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Short Communication

Experimental Evidence for American Bullfrog (*Lithobates catesbeianus*) Susceptibility to Chytrid Fungus (*Batrachochytrium dendrobatidis*)

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Abstract: The emerging fungal pathogen, *Batrachochytrium dendrobatidis* (Bd), has been associated with global amphibian population declines and extinctions. American bullfrogs (*Lithobates catesbeianus*) are widely reported to be a tolerant host and a carrier of Bd that spreads the pathogen to less tolerant hosts. Here, we examined whether bullfrogs raised from eggs to metamorphosis in outdoor mesocosms were susceptible to Bd. We experimentally exposed metamorphic juveniles to Bd in the laboratory and compared mortality rates of pathogen-exposed animals to controls (non-exposed) in two separate experiments; one using a Bd strain isolated from a Western toad and another using a strain isolated from an American bullfrog. We wanted to examine whether metamorphic bullfrogs were susceptible to either of these strains. We show that bullfrogs were susceptible to one strain of Bd and not the other. In both experiments, infection load detected in the skin decreased over time, suggesting that metamorphic bullfrogs from some populations may be inefficient long-term carriers of Bd.

Keywords: chytrid, tolerance, rana, boreas, resistance, vector

Emerging diseases are increasing globally and are a threat to biodiversity (Jones et al. 2008; Fisher et al. 2012; McCallum 2012). High virulence of some pathogens combined with a broad host-range and the presence of biotic or abiotic pathogen reservoirs (Fisher et al. 2012; McCallum 2012) can lead to population-level effects, including extinctions (Fisher et al. 2012; McCallum 2012; Telfer and Brown 2012; Best et al. 2012). Further, the presence of competent reservoir species may facilitate the long-term maintenance and spread of pathogens in ecological assemblages (Telfer and Brown 2012; Keesing et al. 2006; Keesing et al. 2010) and drive the extinction of less tolerant or less resistant species (McCallum 2012; Telfer and Brown 2012; Best et al. 2012; Reeder et al. 2012). Disease-driven loss of biodiversity is a global phenomenon, and is exemplified by the amphibian–chytrid fungus system. *Batrachochytrium dendrobatidis* (Bd), which causes the disease chytridiomycosis, infects keratinized tissues (epidermis) of metamorphic amphibians (Brutyn et al. 2012; Greenspan et al. 2012) and impairs

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osmoregulation (Voyles et al. 2009). The fungus has decimated amphibian populations worldwide (Crawford et al. 2010; Skerratt et al. 2007). Since its discovery in 1998 (Berger et al. 1998; Lips 1998), the number of studies on Bd has risen dramatically (Blaustein et al. 2011), yet, the key factors that drive emergence, spread, and maintenance of Bd are just beginning to be understood (Lips et al. 2003; Morgan et al. 2007; Briggs et al. 2010; Rohr and Raffel 2010; Vredenburg et al. 2010; Bancroft et al. 2011; Farrer et al. 2011; Savage and Zamudio 2011). Bullfrogs (*Lithobates catesbeianus*) have been widely suggested as a tolerant carrier of Bd. That is, they may harbor the pathogen without signs of morbidity or mortality (Daszak et al. 2004; Garner et al. 2006), suggesting they may be an important reservoir species. International trade in bullfrogs is implicated as a major driver of the spread of Bd, both locally and globally (Schloegel et al. 2012; but see Liu et al. 2013). If bullfrogs serve as infection-tolerant reservoir species for Bd in native and non-native habitats, it could have profound effects on disease dynamics.

American bullfrogs are native to central and eastern parts of the United States and were introduced west of their historic range for bullfrog farming in the late 1800s (Kats and Ferrer 2003) and to other regions of the world in the 1930s (Schloegel et al. 2012). In the wild, bullfrogs have tested positive for Bd (Hanselmann et al. 2004; Garner et al. 2006; Bai et al. 2012) although quantitative estimates of Bd infection load in bullfrogs are largely unknown (but see Garner et al. 2006). Infection loads in commercially farmed (and released/escaped) bullfrogs may be unusually high because of housing at high densities, which could facilitate host-to-host transmission (Rodriguez-Serna et al. 1996).

