-preservation Bd load, we conducted two separate linear regressions (one each for PM and MN), using $\log_{10}$-transformed ZE data (all nonzero values). To compare results of Bd detection success and post-preservation ZE ($\log_{10}$-transformed) across all swab events and a subset of them (Events A-E, and A-C, respectively, Table 2.1), we used singlicate data in GLMMs (for Bd detection success) and a linear model (LM) (for post-preservation ZE). In the GLMMs and LM, we treated swab event as a fixed effect and the identity of each individual frog as a random effect and used likelihood ratio tests to evaluate the models with and without swab event included in the model. We conducted a post-hoc Tukey test on the LM to determine degree of difference across all singlicate (Events A-E) ZE results. All analyses were performed in the R programming environment (R Development Core Team 2012).

RESULTS

PM: extraction optimizations

None of the optimizations conducted on PM-extracted swabs increased Bd detection success or ZE values. Reducing the PM extract dilution from 1:10 to 1:5 (within Event D, Table 2.1) had no effect on ZE ($t = -1.559$, $df = 8$, $p = 0.158$) or Bd recovery rate (McNemar's $X^2 = 0.067$, $df = 1$, $p = 0.796$). Within Event D, there was no significant

difference in ZE values ($t = -0.047$, $df = 4$, $p = 0.964$) or recovery rate (McNemar's $X^2 = 0.111$, $df = 1$, $p = 0.739$) when the Genereleaser was used. Within Event C, ZE was significantly reduced when extracts were treated with Genereleaser ($t = -3.809$, $df = 9$, $p = 0.004$); yet there was no significant difference in recovery rate with the treatment (McNemar's $X^2 = 0.067$, $df = 1$, $p = 0.796$). When swabs were rehydrated with TE during the extraction step (Event C), neither ZE ($t = 2.263$, $df = 5$, $p = 0.073$) nor recovery rate were affected (McNemar's $X^2 = 0.727$, $df = 1$, $p = 0.394$).

DNA extraction comparison: PM and MN

The MN extraction significantly increased the likelihood of Bd detection from formalin-fixed specimens over PM-extracted samples (McNemar's $X^2 = 13.5$, $df = 1$, $p = 0.0002$, Figure 2.1), an overall increase in Bd detection of 31%. The regression analysis also indicated the importance of extraction method, and identified pre-preservation Bd load and individual frog mass as influencing Bd detection success, as evidenced by the best-fit models with the lowest AIC values (Tables 2.2 & 2.3). Based on the best-fit models, the probability of Bd detection is higher for MN extractions, with the greatest difference between PM and MN Bd detection success at low to moderate Bd loads; as loads get higher, the probabilities of Bd detection from MN- and PM-extracted swabs become more similar (Figure 2.2).

There was no significant relationship between Bd loads (ZE) prior to and after formalin fixation for either the MN or the PM extractions (MN: $F = 0.023$, $df = 38$, $p = 0.879$; PM: $F = 0.067$, $df = 20$, $p = 0.798$; Figure 2.3), but pre-fixation Bd load was included in all of the best-fit GLMMs describing the probability of Bd detection, so its relevance cannot be completely discounted. The lowest ZE level on a live frog that was successfully detected after formalin fixation was 0.37.

Increasing the number of swab strokes per frog (Events A-C, Table 2.1) increased Bd detection success within the PM-extracted swabs, though not significantly so ($X^2 = 2.90$; df = 2; p = 0.235, Figure 2.4a). Post-preservation Bd detection success and ZE comparisons of singlicate results for all swab events (A through E) are shown in Figure 2.4. There was a significant effect of swab event on both Bd detection success ($X^2 = 41$; df = 4; $p < 0.0001$, Figure 2.4a) and post-preservation ZE ($X^2 = 14.91$; df = 4; p = 0.005, Figure 2.4b). Resulting post-preservation ZE across all PM-extracted swabs (Events A through D; nonzero values only) were not significantly different from each other, with the exception of Events C and D (Tukey's HSD, p = 0.01). ZE from MN-extracted swabs (Event E) were significantly greater than the PM-extracted D event swabs (Tukey's HSD, p = 0.003, Figure 2.4b). Bd detection did not necessarily increase successively with each swab event of equal swab strokes, as evidenced by the decrease in ZE and the proportion of Bd positive swabs detected (see Events C and D; Figure 2.4). This reduction between Events C and D could have been the result of reduced Bd DNA on the frogs as a consequence of previous swab events. None of the samples collected from the four Bd-negative control animals produced a (false) positive result in any of the replicates.

DISCUSSION

We compared two DNA extractions and various optimizations to improve Bd detection from formalin-fixed and ethanol-preserved frog skin. The greatest increase in Bd detection resulted from using the MN spin column-based DNA extraction kit. The MN extraction increased Bd detection by as much as 50% over PM-extracted samples, suggesting that its lack of inhibitors and increased quality of resultant DNA allows for increased DNA detection over PM. This is a similar result to other studies, in which

samples extracted with spin column-based extraction kits such as Qiagen DNeasy and Qiagen Blood and Tissue increased Bd detection over PM by 7% and 23%, respectively (Cheng et al. 2011, Talley et al. 2015). In the standard Bd assay using PM, the extract must be diluted 1:10 prior to qPCR because PM is a relatively rudimentary extraction method that allows proteins from the DNA into the extract, which inhibit PCR reactions (Boyle et al. 2004, Kosch and Summers 2013). All of the optimizations of the PM protocol attempted in this study were unsuccessful. Our results support those of Cheng et al. (Cheng et al. 2011): PM-extracted swabs from formalin-fixed specimens with high pre-fixation fungal load can accurately detect Bd 80-90% of the time (Figure 2.2). However, as Bd loads decrease, Bd detection success also decreases, and considerably more so for PM-extracted swabs than for MN-extracted swabs (Figure 2.2). This is a critical consideration, since the large majority of amphibians in museums are not likely to have high Bd loads.

Although increased Bd load on live frogs increases the probability of detection after formalin fixation, there is likely not an identifiable pre-fixation load below which Bd cannot be detected post-fixation. The lowest Bd load from a live frog that was detected post-preservation was 0.37 ZE. The lack of a relationship between the Bd load of live animals and the Bd load detected after formalin fixation, as shown by this study (Figure

2.3) and others (Cheng et al. 2011, Richards-Hrdlicka 2012) show that post-preservation ZE cannot be used to accurately infer pre-fixation Bd loads. Similarly, historical Bd prevalence should be deduced with caution, considering the relatively low Bd recovery rates for both the PM and MN extraction methods at low Bd loads (Figure 2.2). The percentage of Bd-positive specimens in a given sample cannot be considered an accurate measure of actual historical pathogen prevalence for most historical amphibian

populations. Nonetheless, describing Bd prevalence through time can provide a relative measure of the pathogen's incidence in the landscape (Talley et al. 2015), although we did not specifically test the impact of time since fixation on Bd detection. The multiple swab events conducted may have reduced the amount of Bd DNA on the frogs to be sampled in successive swab events, as indicated in the reduced ZE loads between the PM-extracted Events C and D (Figure 2.4). Consequently, the difference in Bd detection success between PM- and MN-extracted swabs is potentially much greater than shown by this study.

Incidence of false positives is an extremely important factor to consider when using qPCR to detect Bd in formalin-fixed museum specimens, due to the higher sensitivity of qPCR over standard end-point PCR. Here, four Bd-negative control animals that were stored with known Bd-positive animals produced negative runs through all of the replicates, showing that thorough rinsing with fresh ethanol prior to sampling may be an adequate method for reducing false positives from Bd-contaminated ethanol in shared storage jars. If careful sampling hygiene and adequate rinsing with fresh ethanol are conducted, and strict PCR laboratory decontamination protocols are adhered to, false positives in a sample can be avoided. We recognize that some may consider four negative controls out of 62 total animals a relatively small number on which to base this conclusion; however, it is important to note that these 4 negative control animals are represented by 28 individual qPCR runs that did not amplify. Not all preserved specimens in a single jar will be positive for Bd; therefore, these results show that mass contamination within a museum jar does not always happen (Richards-Hrdlicka 2012).

Contemporary researchers examining historical Bd occurrences should be cognizant of the risks of relying solely on qPCR results for Bd detection and consider combining them

with other methods (e.g., histology) in cases of equivocal or enigmatic results. Yet, because of the uneven distribution of chytridiomycosis on amphibian skin (Boyle et al. 2004), histology is not a 100% accurate diagnostic, is laborious when attempting to assess large numbers of animals, and damages specimens. In at least one previous study, an animal that was confirmed Bd positive with PCR was determined negative by histology (Mendelson III et al. 2014). Given low detectability from specimens with low pre-fixation Bd loads (Figure 2.2), researchers should take replicates into account as compared to both positive and negative controls. The extremely small amount of intact Bd DNA remaining after formalin fixation increases the likelihood that each qPCR well will not receive a sample containing Bd. Instead of counting singlicate positives as negatives, researchers should consider re-running the extract and attempting detection again. In addition, the same specimen could be re-swabbed and re-analyzed. The reduction in detection between swab Events C and D in this study suggests that multiple swabbing events can reduce Bd DNA on frogs, although our results also show that when a spin column-based extraction is used, detection probability is relatively high even after frog skin has encountered multiple previous swabbing events.

Based on the results of this study, we make the following recommendations to maximize Bd DNA recovery rates from formalin-fixed museum specimens using qPCR: 1) Use MN or a similar spin-column based DNA extraction method, and/or one specifically designed for formalin-fixed sample types. Like previous research (Garland et al. 2010, Cheng et al. 2011, Kosch and Summers 2013, Talley et al. 2015), this study also finds that qPCR inhibition leads to under-estimates of Bd prevalence. The higher quality of MN-extracted DNA can facilitate future use of the samples in studies aimed at investigating Bd's deeper molecular patterns, including genetics relating to strain differences, variations in

virulence, and population genetics, in addition to allowing for increased Bd DNA detection using qPCR. We have estimated that switching from a PM to an MN extraction would cost approximately one dollar more per extraction, and both methods require equal amounts of labor; 2) When using a "clean" extraction method such as MN, use as much of the DNA extraction template as the master mix reaction volume allows to increase probability of Bd detection by replacing water in the reaction with template (Richards-Hrdlicka 2012); 3) Collect multiple swabs from the same animal so that equivocal results can be re-extracted and re-run to confirm equivocal positives (Richards-Hrdlicka 2012); 4) Run samples in duplicate, and if a sample amplifies in one of the duplicate reactions, run the sample a third time to determine if it should be considered positive or not. This recommendation is also supported by the work of Cheng et al. (Cheng et al. 2011), which found that runs in triplicate or quadruplicate did not significantly differ in their ability to ascertain Bd infection status, as determined by histology, over duplicate runs of the same sample.

CONCLUSION

DNA detection from formalin-fixed tissues is challenging (Wandeler et al. 2007). Establishing clear protocols for most effectively and reliably detecting Bd DNA with qPCR from formalin-fixed specimens will significantly reduce the number of specimens damaged by histological methods. These methods can be used to more accurately sample a large number of specimens and evaluate landscape-scale questions, such as the emergence and prevalence of Bd in threatened amphibian populations. Stemming amphibian declines and extinctions worldwide calls for an extraordinary, coordinated response effort (Mendelson et al. 2006). Critical to guiding these efforts is establishing protocols that are widely used and agreed upon to ensure judicious and comparable deductions from historical Bd data.

TABLES

Table 2.1. Treatments, replicate numbers, and optimizations for each specimen swabbing event, chronological from left to right.

	Specimen swabbing event				
	A	**B**	**C**	**D**	**E**
Swab sequence	5x6	15x6	25x6	25x6	25x6
Total swab strokes	30	90	150	150	150
DNA extraction	PM	PM	PM	PM	MN
TE rehydration	Yes	Yes	Yes	No	No
Dilution & replicate #	1:10: singlicate[a]	1:10: singlicate[a]	1:10: singlicate[a]	1:10: duplicate[a]	No dilution; duplicate[a]
Additional dilution & replicate #	-	-	-	1:5: singlicate[b]	-
Additional treatment & replicate #	-	-	GR singlicate[c]	GR singlicate[d]	-

PM= PrepMan; MN=Macherey-Nagel DNA FFPE; GR=Genereleaser

[a]n=62; [b]n=47; [c]n=50 [d]n=48

Table 2.2. Results of generalized linear models of post-preservation Bd detection success. All models included a random effect of individual frogs. The top three models included extract (PM and MN), pre-preservation Bd load (Live ZE), and individual frog mass.

