

Short Communication

American Bullfrogs (*Lithobates catesbeianus*) Resist Infection by Multiple Isolates of *Batrachochytrium dendrobatidis*, Including One Implicated in Wild Mass Mortality

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Abstract: The emerging amphibian disease chytridiomycosis varies in severity depending on host species. Within species, disease susceptibility can also be influenced by pathogen variation and environmental factors. Here, we report on experimental exposures of American bullfrogs (*Lithobates catesbeianus*) to three different isolates of *Batrachochytrium dendrobatidis* (*Bd*), including one implicated in causing mass mortality of wild American bullfrogs. Exposed frogs showed low infection prevalence, relatively low infection load, and lack of clinical disease. Our results suggest that environmental cofactors are likely important contributors to *Bd*-associated American bullfrog mortality and that this species both resists and tolerates *Bd* infection.

Keywords: chytridiomycosis, resistance, tolerance, susceptibility, carrier, *Rana catesbeiana*

The emerging disease chytridiomycosis, which results from infection of amphibian skin by the aquatic fungus *Batrachochytrium dendrobatidis* (*Bd*), has been implicated in mass amphibian mortalities that have caused the decline and extinction of hundreds of species globally (Berger et al. 1998; Skerratt et al. 2007; Kilpatrick et al. 2010; Eskew and Todd 2013). Despite these devastating impacts on amphibian biodiversity, *Bd* infection often has variable disease outcomes in different host species (Searle et al. 2011; Gahl et al. 2012; Gervasi et al. 2013a). For example, a number of hosts, including American bullfrogs (Daszak et al. 2004; Hanselmann et al.

2004), Pacific chorus frogs (Reeder et al. 2012), African clawed frogs (Weldon et al. 2004), and Japanese giant salamanders (Goka et al. 2009), have been reported to be aclinical *Bd* carriers.

The carrier status of some species, however, has been increasingly called into question. For example, there is evidence for *Bd*-associated disease in American bullfrogs in food production settings (Mazzoni et al. 2003) and after exposure to specific *Bd* isolates (Gervasi et al. 2013b). Furthermore, a recent report from Finley Lake in the Sierra Nevada foothills of California also attributed mass mortality of wild American bullfrogs to chytridiomycosis (Clifford et al. 2012). Our primary aim in this study was to investigate American bullfrog responses to a range of *Bd* isolates that vary in virulence, including an isolate from Finley Lake. We predicted that the Finley Lake *Bd* isolate in particular would cause chytridiomycosis.

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We obtained 26 recently metamorphosed American bullfrogs from Niles Biological, Inc. (Sacramento, CA) in July 2013. We housed frogs individually in 5.7 L plastic containers on a 12 h:12 h light:dark cycle at 20°C, within the optimal temperature range for *Bd* growth (Piotrowski et al. 2004). We fed all animals ~5% of their body mass in crickets three times weekly and cleaned animal housings after each feeding.

We initially conducted experimental *Bd* exposures using two isolates: Finley Lake (hereafter FL) *Bd* and Carter Meadow (CM) *Bd*. FL *Bd* was originally isolated from dead, naturally infected American bullfrogs from the mass mortality event at Finley Lake (Clifford et al. 2012). CM *Bd* was cultured from *Rana cascadae* at Lassen National Forest in the southern Cascades mountains and was included here for comparison to FL *Bd* because CM *Bd* appears to have only moderate virulence (Piovia-Scott et al. 2014). Although all *Bd* isolates used in this study were collected in 2011, samples were cryopreserved and revived just prior to our exposure treatments and thus had relatively low passage numbers (10–15). We cultured *Bd* zoospores in TGhL broth and, after filtration (Whatman Grade 4 filters), quantified zoospore density using a hemocytometer. We then diluted the inoculum with TGhL broth to a concentration of 2×10^5 zoospores/mL. For experimental exposures, frogs were placed in a bath of 5 mL of zoospore inoculum (total inoculum dose of 10^6 zoospores) and 95 mL water overnight (~20 h). We exposed frogs individually in 750 mL plastic containers that limited frog movement to ensure contact with the *Bd* inoculum. Exposure treatment groups were as follows: nine FL *Bd*-exposed frogs, nine CM *Bd*-exposed frogs, and eight sham control frogs exposed only to 100 mL of TGhL broth.