Here, we experimentally examined whether newly metamorphic bullfrogs were susceptible to Bd. We reared American bullfrogs from eggs to metamorphosis in outdoor mesocosms to ensure no previous exposure to Bd (see Supplementary Methods). In two separate experiments, we randomly assigned bullfrogs to a control or a Bd-exposed treatment. In the first experiment, bullfrogs were exposed to either Bd strain JEL 274, isolated from a Western toad (*Anaxyrus boreas*) from Colorado or sham (control) inoculate. In a second experiment, bullfrogs were exposed to Bd strain JEL 630, isolated from an American bullfrog in Oregon or sham (control) inoculate. We compared the rate of mortality between Bd-exposed and control animals *within* each experiment. We did not compare the results between the different experiments.

Experiments were run approximately 2 weeks apart in two different cohort "waves" of animals from experimental mesocosms that were similar in age since metamorphosis (Gosner 1960). Thus, we do not present or discuss comparisons of Bd-exposed animals between pathogen strains. Within both experiments, we exposed newly metamorphic animals (2–4 weeks post-metamorphosis) to Bd at a concentration of 1.7×10^4 zoospores/ml in 15 ml of total inoculate. Control animals were exposed to sham inoculate prepared identically except for the presence of the pathogen (see Supplementary Methods). We monitored survival for 30 days and quantified infection load of all Bd-exposed animals as well as a random sample of 10 control animals (5 controls within each strain experiment) using quantitative-PCR methods of Boyle et al. (2004) (See Supplementary Methods). Animals that died during the experiment were immediately preserved in 95% ethanol. Animals surviving the full experimental duration were humanely euthanized in MS-222 in accordance with approved institutional animal care and use protocol and then immediately preserved in 95% ethanol. All experimental animals were swabbed for infection just before qPCR extractions, which took place several weeks after the end of the experiment. Preservation of animals in ethanol before swabbing for infection load helped to ensure that we were sampling infection load in the skin, rather than zoospores on the skin (from experimental Petri dishes). We sampled animals for Bd infection by firmly wiping fine tipped sterile rayon swabs (Medical Wire and Equipment-MW&E 113) along the ventral surface of animals (10 full swipes down the entire left side of each animal, from armpit to toes).

We used Kaplan–Meier (or product-limit) analyses in S-plus version 8.0 for Windows to generate "survival curves" to compare animals in control versus pathogen-exposed treatments for each strain experiment (JEL 274 and JEL 630). To statistically compare survival curves, we used a Cox's proportional hazards model (which also controlled for body mass). The Cox proportional hazards model gives an overall p value (likelihood ratio test) which assesses the validity of the model, as well as p values for each factor and an associated "hazard ratio". The hazard ratio represents a comparative indicator of the risk or probability of mortality associated with a given factor (a hazard ratio >1 indicates an increase in the probability of mortality). Cox proportional hazards models were performed in *R*, statistical computing environment (version 2.9.0, Institute for Statistics and Mathematics, Vienna) with the "coxph" function and the Survival package for survival

analyses. We used a linear regression model to look at the relationship between the response variable (infection load) and time (days survived) in each experiment.

We show for the first time that bullfrogs were susceptible to JEL 274; the rate of mortality in Bd-exposed animals was significantly greater than the rate of mortality in control animals (Figure 1; Cox proportional hazards model $p < 0.001$; hazard ratio = 12.1). 13 of 17 Bd-exposed animals died. In comparison, bullfrogs were not susceptible to JEL 630; the rate of mortality in Bd-exposed animals was not different from the rate of mortality in control animals (Figure 1; Cox proportional hazards model $p = 0.440$; hazard ratio = 0.658).