Fixed effects	**AIC**	**Δ AIC**
Live ZE + Extract	127.2	
Live ZE * Extract	128.4	1.2
Live ZE + Extract + Frog Mass	128.5	1.3
Live ZE + Extract * Frog Mass	129.6	2.4
Live ZE * Extract + Frog Mass	129.8	2.6
Live ZE * Extract * Frog Mass	131.9	4.7
Extract	133.1	5.9
Extract + Frog Mass	134	6.8
Extract * Frog Mass	134.9	7.7
Live ZE	141.4	14.2
Live ZE * Frog Mass	142.1	14.9
Live ZE + Frog Mass	142.8	15.6
Frog Mass	147.9	20.7

Table 2.3. Results of model averaging for three best-fit (within 2 AIC) models for the dependence of frog parameters on the probability of Bd detection as response variable in generalized linear mixed models, (Table 2.2; n=52 frogs, 104 samples). Individual frog ID was included as a random factor. Individuals without mass data available (n=6) were excluded from the analysis. $^{**}p<0.01$

Parameter(s)	Estimate	SE	Z	Pr(>|z|)
(Intercept)	0.513	0.651	0.780	0.435
Live ZE	0.082	0.045	1.810	0.070
Extract (PM)	-2.274	0.782	2.874	0.004**
Extract (PM) * Live ZE	0.013	0.038	0.351	0.725
Frog Mass	0.0004	0.002	0.319	0.750

FIGURES

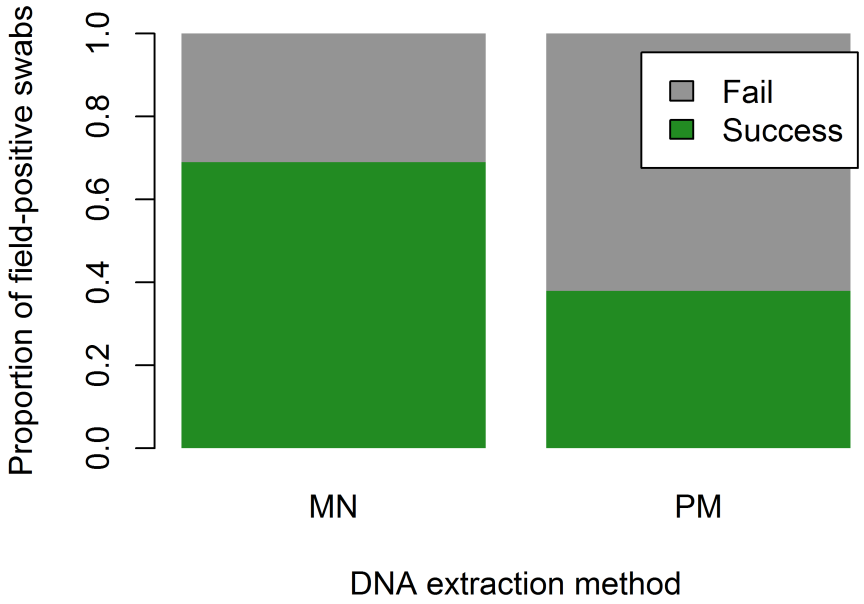

Figure 2.1. Comparison of overall Bd recovery success of Macherey-Nagel DNA FFPE extraction (MN) vs. PrepMan (PM). MN-extracted swabs were 31% more effective than PM at detecting Bd from formalin-fixed specimens that had been previously identified as Bd-positive in the field.

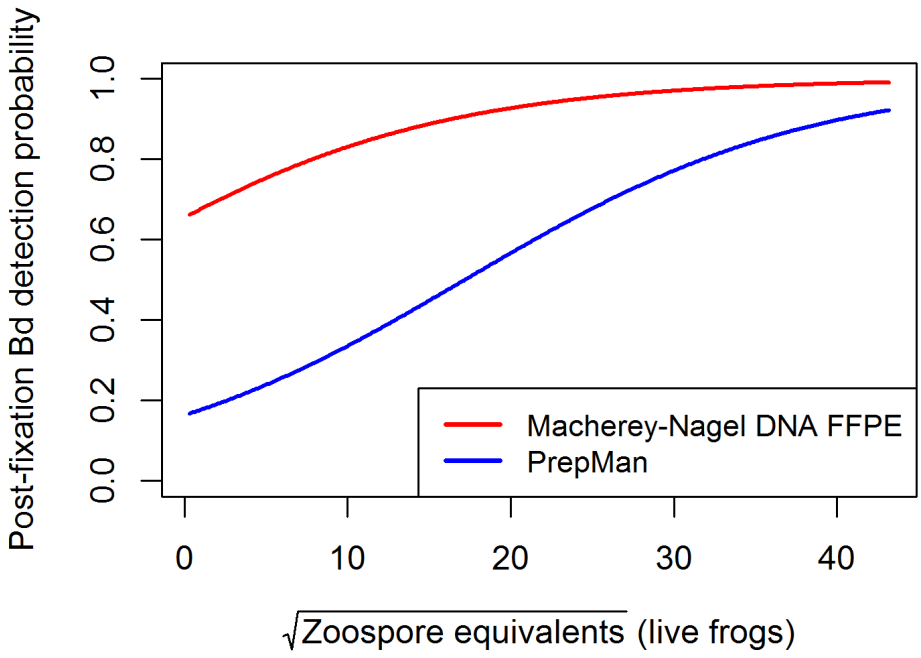

Figure 2.2. Probability of *Batrachochytrium dendrobatidis* (Bd) detection after formalin fixation. We used parameters from the best-fit generalized linear mixed models (Table 2.3) to predict Bd detection probabilities of the two different extraction methods, Macherey-Nagel DNA FFPE (MN), and PrepMan (PM). The differences in Bd detection probability between MN and PM are greatest at low and moderate Bd loads (as much as 50% at the median infection level of this study) and become more similar at higher Bd loads.

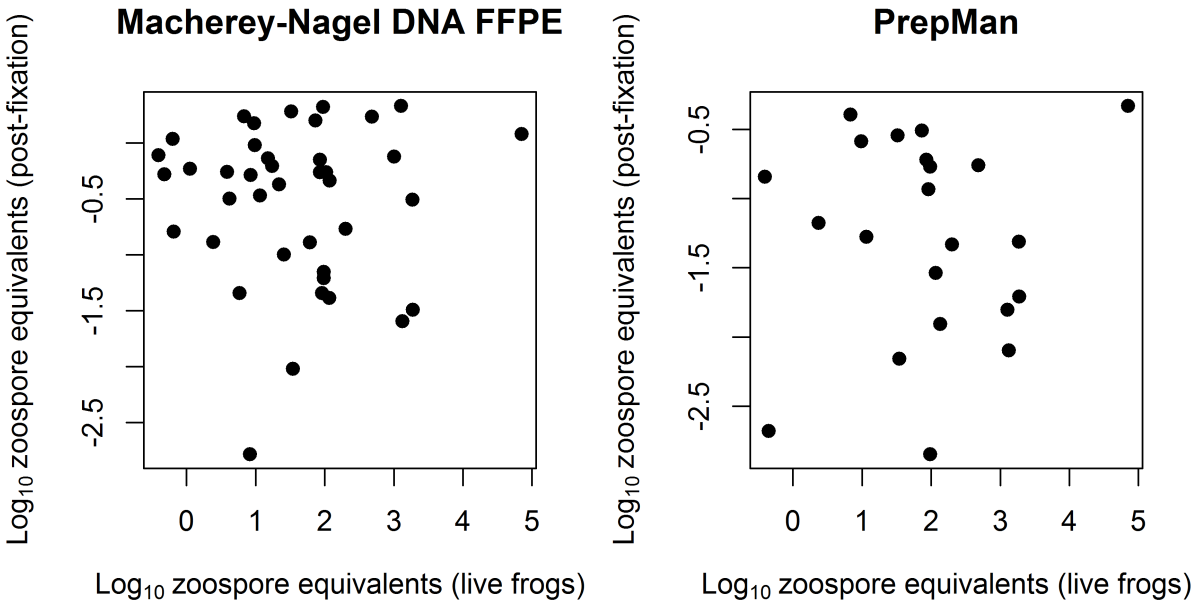

Figure 2.3. Nonzero zoospore equivalent values before and after formalin fixation for MN and PM-extracted swabs. There was no significant relationship between pre-and post-preservation Bd loads on individual swabs for either the MN ($R^2 = 0.0006$; R^2 (adj.) = -0.026; $p = 0.879$) or the PM ($R^2 = 0.003$; R^2 (adj.) = -0.046; $p = 0.798$) extracted specimen swabs.

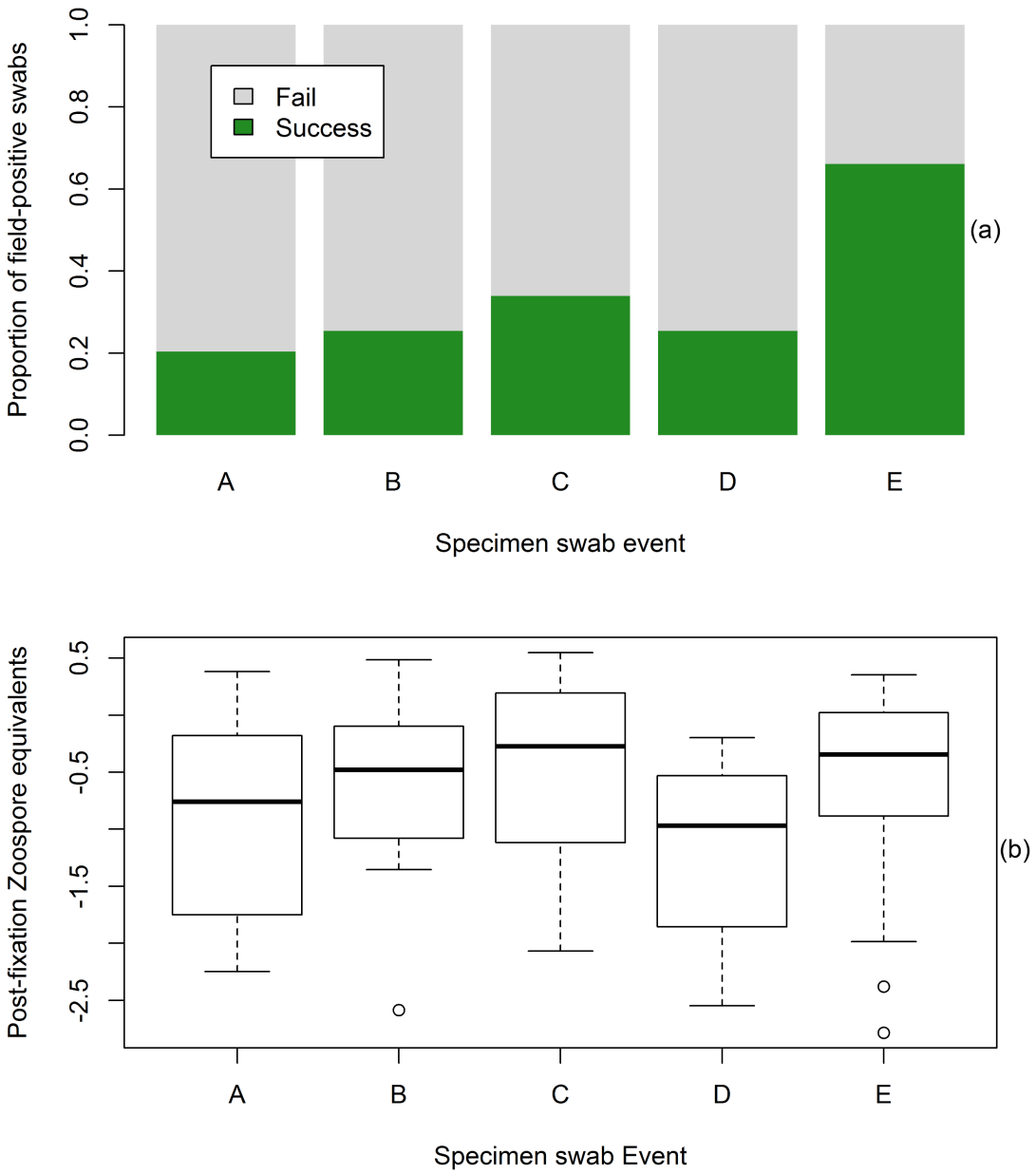

Figure 2.4. Post-fixation Bd detection success (top) and zoospore equivalents (bottom) across all swab events (n=58). (a) Bd detection success was significantly greater for swab event E as compared to all other swab events ($p < 0.0001$). (b) The two ZE outliers in Event E are from specimens that had pre-preservation Bd loads of less than 1 zoospore equivalent and were not detected, in any number of runs, by any of the PM-extracted swabs. Results are based on analysis of singlicate data.