Because we observed low infection prevalence following this exposure ($\leq 25\%$ for both *Bd* treatment groups), we conducted a second exposure 26 days after the first in an attempt to induce greater prevalence and heavier infections. For the second exposure, we divided the remaining study animals into two treatment groups: 12 Section Line (SL) *Bd*-exposed frogs and 11 control frogs. We chose SL *Bd* for the second exposure based on previous results that indicated it is a relatively virulent *Bd* isolate that causes significant mortality in other frog species (see Piovia-Scott et al. 2014 for more detail). Exposure procedures followed the previously described methods except that dosage per animal was increased from 10^6 to 2×10^6 zoospores and exposure was extended over two nights (~44 h total).

Just before the first *Bd* exposure and approximately weekly thereafter, we swabbed frogs for *Bd* infection as previously described in Piovia-Scott et al. (2014). We monitored animals daily, and any frogs lacking a righting reflex or that had other health issues (e.g., untreatable lesions incidental to husbandry) were humanely euthanized via overdose of MS-222. Zoospore loads were calculated from quantitative PCR results as in Piovia-Scott et al. (2014) except that samples were run in duplicate. Swab samples were only considered positive when both sample runs indicated *Bd* presence, and infection load was calculated by averaging results from the two positive wells (all raw qPCR data are available as Supplemental Material). We conducted all data visualization and analysis in R (R Core Team 2015), and tests for survival differences among experimental treatments groups were implemented using the 'survdif' function within the 'survival' package (Therneau 2014).

One animal from the FL *Bd* treatment tested positive for *Bd* prior to the first exposure and was therefore excluded from all further analyses. Following first exposure, prevalence in the FL *Bd* treatment group peaked at 25% 2 days after exposure before falling to zero after 26 days (Fig. 1a). Average zoospore loads on *Bd*-positive animals in the FL *Bd* treatment were always $< 10^3$ zoospore equivalents (ZE) (Fig. 1b). Infection prevalence peaked at 25% in the CM *Bd* treatment 26 days after first exposure, and average zoospore loads of positive animals never exceeded 10^3 ZE (Fig. 1). All control animals remained *Bd*-negative.

No animals developed clinical signs of chytridiomycosis after the first exposure treatments. At 19 days post-exposure, we sacrificed one animal each from the FL *Bd* and CM *Bd* treatments that had consistently tested positive for *Bd*. Histological analysis of these individuals revealed no evidence of clinical chytridiomycosis, including no epidermal hyperplasia or *Bd* thalli. One animal in the control group was euthanized due to bloating and ulcers on the front feet. There were no differences in mortality attributable to the first exposure treatments ($\chi^2 = 1.9$, $df = 2$, $P = 0.392$).

Our second exposure using the SL *Bd* isolate did not result in any detectable infection, and control animals remained *Bd*-negative (Fig. 1). During the second exposure period, we euthanized one animal from the SL *Bd* treatment and two animals from the control treatment for health issues unrelated to chytridiomycosis, resulting in no difference in mortality by treatment ($\chi^2 = 0.4$, $df = 1$, $P = 0.507$). We terminated the study 51 days after first