Infection loads detected in the skin of bullfrogs within each experiment varied over time. In both experiments, infection load of experimental animals decreased over time (Figure 2) and days survived was a significant predictor of infection load in regression models for each strain experiment (strain JEL 274 adjusted $R^2 = 0.74$, $p < 0.0001$; strain JEL 630 adjusted $R^2 = 0.29$, $p = 0.016$). We present the raw infection load values in genome equivalents for all Bd-exposed animals for each experiment as opposed to an average quantitative infection load value (Table 1). Several pathogen-exposed animals (2 of the 17 bullfrogs exposed to JEL 274 and 3 of the 16 bullfrogs exposed to JEL 630) that survived the entire 30-day trial tested negative for infection at the end of the experiment (Table 1). Given that bullfrogs were exposed to four, weekly, inoculations of 1.7×10^4 zoospores, these results suggest that some bullfrogs either

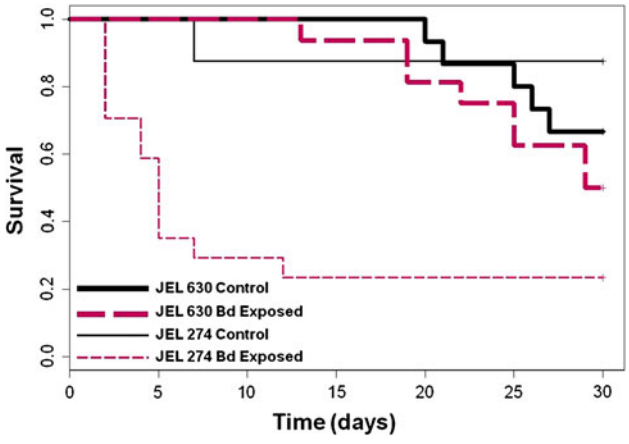

Figure 1. Survival of bullfrogs after exposure to amphibian–chytrid fungus strains JEL 274 (*thin lines*) and JEL 630 (*thick lines*). Survival was reduced in the pathogen treatment for JEL 274. No differences in survival occurred between control and pathogen-exposed animals in the JEL 630 treatment.

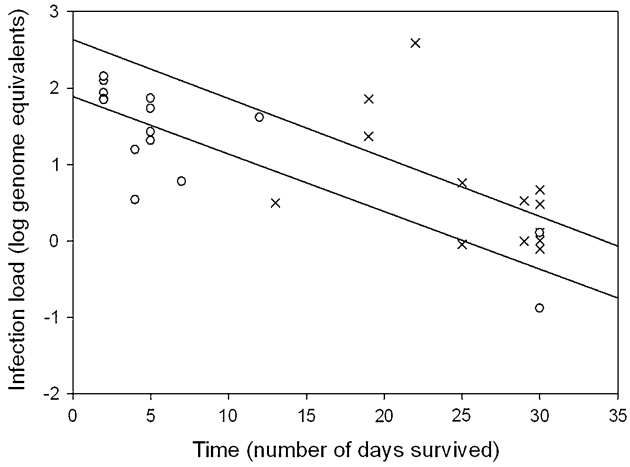

Figure 2. Linear regression lines showing the relationship between infection load and time. *Open circles* and the *lower line* represent animals exposed to JEL 274; *X marks* and the *upper line* represent animals exposed to JEL 630. For both pathogen strains, time (number of days survived) was a significant predictor of infection load. Animals that died earlier in the experiment had higher infection loads than animals that died later or animals that survived to the end of the experiment. Only infection positive animals were included in the analysis.

lose their infection or that some animals are innately resistant to infection. That is, some bullfrogs may actively decrease pathogen loads in epidermal tissue. Average infection load in bullfrogs, regardless of strain, was considerably lower than infection loads of other amphibian species reported from laboratory studies (e.g., Searle et al. 2011; Gahl et al. 2012) and in the field (Garner et al. 2006; Vredenburg et al. 2010; Reeder et al. 2012). The comparatively low infection load values in bullfrogs suggests that, at least under laboratory conditions, there may be a distinct "zoospore threshold" above which mortality occurs in this species, which is lower than the threshold observed in other amphibian species and populations exposed to Bd in field conditions (Vredenburg et al. 2010). Recent evidence shows that zoospore thresholds may be highly species specific (Gervasi et al. 2013). Our results suggest that bullfrogs may be resistant (they may be able to limit or reduce pathogen burden) but may still incur morbidity and mortality during pathogen exposure (i.e., they may not tolerate infection) (Reed et al. 2008; Raberg et al. 2009). Costs of pathogen resistance including energetic investment and collateral damage via immunopathology could contribute to the decline in host survival observed after bullfrog exposure to Bd, even at low infection loads. Rohr et al. (2010) showed that costs of pathogen exposure (in the absence of infection) may