CHAPTER THREE: Temporal increase in fungal pathogen prevalence coincides with rapid localized extirpation of a North American frog

ABSTRACT

Invasive species—especially pathogens—are an increasingly significant cause of biodiversity loss. As extinctions continue across the globe, repatriation programs are also increasing; however, a fundamental prerequisite for repatriation is determining the causes of declines. We hypothesized that *Batrachochytrium dendrobatidis* (Bd), the causative agent of the deadly amphibian disease chytridiomycosis, was an important factor in the rapid localized extirpation of a North American frog (*Rana boylii*), which is purported to have occurred over a period of less than 10 years. We used an interdisciplinary approach, combining interviews with herpetological experts, field notes, and museum specimen collections to examine 1) historical relative abundance of *R. boylii*; 2) potential causes of *R. boylii* declines; and 3) historical prevalence of Bd. Increase in Bd prevalence coincided with *R. boylii* declines and a time of rapid change in the region, wherein fish stocking, backcountry recreation, urban development, and the amphibian pet trade were all on the rise. In addition, extreme flooding during the winter of 1969 marked localized extirpations in *R. boylii* populations observed by respondents. We conclude that Bd likely played an important role in the rapid extirpation of this species from southern California, and that multiple natural and anthropogenic factors may have worked in concert to make this possible in a relatively short period of time. This study emphasizes the importance of the historical context of host-pathogen systems in making future management decisions.

INTRODUCTION

The spread of non-native and invasive species, including pathogens, represents one of the most pressing problems in conservation biology. Critical to mitigating these effects is an understanding of the factors causing the geographical emergence and expansion of novel pathogens in landscapes. Reconstructing historical pathogen distributions and emergence can inform predictions of future pathogen trajectory and the development of repatriation programs where pathogens have driven localized species extinctions (Kauffman and Jules 2006, Sainsbury and Vaughan-Higgins 2012). Amphibian declines are at the forefront of the global biodiversity crisis, with approximately one-third of species threatened with extinction—making amphibians the most vulnerable vertebrate group (Stuart et al. 2004). Yet, for decades, many declines remained enigmatic. *Batrachochytrium dendrobatidis* (Bd), the fungal pathogen responsible for the amphibian disease chytridiomycosis, was not identified and described as a causative factor until the late 20th century, owing in part to its unique role, within the Phylum Chytridiomycota, as a pathogen of vertebrates (Berger et al. 1998, Longcore et al. 1999). Since then, chytridiomycosis has been implicated in declines or extinctions of hundreds of amphibian species globally (Berger et al. 1998, Skerratt et al. 2007, Fisher et al. 2012).

In general, it is rare for disease to be the sole cause of species extinctions (Smith et al. 2006). Until the discovery of chytridiomycosis, disease was largely overlooked as a primary conservation concern in amphibians, as the pathogens known to cause severe declines and extinctions were limited (Collins et al. 2009). Many theoretical models predict that host populations cannot be driven to extinction by disease alone (Anderson et al. 1992), although host extinction can occur if a reservoir for the pathogen exists, and pathogens can

amplify the extinction risk of small host populations (De Castro and Bolker 2005, Briggs et al. 2010). As a result, initially, scientists were hesitant attribute widespread amphibian declines to epidemic disease (Alford and Richards 1997, Hero and Gillespie 1997).

Lack of historical information on pathogens may also be a reason why infectious disease has not traditionally been regarded as an important driver of extinctions (Smith et al. 2006). Historical ecological knowledge and information, combined with interpretation of ecological change, can make an important contribution to informed decision making and addressing contemporary conservation issues (Swetnam et al. 1999, Muths et al. 2016). Diverse historical resources can be drawn upon to measure environmental change, from interviews (Jennings and Hayes 1994b, Golden et al. 2014), to specimens (Lips 2011), and even works of art (Zerefos et al. 2013). In addition, slow changes over the timespan of a human career allow for a "gradual accommodation of the creeping disappearance of resource species", a phenomenon referred to as shifting baselines syndrome (Pauly 1995), which can lead to inaccurate assumptions about historical abundances and trends, and result in poorly informed management decisions. Bringing historical ecological information to the forefront of contemporary ecological analysis can help guard against this phenomenon.

Here, we combined historical and contemporary approaches to determine the timing of extirpation of the foothill yellow-legged frog, *Rana boylii*, from southern California, USA, and examine its potential causes. *R. boylii* exemplifies many amphibian species in western North America and around the world that experienced marked declines beginning in the 1970s (Blaustein and Wake 1990, Corn 2000, Houlahan et al. 2000, Green and Kagarise Sherman 2001, Lips et al. 2004, Whitfield et al. 2007, Berger et al. 2016). With *R. boylii* extirpated from much of its range, ecologists have begun planning for future reintroduction

efforts (Lind 2005, Lind et al. 2011). To evaluate the relevance of a reintroduction program and increase the chances of its success, a comprehensive understanding of the original causes of the decline is essential. Often, for amphibian species that have been extirpated from relatively pristine environments, the best-supported evidence suggests that chytridiomycosis was a significant factor (Berger et al. 1998, Rachowicz et al. 2006, Skerratt et al. 2007). Because the southern California *R. boylii* habitats were relatively remote and unaltered at the time declines occurred, we hypothesized that chytridiomycosis was a significant causative factor in *R. boylii*'s decline from the region. To test this hypothesis, we sampled for Bd in archived museum collections and in live amphibians in the field and combined these data with field notes and interviews with experts. We also aimed to determine how quickly the species was extirpated in order to compare trends in *R. boylii* occupancy with the timing of Bd emergence. Bd is capable of driving species to extinction without the involvement of any associated stressor; however, species are at more risk of extinction from disease once they have been impacted by other, non-disease related factors (Heard et al. 2013), so we also used interviews with experts to examine other threats that may have contributed to declines.

METHODS

Study Species

R. boylii is a largely stream-dwelling and obligate stream-breeding species that historically inhabited foothill streams from southern Oregon to Los Angeles County, California, USA, from near sea level to 1,219 m (Stewart et al. 2005). Listed as a Species of Special Concern in the state of California and currently proposed for federal Endangered Species Act listing (Jennings and Hayes 1994a, U.S. Fish and Wildlife Service 2015), *R.*

boylii has been extirpated from approximately half of formerly occupied sites, with the most pronounced declines and local extirpations concentrated in northern Oregon and southern California, although the extent of disappearances from historical localities may be underestimated (Davidson 2004, Lind 2005, Hayes et al. 2016). Most *R. boylii* populations in southern California were apparently stable until the mid-1970s, when populations collapsed (Jennings and Hayes 1994a, Jennings 2005). *R. boylii* persistence at historical sites has been positively associated with latitude and precipitation, and negatively associated with both upwind and surrounding agriculture (Davidson et al. 2002). Elsewhere in the range, dams and diversions have been identified as primary causes of the species' decline (Hayes et al. 2016); however, at the time that *R. boylii* disappeared from southern California, the streams where the species occurred were largely undammed or where dams existed *R. boylii* had persisted for decades at high abundances despite flow regulation.

Until recently, little was known about the susceptibility of *R. boylii* to Bd. Experimental infections in the laboratory resulted in either mortality of Bd-positive individuals not significantly different from those of controls (Davidson et al. 2003) or reduced growth in Bd-positive juveniles but no mortality (Davidson et al. 2007). In a repeat experiment of Davidson et al. (2007), however, animals from the same source population exposed to the same Bd isolate experienced 100% mortality (C. Davidson, unpublished data). In 2013, a central California population of *R. boylii* experienced a chytridiomycosis-induced mortality event (Adams et al. 2017), the first such record for this species in the wild.

Interviews and Field Notes

We used interviews and field notes to determine pre-extirpation *R. boylii* abundance and to assess the rapidity with which the species disappeared from the region. We conducted semi-directive interviews, which enable the interviewee to guide the conversation beyond a

few basic questions, and for which there is no time limit. Semi-directive interviews are powerful tools for accessing and documenting ecological knowledge that is not available from any other source (Huntington 1998).

To identify candidate interviewees, we used VertNet (National Science Foundation 2016) records to identify the collectors of southern California *R. boylii* specimens prior to its extirpation. We identified additional interviewees through conversations with these and other experts in the herpetological and California natural history fields. Candidate interviewees were usually contacted via email, except for a few that were contacted through Field Herp Forum (Herp Nation Media 2016) when no email was readily available. If they consented to an interview, respondents were given an information sheet and oral history interview agreement. The information sheet formally invited the interviewee to participate in the study, briefly described the objective of the study, explained why they were selected as a participant, and explained that participation was entirely voluntary. The interview agreement provided for formal consent to the interview and gave options (via checkboxes) for whether interviewees allowed for the interview to be recorded, transcribed, or archived. These instruments were reviewed and approved by the University of California Santa Barbara Human Subjects Committee (protocol # 1-14-0245). Interviews took place in person, over the telephone, or via email. Interviewees were asked a series of general questions about their herpetological expertise, observations of amphibian mortality events and declines, *R. boylii* natural history and abundance observations, and perceived causes of amphibian declines and *R. boylii* extirpation.

When field notes accompanied specimens or observations, we reviewed them for abundance information. A collection of field notes were accessed online through the

Museum of Vertebrate Zoology EcoReader (Museum of Vertebrate Zoology 2016) and the California Academy of Sciences Herpetology Collection Database (California Academy of Sciences 2016); others were reviewed at natural history museums where they were archived or were provided directly by interviewees. Responses to interview questions and relative abundance information from field notes were transcribed and entered into a database for later analysis. We also used the size of *R. boylii* specimen collections to estimate minimum abundance. To account for the differences between the reliability of primary sources (i.e., specimen collections and field notes) versus interviews that rely solely on memory (Alagona et al. 2012), we coded interviews, field notes, and specimen collections as separate information categories.

Specimen Sampling and Analysis

Retrospective surveys of museum specimens can reveal signatures of chytridiomycosis effects on populations, even when observations of die-offs and mass mortality are lacking (Burrowes et al. 2004). We sampled post-metamorphic amphibian specimens and used quantitative PCR (qPCR) to detect Bd DNA in order to determine infection status. To identify desired specimens, we searched for species and county records in VertNet (National Science Foundation 2016), Specify (University of California Santa Barbara 2016), and via interviews. In addition to *R. boylii* specimens from the southern California study area (defined as Ventura, Santa Barbara, and Los Angeles Counties), and because few *R. boylii* were collected relative to other species, we sampled all amphibian species that occupied the same streams in the region: *Anaxyrus boreas halophilus* (California toad); *Anaxyrus californicus* (arroyo toad); *Rana catesbeiana* (bullfrog—a non-native species that now occupies some former *R. boylii* sites); *Rana draytonii* (California red-legged frog); *Hyliola cadaverina* (California tree frog); *Hyliola regilla* (Pacific chorus

frog); and *Taricha torosa* (California newt). Specimens were considered desirable if they were one of the species described above and occurred within the same watershed or stream as *R. boylii* before it was extirpated.

Specimen sampling, DNA extraction using Macherey-Nagel DNA FFPE, and qPCR protocols followed Adams et al. (2015), with the exception that 10uL of extract were used per qPCR reaction. All samples were run in triplicate, with sample replicates run on separate qPCR plates. Specimens were considered positive when two or more samples exhibited qPCR amplification (i.e., as evidenced by an exponential amplification curve). In addition, 57 *T. torosa* specimens were tested for both Bd and *Batrachochytrium salamandrivorans* (Bsal) using duplex PCR following Blooi et al. (2013). These specimens were sampled and DNA extracted as described above.

In addition to qPCR, we conducted histology on select specimens to examine chytridiomycosis status, following Reeder et al. (2012). This allowed us to confirm the Bd status of 13 specimens collected prior to 1970 that tested positive for Bd via qPCR (i.e., >2 amplified replicates in the sample). We also conducted histological examination on 6 *R. boylii* specimens collected from Clear Creek, San Benito County in 1989 that died in captivity at a university laboratory within six weeks of their collection from the wild, and an *Anaxyrus californicus* metamorph from Santa Barbara County that also died in the same laboratory during that time.