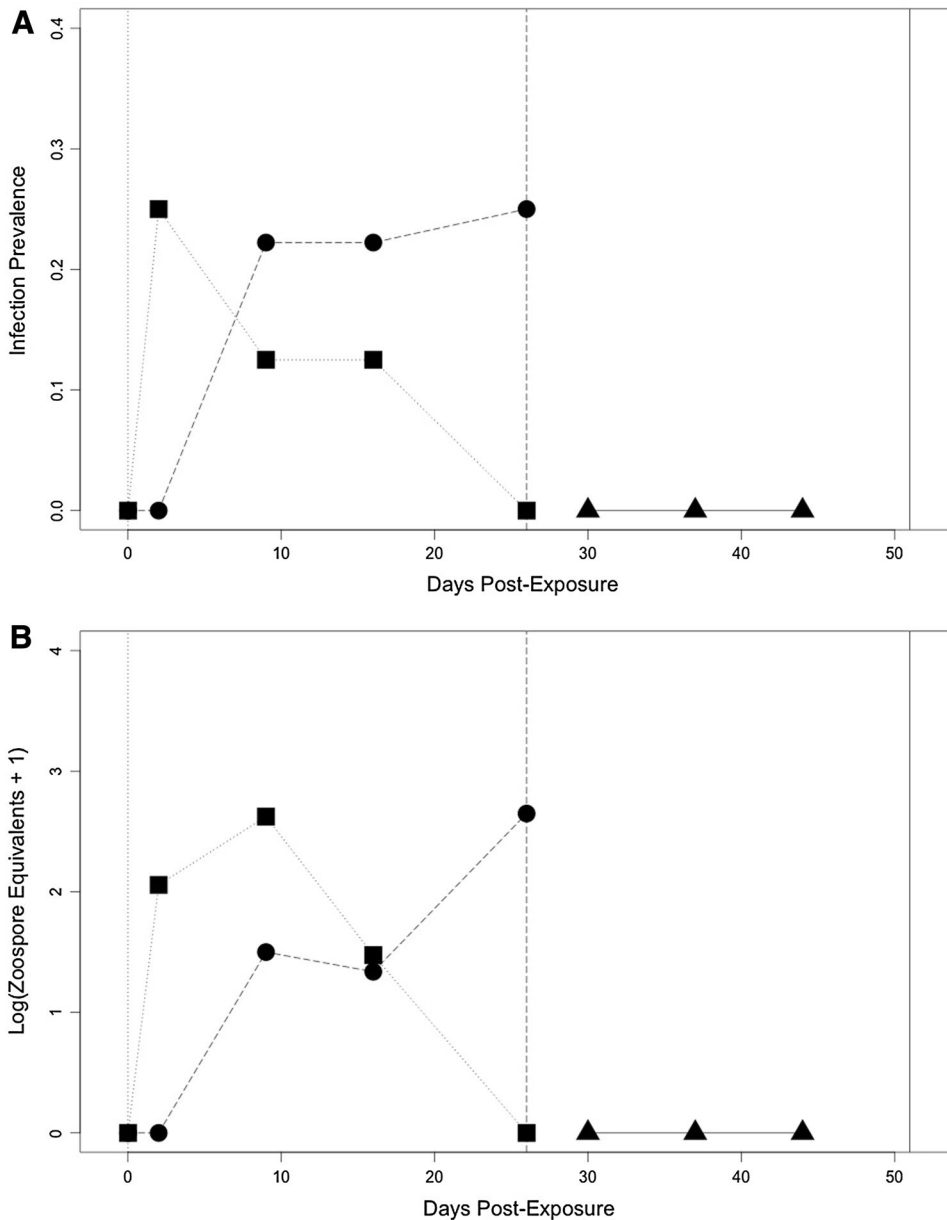


Fig. 1. *Batrachochytrium dendrobatidis* (*Bd*) infection prevalence (a) and average infection load of *Bd*-positive animals (b) throughout the study duration. The first exposure, second exposure, and study endpoint are illustrated with the vertical dotted, dashed, and solid lines, respectively. Days post-exposure are relative to the first exposure. Infection prevalence and load were evaluated using a quantitative PCR assay. Data from Finley Lake *Bd*-exposed animals are shown as squares connected by dotted lines, Carter Meadow *Bd*-exposed animals as circles connected by dashed lines, and Section Line *Bd*-exposed animals as triangles connected by solid lines. Data from control animals are not shown as they remained *Bd*-negative throughout the course of the study.

exposure (25 days after second exposure). Initial exposure treatments (e.g., those applied on day zero) failed to explain variation in mortality observed throughout the entire study duration ($\chi^2 = 0.5$, $df = 2$, $P = 0.786$).

The mass mortality of wild American bullfrogs at Finley Lake that was attributed to chytridiomycosis (Clifford et al. 2012) conflicts with our experimental findings of low infection prevalence, relatively low infection load, and lack of clinical disease in American bullfrogs exposed to FL *Bd* in lab conditions. Our results agree, however, with several other studies of American bullfrog response to *Bd*. Hanselmann et al. (2004), for example, described American bullfrogs with no clinical disease despite evidence of *Bd* infection. Another study used six different *Bd* isolates over