Table 1. Quantitative infection load summaries for Bd-exposed animals exposed to JEL 274 (top) or JEL 630 (bottom).

Bd strain: JEL 274	Number of days survived	Bd-infection load (raw genome equivalents)
1	12	40.98
2	30	Negative (zero infection)
3	4	3.411
4	2	123.6
5	5	20.41
8	30	Negative (zero infection)
12	5	72.86
13	2	85.83
14	2	71.91
16	2	141.3
17	2	69.61
18	30	0.1293
20	5	53.62
23	4	15.46
25	5	26.42
29	7	5.932
32	30	1.257

Bd strain: JEL 630	Number of days survived	Bd-infection load (raw genome equivalents)
1	30	Negative (zero)
2	13	3.124
3	30	Negative (zero)
4	19	23.42
5	30	0.7758
6	30	Negative (zero)
9	25	0.8976
14	25	5.738
15	29	3.330
19	19	71.74
23	29	0.9843
24	22	388.7
25	30	1.287
26	30	0.9988
28	30	2.991
31	30	4.622

also have important fitness consequences for hosts. This concept is especially relevant in the amphibian–Bd system given recent evidence that Bd releases a compound that can cause rapid host mortality in the absence of infection (McMahon et al. 2013), which likely explains similar phenomena reported previously (Blaustein et al. 2005; Searle et al. 2011).

Our results show that American bullfrogs were susceptible to one strain of Bd but not to another. Thus, in some populations, depending upon the Bd strain they are exposed to, bullfrogs may be inefficient carriers or reservoir species for Bd. Since bullfrogs were susceptible to at least one strain of Bd, they may be susceptible to others. However, in other populations, bullfrogs may persist with infection that can be disseminated to less tolerant amphibian species. Our data support these conclusions for post-metamorphic bullfrogs, but further research is needed to determine if these results hold for adult bullfrogs.

Our results show that American bullfrog exhibit differential sensitivity to Bd. Differences in host–pathogen co-evolution with certain strains of Bd could explain consistently cited innocuous effects of pathogen exposure and infection in this species. For example, genotypic differences among pathogen strains could account for differences in virulence (Schloegel et al. 2012) and other pathogen characteristics (e.g., reproductive rate and zoospore size) and thus, alter host–pathogen interactions (Rosenblum et al. 2008, 2012; Fisher et al. 2009). In addition to mortality we observed during exposure to JEL 274, we also observed low infection loads and a decrease in infection load, over time (Figure 2). There is a lack of data on quantitative infection load in bullfrogs across the globe. At least one study has highlighted the vast differences in infection load in populations of bullfrogs in different regions of the world (Garner et al. 2006). Our experimental results have implications for the general belief that bullfrogs maintain high infection with Bd without signs of morbidity or mortality. Across ecological systems there may be variation in which species represent the most relevant reservoir species for this pathogen (Reeder et al. 2012). While adult bullfrogs may be important carriers of Bd in some systems, alternative hosts may be just as or more important for the maintenance and spread of this pathogen (Reeder et al. 2012; Gervasi et al. 2013; McMahon et al. 2013). Further, recent evidence suggests that there are important non-amphibian carriers of Bd including crayfish (McMahon et al. 2013) and that international trade of both amphibian and non-amphibian carriers could drive the global pathogen distribution (Liu et al. 2013). This study underscores how experimental studies may help us understand the often complex and context-dependent dynamics of host–pathogen systems.

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