Field Sampling

To characterize the current status of Bd in extant stream-dwelling amphibians within the former range of *R. boylii*, we conducted field sampling from 2011-2014 in formerly occupied habitats of *R. boylii* in Ventura and Santa Barbara counties. We sampled all species described above for specimen sampling except for *T. torosa*, because very few

individuals of this species were encountered during the nocturnal surveys. Bd sampling and qPCR protocols for field-collected swabs followed Hyatt et al. (2007) and Boyle et al. (2004), respectively.

Statistical analysis

To test various hypotheses for Bd infection in museum specimens, we used an information-theoretic approach and generalized linear model (GLM). The GLM included a binomial (Bd presence/absence) response and a logit-link function. To avoid pseudoreplication, because we sampled animals that were collected from the same site on the same day, we removed samples that were already represented by at least one sample on a collection date at a collection locality. When multiple species were collected on the same day at the same site, we treated each individual species independently. If at least one animal at a site was found to be Bd-positive (≥2 amplified qPCR replicates), we considered that site Bd-positive at that time for the purposes of the GLM. We sampled 1561 specimens for Bd and used 632 of these samples for the GLM analysis.

Based on other studies of historical Bd prevalence in California (Padgett-Flohr and Hopkins 2009, Sette et al. 2015) and our hypothesis that Bd was a causative factor in *R. boylii*'s extirpation in the middle of the 20th century, we expected spatial and temporal variables to be significant predictors of Bd infection in museum specimens. Therefore, we included decade, year, month, 20-year time interval, and county as categorical predictor variables in the GLM (Table 3.1). Because temporal variables were correlated with each other, we only included one temporal variable in each model at one time. We also created a spatiotemporal variable, distance from earliest positive specimen detected, which enabled us to test the hypothesis that Bd could have spread spatiotemporally from an early Bd introduction (Table 3.1). Distance from first positive specimen was calculated, in kilometers,

from latitude and longitude in decimal degrees using the Vincenty formula for ellipsoidal geodesic distances. Since Bd susceptibility can be highly variable between taxa (Searle et al. 2011), we also included two biological variables—family and species—to determine if there were taxonomic trends in historical Bd status.

We conducted all analyses using R (R Core Team 2016), and used a forward selection procedure to determine the predictor variables that were the best fit to the data. To check for multicollinearity among predictors, we used "vif" function in package "car" to ensure that variance inflation factor values were less than 3 in candidate models that contained more than one predictor (Zuur et al. 2010). We used likelihood ratio tests to compare nested models and added predictor variables sequentially in the order presented in Table 3.1. 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 ΔAIC >2 as compared to the model with the lowest AIC were considered not as well-supported by the data (Burnham and Anderson 2004).

RESULTS

Field sampling

Between 12 March 2011 and 9 September 2014, we captured and sampled 366 post-metamorphic anurans. Only *Anaxyrus californicus* and *Hyliola cadaverina* did not test positive for Bd; infection prevalence (Figure 3.1A) for all species combined was 15% (55 of 311 samples). Among species, Bd prevalence and load were highest in the two ranid species sampled (*Rana draytonii* and *Rana catesbeiana*) and *H. regilla* (Figure 3.1B).

Interviews

We contacted 29 candidate interviewees and conducted interviews with 21 of them. Of the respondents, 4 communicated exclusively via email; the remainder were interviewed over the phone or in person. Average interviewee age was 69, and respondents collectively represented 873 years (mean 42 years) of herpetological field experience. Mean verbal interview duration was 1.2 hours.

Reasons for R. boylii decline. Most respondents declined to speculate what could have caused *R. boylii* extirpations from southern California. Responses centered around several common hypotheses of amphibian declines, including chytridiomycosis, pet trade, exotic species, and climate change (Catenazzi 2015); however, two relatively unique threats emerged: increased recreational use of habitats and extreme flooding (Table 3.2). Three respondents reported observing the localized extirpation of once-abundant *R. boylii* populations after extreme flood events: 1) Tulare County in the late 1960s (Interview 9); 2) Caliente Creek (Kern County) in the mid-1970s (Interview 11); and 3) Evey Canyon in the San Gabriel Mountains (Los Angeles County) in the late 1960s (Interview 9). The Evey Canyon site shifted from having a very abundant population to no *R. boylii* found, according to the interviewee:

> *"The frogs* [R. boylii] *were extremely abundant there…you'd walk along the creek for a couple hundred yards and collect a sample of 15 or 20…the frogs were just all over the place. But after the '69 floods…I went back in '70…there weren't frogs in that creek at all and there haven't been since." (Interview 9)*

R. boylii *occurrence & abundance in southern California.* Despite the number of naturalists frequenting various portions of the study area, none were able to produce a record of *R. boylii* from later than 1977. One interviewee confirmed his sighting of a *R. boylii* in Piru Creek, Los Angeles County, on 6 July 1977, which is the last known record of the species in

southern California (Jennings and Hayes 1994a, Hayes et al. 2016). Extensive *R. boylii* resurveys were conducted in southern California by two independent research teams from 1981-1993 and from 1988-1991; however, none encountered the species. In addition, we did not encounter the species during our field surveys from 2011-2014. Several *R. boylii* collections were made in the 1940s and 1950s, along with field notes containing abundance information such as, "very common" (R. Zweifel field notes, 20 March 1948) and "fairly abundant" (Interviewee 20 field notes, 3 May 1970); only one field note entry was found that reported a negative survey for *R. boylii* prior to the late 1970s (R. Zweifel field notes, 2 April 1950). Some interviewees reported abundance information such as, "15-20 individuals per mile of stream" (Interview 8).

Chytridiomycosis in Rana boylii

Interviews revealed two previously unknown *R. boylii* mortality events attributable to chytridiomycosis, both outside of the immediate study area. In 1989, 6 live *R. boylii* individuals collected from Clear Creek, San Benito County (central California) were taken to a university laboratory where they were housed in a 60-gallon tank with recirculating water. Within 6 weeks, all frogs succumbed to an unknown infection, which we confirmed to be chytridiomycosis (see "Museum specimens" section below). Histological examination of these frogs revealed hyperkeratosis and hyperplasia in all 6 in animals, consistent with chytridiomycosis, and Bd organisms in one (Table 3.3). Necropsy conducted on one of the individuals suggests that this animal died of chytridiomycosis. These animals appeared healthy when collected from the stream where they occurred, although they were taken from a stream that was actively being used by off-road vehicles (Interview 19). It is unknown whether they contracted Bd from the field or in the lab, as this event occurred a decade before Bd was described (Longcore et al. 1999). The second mortality event was a *R. boylii*

die-off in a stream in Stanislaus County (central California) in 1986. Approximately 85 dead individuals were observed over a period of 2 weeks, until there were only a few animals left (Interview 12). Retrospective histology on these specimens confirmed that they were infected with Bd and showed symptoms of chytridiomycosis (Interviews 10 & 12). Although in a part of California where *R. boylii* is still extant, the *R. boylii* population at this locality has not recovered to the level of abundance present prior to this chytridiomycosis outbreak (Interview 12).

Museum specimens

We sampled 1561 museum specimens from eight institutional collections. All species tested positive for Bd, and overall infection prevalence was 10.6% (149 of 1561 samples). Bd-positive individuals were present in all counties sampled. The earliest Bd positives detected with qPCR were two *Anaxyrus boreas* specimens collected from Los Angeles County in 1915. Bd prevalence varied temporally, and showed a steady increase beginning in the 1970s and continuing through the 1990s, followed by a decline in prevalence after the 1990s (Figure 3.2). California newts (*Taricha torosa*) and non-native American bullfrogs (*Rana catesbeiana*) had the highest prevalence of infection among species (Figure 3.3A). Bd prevalence in *R. boylii* was among the lowest (with the possibility of higher prevalence in the 1970s); however, samples from this species were limited to the period prior to its extirpation, with prevalence in other species increasing during the 1990s, by which time *R. boylii* was extirpated from the study area (Figures 3.3B & 3.4). The GLM analysis indicated that Bd presence-absence in museum specimens was best predicted by 20-year time interval and distance from the first positive specimen (Tables 3.4 and 3.5), suggesting that Bd spread spatiotemporally from the area that is now the greater Los Angeles Area in the early 1900s (Figure 3.5). Species, month, year, decade, family, and

county were not important predictors in the GLM. The importance of 20-year time interval despite the relative unimportance of decade indicates that longer time intervals are required to observe a clear trend in the independent samples we collected.

Histologic examination revealed evidence of chytridiomycosis in two of the 13 specimens collected prior to 1970 that were qPCR positive for Bd (Table 3.3)—two *A. boreas* specimens collected from Los Angeles County in 1915 (CAS 39865, 39867). Both individuals had lesions of hyperplasia and hyperkeratosis (a thickened stratum corneum, consistent with chytridiomycosis infection (Pessier et al. 1999)). All of the *R. boylii* collected from Clear Creek in San Benito County in 1989 that later died in captivity exhibited hyperkeratosis and hyperplasia, as did the *A. californicus* metamorph that died in the same university lab at the same time as the *R. boylii* collected from Clear Creek. No visceral lesions of other infectious diseases known to cause mortality events of amphibians (e.g. Ranavirus) were observed in any of the animals histologically examined. None of the 59 specimens additionally tested for Bsal were positive for that pathogen, consistent with sampling efforts that have not yet identified Bsal infection in North America (Bsal Task Force 2016).

DISCUSSION

Bd prevalence coincident with R. boylii *extirpation*

One of the most important findings of the current study is that the proliferation of Bd in southern California coincided with the rapid extirpation of *R. boylii* from the region (Figures 3.2 & 3.4). Many declines ascribed to Bd have been unusually rapid as compared to those attributed to other causes, and populations that experience Bd-induced declines can experience increased Bd prevalence in years immediately before, during, or after declines

are observed (Berger et al. 1998, Muths et al. 2003, Lips et al. 2006, Vredenburg et al. 2010, Cheng et al. 2011). In retrospective disease-related analyses of museum specimens, high Bd prevalence is often observed during and after declines, but not before (Gillespie et al. 2015). When Bd first arrives in a naïve population of susceptible hosts, infection prevalence rapidly increases, followed by either extirpation or transition to an eventual enzootic steady state of disease dynamics, wherein disease prevalence decreases (Briggs et al. 2010, Vredenburg et al. 2010).

The spatial pattern of *R. boylii* decline also mirrors patterns of Bd emergence. The gradual, progressive south-to-north trend of *R. boylii* declines and extirpations in California was mentioned by two *R. boylii* experts during their interviews (9 & 10) and is supported by the literature (Davidson et al. 2002, Lind 2005). In addition, the wave-like pattern of Bd emergence and proliferation observed in this study (Figure 3.5) is consistent with observations elsewhere in California (Padgett-Flohr and Hopkins 2009, Vredenburg et al. 2010) and other parts of the world (Laurance et al. 1996, Lips et al. 2008). Abundance information estimated from interviews, specimen collections, and field notes suggest that *R. boylii* populations in southern California prior to the proliferation of Bd were not small (Figure 3.4). While these are only relative abundance estimates and not the results of field surveys specifically targeted for quantifying *R. boylii* occupancy, they suggest that the species was relatively abundant shortly before it was extirpated (Figure 3.4).

Pathogen detection from specimens

Because of the large sample sizes in every decade from 1940 to 1980, we can be quite confident that Bd prevalence was initially low and then increased markedly. Because of the smaller sample sizes in the early decades and the failure of histology to detect Bd in early-collected specimens, we are less confident of the prevalence prior to 1940. The

probability of Bd detection from formalin-fixed, ethanol-preserved specimens increases as pre-fixation (live animal) infection load increases (Adams et al. 2015); therefore, it is likely that higher Bd prevalence observed between the 1970s and 1990s in this study also indicate higher pathogen burden, which is consistent with an epidemic phase. It is important to note that due to the difficulty in recovering Bd DNA from formalin-fixed museum specimens using qPCR (Adams et al. 2015), estimated Bd prevalence from museum specimens will appear lower than is actually the case; therefore, comparing contemporary, field-based Bd data to specimen Bd data should be conducted with care. Although the prevalence estimates for field and specimen samples collected since the 2000s appear similar (Figures 3.1A and 3.3A), prevalences are likely much lower now than they were during the initial epizootic stage from the 1970s through the 1990s, due to better detection of Bd DNA from field samples as compared to formalin-fixed specimens (Figure 3.2). In addition, post-fixation Bd load does not correlate with pre-fixation Bd load (Richards-Hrdlicka 2012, Adams et al. 2015); therefore, we are not able to infer pre-fixation (i.e., live animal) loads from the qPCR results we obtained from the specimens.