the course of four separate exposures with maximum pathogen doses of 10^7 zoospores/day yet failed to establish 100% prevalence or clinically significant chytridiomycosis in American bullfrogs (Daszak et al. 2004). Furthermore, the only previous indication of American bullfrog susceptibility to chytridiomycosis in a laboratory setting (Gervasi et al. 2013b), which used *Bd* strain JEL 274 isolated from Colorado, is atypical in that it attributed mortality to disease at zoospore loads (<10 ZE) far below those commonly thought necessary for severe chytridiomycosis that leads to death ($\sim 10,000$ ZE) (Carey et al. 2006; Voyles et al. 2009; Vredenburg et al. 2010; Kinney et al. 2011). We note that detection of *Bd* via swabbing can be challenging, especially at low infection intensities (Shin et al. 2014), and

thus our prevalence estimates may be conservative. However, even if actual prevalence was higher than reported here, our data still suggest relatively low infection intensities overall, providing further support for the observation that low *Bd* loads seem to be typical of American bullfrogs (Greenspan et al. 2012a).

Although wild frogs collected from Finley Lake showed evidence of significant *Bd* lesions, strongly implicating chytridiomycosis as the cause of mortality (Clifford et al. 2012), it remains possible that additional unidentified factors contributed to disease progression. In another American bullfrog mass mortality event contemporaneous with *Bd* infection in the population, environmental stressors, such as temperature, were posited to have contributed to disease outbreak (Mazzoni et al. 2003). At Finley Lake, poor host body condition, low temperatures that favor *Bd* and limit host immune competence (Eskew and Todd 2013), or some unmeasured environmental factor may have served to exacerbate disease given that our experimental results suggest FL *Bd* is not especially virulent in this host. In fact, in vitro cytotoxicity tests indicate FL *Bd* is relatively benign compared to other *Bd* isolates, including CM *Bd* and SL *Bd* (Piovia-Scott et al. 2014). There also remains the possibility that multiple *Bd* strains were circulating at Finley Lake and that our isolate was not responsible for the severe mortality of American bullfrogs. Alternatively, intraspecific variation in chytridiomycosis susceptibility has been noted for other frogs (Tobler and Schmidt 2010; Savage and Zamudio 2011). The American bullfrog population at Finley Lake may therefore warrant further investigation given the potential that it is susceptible to disease for currently unknown reasons.

Finally, our results suggest that American bullfrogs have mechanisms of both resistance to and tolerance of *Bd* infection (Read et al. 2008; Schneider and Ayres 2008; Medzhitov et al. 2012). Resistance is defined as the ability to limit pathogen burden, whereas tolerance mechanisms enable an organism to limit the negative consequences of a given pathogen burden (Schneider and Ayres 2008; Medzhitov et al. 2012). Few American bullfrogs that we exposed to *Bd* became infected, and among those that did, we generally observed patterns of decreasing zoospore load over time (Fig. 1b). Both results suggest that American bullfrogs have the ability to successfully resist *Bd* infection. The relatively thick epidermis of this species, which allows for rapid skin sloughing and thus shedding of epidermal *Bd* infection, may contribute to resistance (Greenspan et al. 2012a, b). In addition, the complete lack of infection following our second exposure with SL *Bd* might be partially

attributable to primed host defenses that conferred effective resistance to these individuals, all of which had been previously exposed to *Bd* (Shaw et al. 2010; McMahan et al. 2014). However, the lack of clinical signs of chytridiomycosis in the American bullfrogs that did become infected indicates tolerance mechanisms also play a role in bullfrog–*Bd* interactions, as previous work has suggested (Hanselmann et al. 2004; Greenspan et al. 2012b).

The distinction between infection resistance and tolerance is rarely made with respect to *Bd* infection in amphibians (Venesky et al. 2014, but see Savage and Zamudio 2011; Reeder et al. 2012; Gervasi et al. 2013b; McMahan et al. 2014), yet these host defense mechanisms have profoundly different implications for disease risk within the broader amphibian community. If American bullfrogs commonly clear themselves of *Bd* infection or avoid infection altogether as a result of resistance mechanisms, then bullfrogs are less likely to be important *Bd* carriers as is often suggested (Mazzoni et al. 2003; Daszak et al. 2004; Hanselmann et al. 2004; Garner et al. 2006; Schloegel et al. 2010). In contrast, if American bullfrogs do commonly become infected with *Bd* but have mechanisms that allow them to tolerate infection or if they shed heavily infected skin tissues as in Greenspan et al. (2012a), then they may indeed contribute disproportionately to interspecific *Bd* transmission within amphibian communities.

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