Despite not being able to detect chytrid organisms via histology in some qPCR-positive specimens sampled from prior to 1970, we considered these positive for the purposes of this study, based on the following: 1) the 2 *A. boreas* specimens from 1915 (which we consider to the earliest positives detected in this study) had lesions consistent with chytridiomycosis despite lacking Bd organisms; 2) histology can produce false negatives even in highly infected individuals due to the patchy or uneven distribution of infection on amphibian skin or low zoosporangia density (Pessier et al. 1999, Boyle et al. 2004, Reeder et al. 2012); 3) we were limited to sampling a single, small patch of skin on

each specimen by some lending institutions, so the possibility that we missed sampling infected areas, due to the patchy distribution of chytridiomycosis on amphibian skin, is high; 4) swabbing may have removed some of the zoosporangia-containing tissue that is important for histological diagnosis (Fong et al. 2015); 5) when the specimen sampling and qPCR protocols we followed are used, Bd contamination is unlikely (Adams et al. 2015); and 6) considering the pre-1950s specimens positive based on 2 qPCR positives was conservative to our hypothesis that Bd prevalence increased in the middle of the 20th century. Items 1-5 above have implications extending beyond this study—we urge curators of natural history collections and the researchers that use them to communicate with one another regarding the specimens that have been sampled for Bd so that subsequent researchers can be aware of the increased risk of false histology negatives after specimens have been sampled, since the act of swabbing may remove zoosporangia-containing tissue (Fong et al. 2015).

Reservoir hosts and Bd introduction

The presence of a pathogen reservoir can allow a pathogen to lead to disease-induced extinction of some host species. This can be either a reservoir host that can tolerate pathogen infection, or an environmental reservoir that can allow the pathogen to persist following suppression of the host population (De Castro and Bolker 2005, Briggs et al. 2010). In populations for which there is ample indication of pathogen-induced extinction in mammals and birds, there is clear evidence of adequate reservoir hosts in the system (McCallum 2012). Our 2011-2014 field sampling of extant species within the former range of *R. boylii* indicated that Bd prevalence and loads are highest in *R. catesbeiana* (American bullfrog) and *Hyliola regilla*, which are both considered to be vectors of Bd in California (Padgett-Flohr and Hopkins 2009, Reeder et al. 2012, Adams et al. 2017)(Figure 3.3). These

species may have provided sufficient reservoir hosts to allow for Bd persistence in the system, facilitating chytridiomycosis-induced extinction of *R. boylii*. While some laboratory studies have suggested that bullfrogs may not always make suitable reservoir hosts for Bd (Gervasi et al. 2013, Eskew et al. 2015), our observation that bullfrogs have the highest Bd prevalence and loads among species sampled in the field (Figure 3.1), and previous work showing that bullfrog sympatry is an important predictor of Bd infection in *R. boylii* (Adams et al. 2017), supports the hypothesis that bullfrogs are a vector of Bd in coastal California, including where *R. boylii* are extant. Crayfish can also carry Bd, and have been introduced to portions of the study area (Kats and Farrer 2003, McMahon et al. 2013, Brannelly et al. 2015b), but we did not sample crayfish as part of this study.

Bullfrog farming may have played a key role in introducing and spreading Bd in California. An often-tolerant reservoir host for the pathogen, bullfrogs have been implicated in declines of amphibians in places throughout the world where they are not native, including California (Jennings and Hayes 1994a, Daszak et al. 2004, Miaud et al. 2016). Bullfrogs were brought to California in the early 20th century from the eastern U.S., where Bd may have occurred in an endemic state for much longer than in the western U.S. (Talley et al. 2015). Between 1900 and 1930, several farms that originally imported bullfrogs from the eastern U.S. were present throughout California, resulting in bullfrog introductions to the wild (Collins et al. 2009). Around 1912, bullfrogs were introduced to an artificial lake near Topanga Canyon (Los Angeles County) from New Orleans, Louisiana, which provided a source population for subsequent introductions throughout California (Jennings and Hayes 1994b). The earliest Bd positives detected in our study, 2 *A. boreas* collected from Big Tujunga Canyon (Los Angeles County) in 1915, may have been the result of nearby bullfrog

farming operations such as the one in Topanga Canyon. Although Bd appears to have arrived early in California—even pre-dating introductions of the Bd-tolerant African clawed frog, *Xenopus laevis* (Weldon et al. 2004, Huss et al. 2013)—our results suggest that Bd may have experienced multiple failed invasions with limited spread (Sette et al. 2015) prior to its proliferation beginning in the 1970s in southern California (Figure 3.2). In addition, Bd's ubiquity among sites sampled in the 2010s, its relatively low prevalence and pathogen loads among the anuran community, and a post-epizootic pattern of decline in prevalence after the 1990s indicate that it may have reached an enzootic state in the study area (Figures 3.1 & 3.2).

Anthropogenic stressors

The 1970s extirpation of *R. boylii* from southern California is contemporaneous with some of the earliest reports of enigmatic amphibian declines in western North America (Blaustein and Wake 1990, Sherman and Morton 1993, Drost and Fellers 1996, Green and Kagarise Sherman 2001), Puerto Rico (Burrowes et al. 2004), and Australia (Berger et al. 1999a). During the post-World War II era, California's human population grew much faster than most other U.S. regions (Hope 2011), sparking a surge in sprawl-like development. With new developments, roads, and automobiles reaching further into previously remote areas, exotic pathogens that were once restricted to their native habitats or near ports of entry became more mobile. Roads are often associated with increased pathogen transmission; for example, the invasive root pathogen *Phytophthora lateralis* of the Port Orford cedar (*Chamaecyparis lawsoniana*) was rapidly dispersed in the Pacific Northwest after an increase in road building and timber harvest in the 1960s and 1970s (Jules et al. 2002).

As human impacts expand, so do opportunities for Bd to spread through the global amphibian trade and subsequent spread of exotic species into the landscape (Schloegel et al. 2012, Liu et al. 2013). In northern California, Bd may have spread from the Bay Area and Central Valley to more remote areas in the mountains, with early positives occurring closest to a transportation corridor (De León et al. 2016). Cities and increased human activity increase opportunities for direct amphibian pathogen transport (Price et al. 2016) and spread via other hosts (Weldon et al. 2004, Schloegel et al. 2009). Similarly, it is likely that increased human use of natural areas within *R. boylii* habitat during the rapid growth of southern California in the middle of the 20th century was exacerbated by the proliferation and wide availability of the pet trade. At this time in southern California, the pet trade was thriving—wild-caught red-eared sliders (*Trachemys scripta elegans*) and frogs were widely available at department stores such as Grant's in southern California (Interview 12) (Salisbury Post 2014). A burgeoning pet trade and increased use of backcountry areas may have contributed to the spread of chytrid fungus, due to the increased likelihood of captive releases of these pets back into the wild.

Several interviewees cited an increase in human use of habitats immediately prior to *R. boylii* extirpations (Table 3.2). In addition to direct effects of human disturbance to amphibians in habitats such as trampling (U.S. Fish and Wildlife Service 2012), Bd is spread through the commercial amphibian trade and introduction of exotic hosts (Schloegel et al. 2012, Miaud et al. 2016), and other amphibian diseases, such as Ranavirus, are more readily spread in areas of dense human habitation (Price et al. 2016). In the two chytridiomycosis-related *R. boylii* mortality events reported in this study, the habitats of both populations were actively being used by off-road vehicles at the time (Interviews 12 & 19; although the source

population for the captive animals that experienced mortality in the lab did not succumb to chytridiomycosis in the field). This coincidence suggests that increased recreational use by humans could facilitate the spread and proliferation of Bd in these habitats. In addition, environmental disturbances may stress amphibians enough to suppress their immune defenses against Bd (Rollins-Smith et al. 2002) (but see Searle et al. (2014)).

Extreme flooding

In January and February 1969, extreme flooding caused extensive damage to southern California, killing at least 115 people (U.S. Army Corps of Engineers 1969). It has been suggested that the 1969 flood events led to the extirpation of *R. boylii* from southern California (Sweet 1983), and 5 interviewees mentioned flooding as a proximate or ultimate cause of *R. boylii* extirpation (Table 3.2). Although only one of these observations of *R. boylii* extirpations following flood events occurred in the southern California study area (Evey Canyon, Los Angeles County), they suggest that the species could be vulnerable to extreme flood events. *R. boylii* is a stream obligate and rarely found more than a few meters from water (Zweifel 1955, Kupferberg 1996). Radio-tracked *R. boylii* in northern California have been observed up to 40 m from the stream channel during relatively extreme precipitation events, suggesting that they will use uplands to avoid flows that would otherwise sweep them downstream, and will avoid peak flows in the main stream channel by overwintering in tributaries (Bourque 2008). In another study, the magnitude of winter floods did not affect *R. boylii* clutch density (a measure of adult abundance), but dam releases timed asynchronously with environmental cues for flooding caused scouring of clutches and *R. boylii* recruitment losses (Kupferberg et al. 2012).

Evey Canyon is a steep site where frogs may not have been able to retreat to uplands during flooding. The 1969 flood event, however, was not the first of its kind in the region.

The 1969 floods are considered the most damaging on record because they occurred after extensive development, but flooding in 1907, 1914, 1916, and 1938 may have equaled the 1969 floods in magnitude (U.S. Army Corps of Engineers 1969). In addition, sediment deposition indicates that the 1969 floods were predated by a much larger flood events (Ingram 2013, Hendy et al. 2015). Flooding has the potential to cause catastrophic mortality events, affecting population age structure, but is unlikely, on its own, to cause the rapid extirpation of a species from an entire region (Metter 1968, Corn 1994). Therefore, flooding was probably not the sole cause of *R. boylii* extirpation from southern California. Instead, we suggest that the flood event occurred at the same time as when Bd prevalence was on the rise (Figure 3.2), so chytridiomycosis and extreme flooding may have acted in concert to extirpate the species.

Why is R. boylii *still extant elsewhere in its range?*

Phylogeography. R. boylii is more phylogenetically distinct at the edges of its range than at the core—the most distinct lineages occur at the southern portion of its range—suggesting that the now-extirpated southern California populations were more divergent (Lind et al. 2011). Differential outcomes in Bd susceptibility have been observed in the field (Briggs et al. 2010), and under identical conditions in the laboratory (Searle et al. 2011)(C. Davidson, unpublished data). Immunological and genetic responses to Bd infection may reduce frogs' Bd susceptibility, resulting in persistent populations that are able to recover if there is a large enough population to sustain itself while this evolution occurs over generations (Ramsey et al. 2010, Savage and Zamudio 2011, Knapp et al. 2016). Southern California *R. boylii* populations were seemingly abundant (Figure 3.4) but restricted to a relatively small

geographic area, so its phylogenetic distinctness and limited geographic range may have limited its ability to adapt to a novel pathogen.

R. boylii populations in northern California may also have been affected by Bd and flooding; however, their more contiguous metapopulation structure likely makes them more resistant to rapid extirpation than southern California populations. As compared to the southern California metapopulations' fragmented distribution, northern California populations are less isolated, thus providing more opportunities for recolonization after population-level extinction events (Groom et al. 2006). In addition, Bd may not have arrived in central and northern California until later in the 20th century than our observations for southern California: retrospective surveys of museum specimens suggest that Bd arrived in the San Francisco Bay area of California in the late 1950s (Padgett-Flohr and Hopkins 2009)(but see (Huss et al. 2013)) and as late as the mid-70s in parts of northern California (De León et al. 2016). One interviewee (10) reported heavy flooding in northern California in 1956 and 1964, which may have occurred prior to Bd's arrival in that region.

Climate. Bd has a low tolerance for warm temperatures, leading many to hypothesize that latitude and altitude are positively correlated with Bd infection (Kriger et al. 2007, Kriger and Hero 2008, Knapp et al. 2011). Under this assumption, one might not expect southern California *R. boylii* populations to be as susceptible to Bd as those further north. However, southern California's Mediterranean climate is characterized by cool, wet winters and warm, dry summers. While the mean temperature is warmer overall as compared to the rest of California, southern California's streams and microhabitats are often within the temperature threshold for Bd's optimal growth (Piotrowski et al. 2004, Klose et

al. 2012). In an extant population of *R. boylii* in central California, water temperature was not predictive of either Bd prevalence or load in *R. boylii* (Adams et al. 2017).

Bd strain/lineage. Frogs vary in their responses to different strains and isolates of Bd (Piovia-Scott et al. 2015) and novel strains can cause negative impacts to species at the same time that endemic strains do not (Gahl et al. 2012). Yet, separate Bd strains do not always result in different Bd outcomes (Knapp et al. 2016). There is no evidence to suggest that the Bd DNA detected in this study is from a strain endemic to the region. All of the wild Bd isolates genotyped to date from California and the Pacific coast of North America are part of the Bd-GPL (Global Panzootic Lineage), belonging to the rapidly dispersed hypervirulent lineage of Bd, and there is no genetic evidence of any endemic lineages in North America (James et al. 2015). Where endemic and epizootic Bd lineages are sympatric, the endemic Bd shows a much more limited geographic distribution, and Bd-GPL may be better at dispersing in a heterogeneous landscape as well as infecting a broader range of host species (Jenkinson et al. 2016).

CONCLUSION

Emerging infectious diseases are a significant threat to global biodiversity, and information on the introduction, spread, and effects of novel pathogens is essential for enacting appropriate responses to this threat (Roy et al. 2016). Anthropogenic forces have probably played an important role in the spread of hypervirulent lineages of Bd globally (Farrer et al. 2011), and the rapid growth of mid-20th century southern California coinciding with increased Bd prevalence and the extirpation of a once-common anuran is congruent with this hypothesis. This study emphasizes the importance of the human context within which most host-pathogen systems occur, including perceptions of biodiversity loss. Many

interviewees were reluctant to hypothesize why *R. boylii* disappeared in such a short period of time, in part because amphibian populations naturally fluctuate; many did not know that anything unusual was happening at the time. As one interviewee stated:

> *"…I would go to places I had seen them [R. boylii] and they wouldn't be there anymore... I didn't think anything of it, of course—you see those patterns—but I saw 2 or 3 frogs at a spot one year and then went back 5 years later and didn't see anything. It didn't mean anything [at the time], but when you don't see them any more times that you're out there…then you realize that they're gone." (Interview 2)*

Historical information can help guard against inaccurate assumptions about abundance and trends that can lead to poorly informed management decisions. Considering the extent of *R. boylii*'s extirpation from many areas of California, biologists are examining the practicalities of repatriating the species to areas where it has been extirpated (Lind 2005). To date, the possibility of disease resulting from a translocation has not been examined in this species. Non-native microparasites that are able to persist within reservoir hosts with long infection periods and that are generalists (can inflect multiple hosts) are of greatest concern for conservation translocation programs, and Bd meets these criteria (Rideout et al. 2016). Gaining a better understanding of pattern and process of extinctions and extirpations is only the beginning of successfully predicting best management practices for repatriation programs. An important next phase is to describe the immunogenetic and ecological attributes of *R. boylii* populations that are persisting despite ongoing Bd infection, and use insights gained from these studies to design *R. boylii* reintroduction programs that maximize the probability of success. In combination with this information, the current study

provides a solid foundation for *R. boylii* reintroductions into areas in southern California from which this species has long been extirpated.

TABLES

Table 3.1. Variables used in generalized linear model used to determine the best predictors of Bd presence-absence in museum specimens.

Covariate	**Type**	**Range or levels**	**Description**
Decade	Temporal	1910; 1920; 1930; 1940; 1950; 1960; 1970; 1990; 1980; 1990; 2000	Decade that specimen was collected from the wild
20 - year interval	Temporal	1910-1929; 1930-1949; 1950-1969; 1970-1989; 1990-2009	20-year period that specimen was collected from the wild
Year collected	Temporal	1911 - 2009	Year that specimen was collected from the wild
Distance from first positive specimen	Spatiotemporal	0 – 309.9 km	Vincenty distance from the earliest Bd positive detected in this study (z-transformed)
Month collected	Temporal	1 - 12	Month of the year that specimen was collected from the wild
County	Spatial	Los Angeles; San Luis Obispo; Santa Barbara; Ventura	California county where specimen was collected from the wild
Species	Biological	*Anaxyrus boreas*; *Anaxyrus californicus*; *Rana catesbeiana*; *Hyliola cadaverina*, *Hyliola regilla*, *Rana boylii*, *Rana draytonii*, *Taricha torosa*	Species of specimen sampled
Family	Biological	Bufonidae, Hylidae, Ranidae, Salamandridae	Family to which specimen sampled belongs

Table 3.2. Interviewee responses to inquiry of potential causes of *R. boylii* extirpation from southern California.

Cause	**Years**[1]	**Interview #**	**Explanation**
Amphibian pet trade & exotic amphibians	175 [58]	10, 12, 20	Amphibians wild-caught; availability in local department stores; pet releases into the wild.
Bd	195 [49]	3, 10, 12, 20	Chytridiomycosis
Bd + climate change	54	10	Climate change exacerbates conditions when Bd is already present; making species more susceptible
Bd + fish stocking	56	12	Releases Bd zoospores into new waterways
Bd + flooding	56	12	Unusual frequency of rain events & overcast days reduced opportunities for frog basking (which can mitigate chytridiomycosis)
Climate change	54	10	Declines trend from south to north; decline first in drier climates
Drought	60	4	l955-l960, l971-l978 were very dry years
Non-native fish	63	17	Non-native fish introduced; predation
Flooding (1968-69)	210 [53]	9, 11, 12, 19, 20	Extreme flood events scoured frogs to lower elevations/less suitable habitat; permanent change in habitat quality and suitability
Recreation	234 [59]	12, 17, 19, 20	Increased and intensified recreational uses of streams

[1]Cumulative years' herpetological experience represented by respondents; number in brackets shows average years.

Table 3.3. Results of histological examination of formalin-fixed, ethanol-preserved museum specimens.

Institution	Collection #	Species	County	Year	qPCR replicates positive	Histology Result
CAS	39865	*Anaxyrus boreas*	Los Angeles	1915	2	Lesions of hyperplasia & hyperkeratosis, no Bd organisms
CAS	39867	*Anaxyrus boreas*	Los Angeles	1915	3	Lesions of hyperplasia & hyperkeratosis, no Bd organisms
CAS	63049	*Hyliola cadaverina*	Ventura	1927	2	negative
MVZ	27881	*Rana catesbeiana*	Los Angeles	1939	3	negative
MVZ	33668	*Rana boylii*	Ventura	1940	2	negative
MVZ	33672	*Rana boylii*	Ventura	1940	2	negative
LACM	13455	*Rana draytonii*	Los Angeles	1947	2	negative
LACM	13703	*Rana boylii*	Ventura	1954	2	negative
LACM	13496	*Rana draytonii*	Ventura	1954	2	negative
CAS	181067	*Anaxyrus boreas*	Santa Barbara	1961	2	negative
LACM	13457	*Rana draytonii*	Los Angeles	1963	2	negative
CAS	190944	*Anaxyrus californicus*	Santa Barbara	1966	2	negative
LACM	76274	*Rana draytonii*	Ventura	1968	3	negative
private	NA	*Anaxyrus californicus*	Santa Barbara	1989	1	Hyperplasia & hyperkeratosis, no Bd organisms
private	NA	*Rana boylii*	San Benito	1989	0	Hyperplasia & hyperkeratosis with Bd organisms
private	NA	*Rana boylii*	San Benito	1989	0	Hyperplasia & hyperkeratosis, no Bd organisms
private	NA	*Rana boylii*	San Benito	1989	0	Hyperplasia & hyperkeratosis, no Bd organisms
private	NA	*Rana boylii*	San Benito	1989	0	Hyperplasia & hyperkeratosis, no Bd organisms
private	NA	*Rana boylii*	San Benito	1989	0	Hyperplasia & hyperkeratosis, no Bd organisms
private	NA	*Rana boylii*	San Benito	1989	0	Hyperplasia & hyperkeratosis, no Bd organisms

Table 3.4. Candidate mixed effects models used to determine the best predictors of Bd presence/absence in museum specimens. K = number of parameters in the model.

Rank	Model	K	logLik	AIC_c	ΔAIC_c	AIC_w
1	20-year interval	5	-195.39	400.87	0	0.49
2	20-year interval + Distance from first positive specimen	6	-195.11	402.36	1.49	0.23
3	20-year interval + Family	8	-193.37	402.96	2.09	0.17
4	20-year interval + County	8	-194.53	405.29	4.42	0.05
5	Decade	10	-193.46	407.27	6.4	0.02
6	20-year interval + Species	12	-191.49	407.48	6.61	0.02
7	20-year interval + Month	16	-188.61	410.11	9.24	0
8	Year	80	-153.14	489.8	88.93	0
9	Null	1	-255.06	512.13	111.26	0

Table 3.5. Parameter estimates for best-fit model of Bd presence/absence in museum specimens, which includes 20-year time intervals ($R^2 = 0.375$). ‡ = $p < 0.05$.

Parameter	Estimate	SE	z	p
(Intercept) ‡	-2.1401	0.7475	-2.8628	0.0042
1930-1949	-0.8389	0.9535	-0.8798	0.3790
1950-1969 ‡	-1.6474	0.8396	-1.9621	0.0497
1970-1989	1.1055	0.7683	1.4388	0.1502
1990-2009 ‡	1.9990	0.7845	2.5481	0.0108

FIGURES

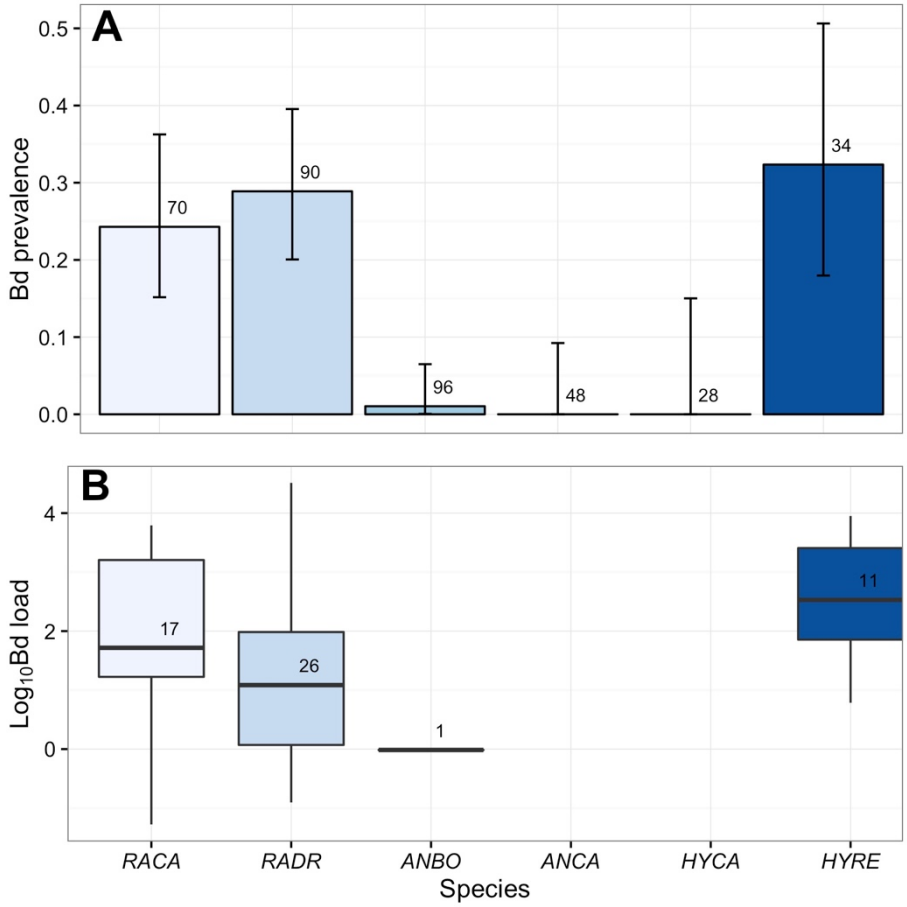

Figure 3.1. Bd infection among live anurans sampled for Bd in the wild during 2011-2014 within the former range of *Rana boylii* in southern California in Ventura and Santa Barbara Counties, indicating (A) Bd prevalence; and (B) pathogen load of Bd-positive individuals. Error bars in (A) represent the 95% Clopper-Pearson binomial confidence intervals. Bold horizontal lines within each boxplot in (B) indicate the median, boxes show the interquartile (IQ) range, and whiskers show the range within 1.5 times the IQ range. Numbers above the bars indicate (A) total sample size for each species or (B) the number of Bd-positive individuals. *RACA = Rana catesbeiana*; *RADR = Rana draytonii*; *ANBO = Anaxyrus boreas*; *ANCA = Anaxyrus californicus*; *HYCA = Hyliola cadaverina*; *HYRE = Hyliola regilla*.

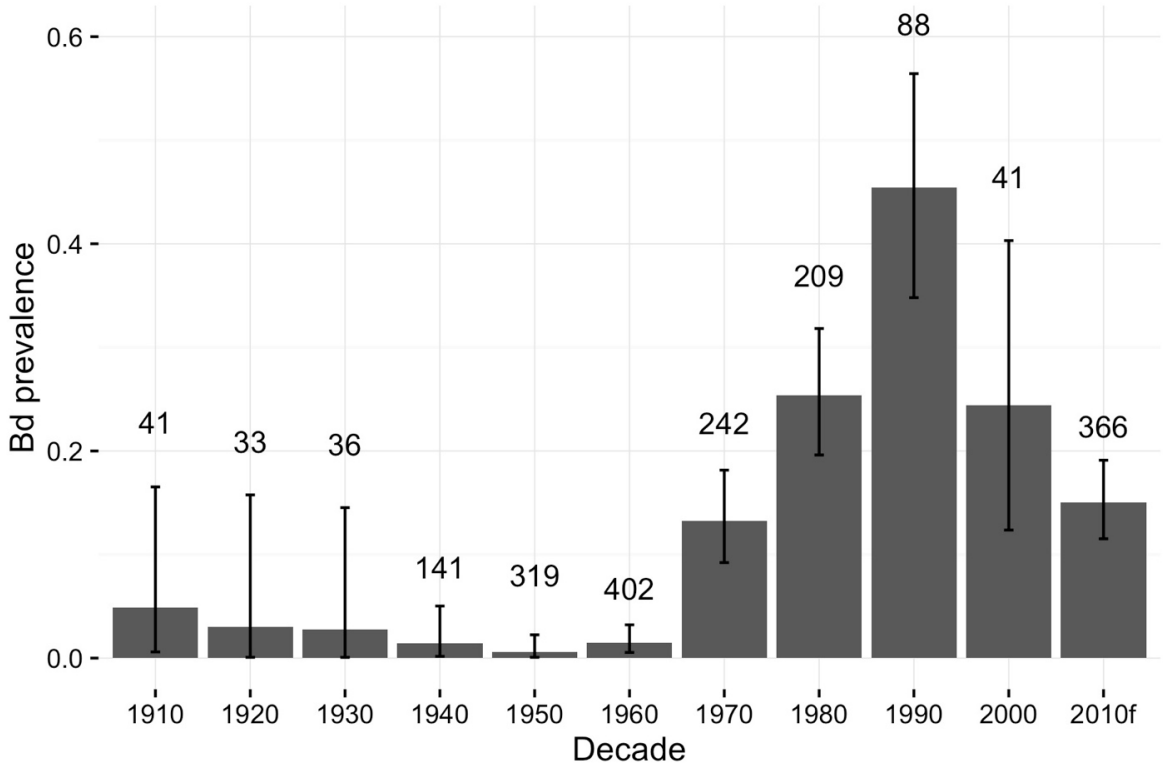

Figure 3.2. Bd prevalence through time for all museum specimens sampled. Each bar represents a decade that begins with the year indicated by the x-axis label. Error bars represent Clopper-Pearson binomial confidence intervals. Numbers above the bars denote sample size. Data are from specimens collected from the study area, including Los Angeles, Ventura, and Santa Barbara Counties. The "2010f" category represents Bd samples collected from live post-metamorphic amphibians sampled in the field from 2011-2014.

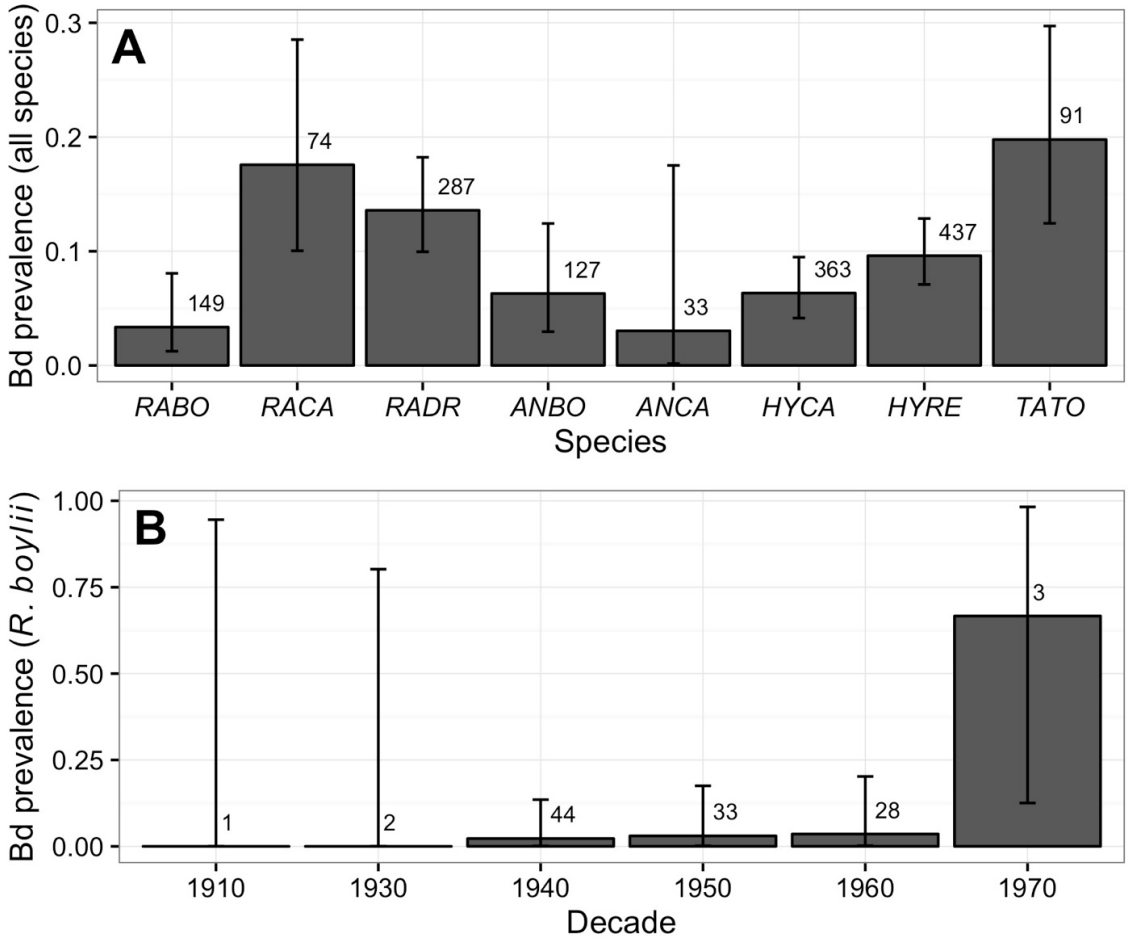

Figure 3.3. Bd prevalence of museum specimens, representing (A) all species; and (B) *R. boylii* only, by decade. Species codes: *RABO = Rana boylii*; *RACA = Rana catesbeiana*; *RADR = Rana draytonii*; *ANBO = Anaxyrus boreas*; *ANCA = Anaxyrus californicus*; *HYCA = Hyliola cadaverina*; *HYRE = Hyliola regilla*; *TATO = Taricha torosa*. Decades are indicated on the x axis in (B) by the first year in each decade.

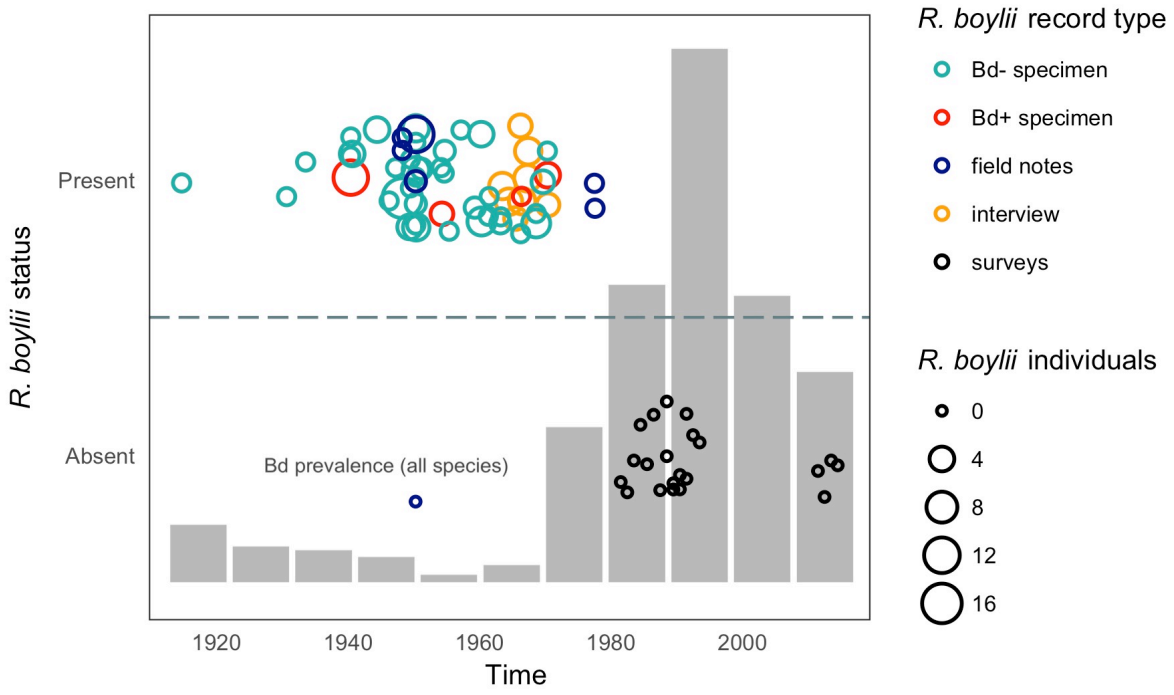

Figure 3.4. *Rana boylii* relative abundance (jittered) by record type through time. Specimen abundances are represented by the number of post-metamorphic individuals collected at a site on a particular date. Background plot shows relative Bd prevalence of all species detected from museum specimens through time (see Figure 3.1 for details). For each Bd-positive *R. boylii* sample shown (i.e., 2 or more qPCR replicates amplified), only one specimen in the collection was positive, even though all of the available individuals from a given collection day & locality were sampled for Bd. Therefore, circle sizes correspond to the number of *R. boylii* individuals and not Bd prevalence. For Bd prevalence though time in *R. boylii*, refer to Figure 3.3B.

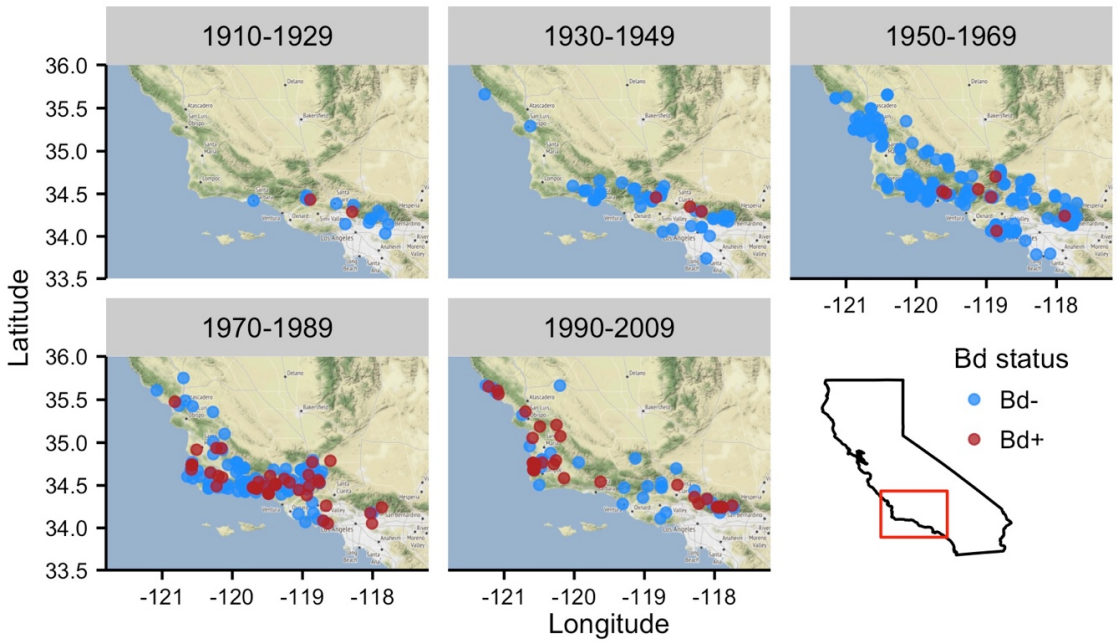

Figure 3.5. Map of localities sampled for Bd in museum specimens from 1915-2009 as used in the GLM. Dates above each map represent the 20-year time interval in which each specimen was collected. Data were arranged prior to mapping so that Bd-positive samples would be superimposed on Bd-negative samples when they overlapped. Data include sampled specimens collected from Ventura, Santa Barbara, Los Angeles, and San Luis Obispo counties.

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APPENDIX 1

TABLES

Table 1. Total field days and number of amphibian individuals swabbed by season.

	Fall	**Winter/Spring**	**Summer**	**Total**
Visits/Field Days	7	19	4	30
Rana boylii swabbed	37	41	64	142
Other species swabbed	25	14	35	74

Table 2. Candidate mixed effects models used to determine the best predictors of Bd presence/absence in post-metamorphic *R. boylii*.

Rank	Model	K	logLik	AIC_c	ΔAIC_c	AIC_w
1	Water year + Sex-stage + Bullfrog Time	7	-52.88	120.71	0.00	0.37
2	Water year + Sex-stage + Bullfrogs†	6	-54.84	122.39	1.68	0.16
3	Sex-stage + Bullfrog Time	6	-54.88	122.45	1.74	0.15
4	Water Year + Sex-stage + Bullfrogs + *R. boylii* Clutches	7	-53.98	122.90	2.19	0.12
5	Water Year × Bullfrogs + Sex-stage	7	-54.30	123.53	2.83	0.09
6	Water Year + Sex-stage	5	-57.39	125.28	4.57	0.04
7	Water Year + Sex-stage + Bullfrog Distance	6	-56.64	125.97	5.27	0.03
8	Water Year + Sex-stage + Crayfish	6	-57.04	126.78	6.08	0.02
9	Water Year + Sex-stage + Length	6	-57.35	127.39	6.69	0.01
10	Water Year + Bullfrogs	4	-60.64	129.60	8.90	0.00
11	Water Year	3	-62.32	130.84	10.14	0.00
12	Water Year + Days Since Peak Stream Flow	4	-61.38	131.09	10.38	0.00
13	Water Year + Drought Index	4	-61.44	131.22	10.51	0.00
14	Water Year + Mean Daily Stream Flow	4	-62.05	132.42	11.72	0.00
15	Water Year + Water Temperature	4	-62.30	132.92	12.22	0.00
16	Water Year + Preceding Peak Stream Flow	4	-62.32	132.98	12.27	0.00
17	Intercept Only	2	-65.18	134.46	13.75	0.00
18	Flow Regime	5	-62.03	134.57	13.86	0.00
19	Water Temperature	3	-64.65	135.50	14.79	0.00
20	Season 2	3	-65.09	136.37	15.66	0.00
21	Season3	4	-64.29	136.90	16.19	0.00

Notes: All models included a random effect of survey event. K indicates the number of parameters. Explanations of fixed effects can be found in Table 1 of the main text.

†A Bayesian model that included an interaction with bullfrogs and sex-stage (Water Year + Bullfrogs × Sex-stage) was also fitted and compared directly to another Bayesian model without the interaction (Water Year + Bullfrogs + Sex-stage). The interaction did not improve the model as shown by AIC (with interaction: 121.30; without interaction: 118.34).

Table 3. Candidate mixed effects models used to determine the best predictors of Bd load in post-metamorphic *R. boylii*.

Rank	Model	K	logLik	AIC_c	ΔAIC_c	AIC_w
1	Season3 + Mean Daily Stream Flow + Bullfrog Time + *R. boylii* clutches	9	-147.68	316.63	0.00	0.33
2	Season3 × Mean Daily Stream Flow + Bullfrogs + *R. boylii* clutches	10	-146.83	317.73	1.10	0.19
3	Season3 × Mean Daily Stream Flow + Bullfrog Time + *R. boylii* clutches	11	-145.55	318.08	1.45	0.16
4	Season3 + Mean Daily Stream Flow + Bullfrog Time	8	-150.36	319.30	2.67	0.09
5	Season3 + Mean Daily Stream Flow + Bullfrogs + *R. boylii* clutches	8	-150.65	319.88	3.25	0.07
6	Season3 + Mean Daily Stream Flow + Bullfrogs	7	-152.44	320.85	4.22	0.04
7	Season3 + Mean Daily Stream Flow + Bullfrog Distance	7	-156.24	328.45	11.82	0.00
8	Season3 + Mean Daily Stream Flow + Bullfrogs × Sex-stage	10	-152.27	328.62	11.99	0.00
9	Season3 + Mean Daily Stream Flow	6	-157.79	329.02	12.39	0.00
10	Season3 + Mean Daily Stream Flow + Crayfish	7	-157.57	331.10	14.47	0.00
11	Season3 + Mean Daily Stream Flow + Length	7	-157.79	331.54	14.91	0.00
12	Season3	5	-161.19	333.40	16.77	0.00
13	Mean Daily Stream Flow + Bullfrogs + *R. boylii* clutches	6	-160.02	333.49	16.86	0.00
14	Season3 + Mean Daily Stream Flow + Sex-stage	8	-157.72	334.01	17.38	0.00
15	Season3 + Water Year	6	-160.62	334.69	18.06	0.00
16	Season3 + Mean Daily Stream Flow	8	-158.39	335.35	18.72	0.00
17	Season3 + Flow Regime	8	-158.39	335.35	18.72	0.00
18	Season3 + Drought Index	6	-161.16	335.77	19.14	0.00
19	Season3 + Days Since Peak Stream Flow	6	-161.19	335.82	19.19	0.00
20	Season3 + Preceding Peak Stream Flow	6	-161.19	335.83	19.20	0.00
21	Mean Daily Stream Flow + Water Temperature	5	-162.79	336.60	19.97	0.00
22	Intercept Only	3	-165.41	337.22	20.59	0.00
23	Season3 + Mean Daily Stream Flow + Sex-stage + Bullfrog Distance × Sex-stage	11	-155.29	337.56	20.93	0.00
24	Season2	4	-165.25	339.16	22.53	0.00
25	Water Temperature + Days Since Peak Stream Flow	5	-164.45	339.92	23.29	0.00

Notes: All models included a random effect of survey event. K indicates the number of parameters. Descriptions of fixed effects can be found in Table 1 of the main text.

Table 4. Candidate mixed effects models used to determine the best predictors of Bd presence-absence in bullfrogs.

Rank	Model	K	logLik	AIC_c	ΔAIC_c	AIC_w
1	Water Temperature + Stage	4	-6.97	23.37	0.00	0.71
2	Water Temperature + Drought Index	4	-9.26	27.95	4.58	0.07
3	Water Temperature + Length	4	-9.26	27.95	4.58	0.07
4	Water Temperature × Preceding Peak Stream Flow	5	-8.04	28.31	4.94	0.06
5	Water Temperature × Drought Index	5	-9.15	30.52	7.15	0.02
6	Water Temperature × Length	5	-9.15	30.52	7.15	0.02
7	Water Temperature × *R. boylii* Clutches	5	-9.49	31.21	7.84	0.01
8	Water Temperature + *R. boylii* Clutches	4	-11.53	32.48	9.12	0.01
9	Water Temperature	3	-13.21	33.25	9.88	0.01
10	Water Temperature + Days Since Peak Stream Flow	4	-12.35	34.13	10.76	0.00
11	Water Temperature + Preceding Peak Stream Flow	4	-12.50	34.44	11.07	0.00
12	Water Temperature + Mean Daily Stream Flow	4	-13.09	35.61	12.25	0.00
13	Length	3	-14.94	36.71	13.35	0.00
14	Drought Index	3	-14.94	36.71	13.35	0.00
15	Water Temperature × Days Since Peak Stream Flow	5	-12.32	36.87	13.50	0.00
16	Stage	3	-15.25	37.33	13.96	0.00
17	Water Temperature × Mean Daily Stream Flow	5	-13.03	38.28	14.91	0.00
18	*R. boylii* Clutches	3	-16.01	38.86	15.49	0.00
19	Intercept Only	2	-17.28	38.95	15.59	0.00
20	Season2	3	-17.24	41.30	17.93	0.00
21	Flow Regime	4	-16.60	42.64	19.27	0.00

Notes: All models included a random effect of survey event. K indicates the number of parameters. Descriptions of fixed effects can be found in Table 1.1 of the main text.

Table 5. Candidate mixed effects models used to determine the best predictors of Bd load in bullfrogs.

Rank	Model	K	logLik	AIC_c	ΔAIC_c	AIC_w
1	Intercept Only	3	-25.93	60.85	0.00	0.27
2	Calendar Year	4	-25.15	64.01	3.16	0.06
3	Season 2	4	-25.23	64.17	3.31	0.05
4	Mean Daily Stream Flow	4	-25.50	64.70	3.85	0.04
5	Days Since Peak Stream Flow	4	-25.60	64.91	4.06	0.04
6	Drought Index	4	-25.65	65.01	4.16	0.03
7	Preceding Peak Stream Flow	4	-25.77	65.25	4.40	0.03
8	Water Temperature	4	-25.91	65.54	4.68	0.03
9	Length	4	-25.91	65.54	4.68	0.03
10	*R. boylii Clutches*	4	-25.92	65.56	4.71	0.03
11	Crayfish	4	-25.93	65.57	4.71	0.03
12	Season 3	5	-24.97	69.94	9.09	0.00
13	Flow Regime	5	-25.15	70.30	9.44	0.00
14	Sexstage	5	-25.80	71.59	10.74	0.00

Notes: All models included a random effect of survey event. K indicates the number of parameters. Descriptions of fixed effects can be found in Table 1.1 of the main text.

FIGURES

Figure S1. Photo showing cryptic nature of *Rana boylii* in the stream channel relative to a *R. boylii* egg mass. The arrow points to a male near the bank with its head out of the water. Photo credit: Sarah Kupferberg.

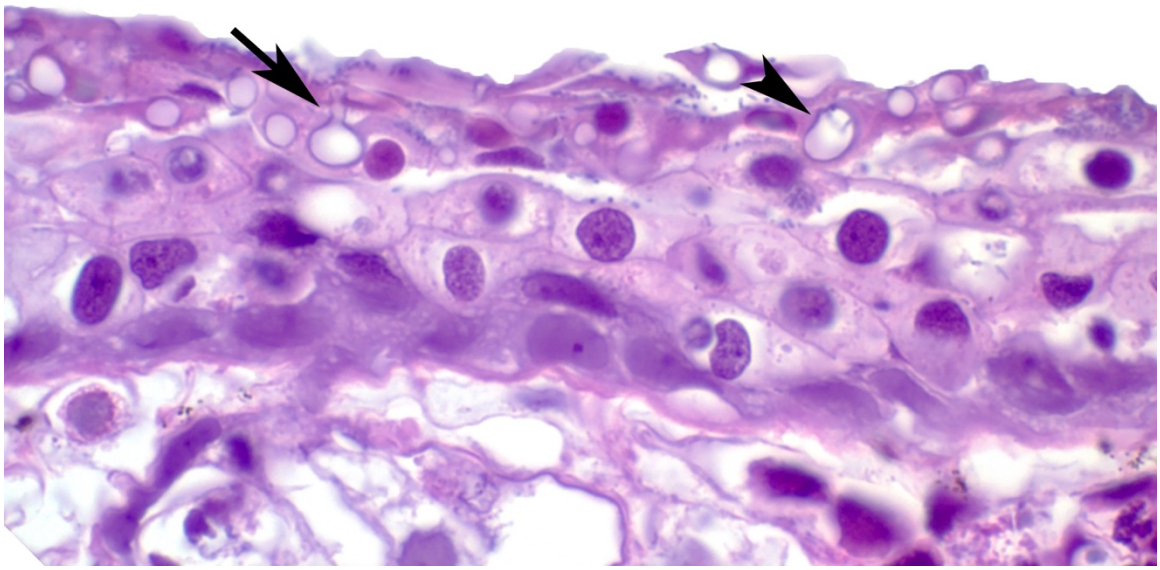

Figure S2. Photomicrograph of *R. boylii* skin from a dead metamorph collected at the die off in Alameda Creek in November 2013. The epidermis is thickened and disorganized (hyperplasia) with numerous chytrid thalli in the keratinized layers. Characteristic thallus forms include zoosporangia with a prominent discharge tube (arrow) and colonial thalli with evidence of internal septation (arrowhead). Photo credit: Allan Pessier.