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Batrachochytrium dendrobatidis (*Bd*) exposure damages gill tissue and inhibits crayfish respiration

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ABSTRACT: *Batrachochytrium dendrobatidis* (*Bd*) is a pathogenic fungus known to infect amphibians and crayfish. In crayfish, *Bd* causes gill tissue damage, and in some cases, mortality. Most research has focused on the amphibian–*Bd* system, so to date, little is known about the effects of *Bd* on the crayfish host. Here, we studied the effects of sublethal exposure to *Bd* and the metabolites produced by *Bd* on crayfish *Procambarus alleni* survival, gill damage, and oxygen consumption (as a proxy for mass-specific metabolic rate). Oxygen consumption increased 24 h post-exposure to live *Bd*, indicative of a stress response, followed by a decrease in oxygen consumption over time ($\chi^2_1 = 6.39$, $p = 0.012$). There was no difference in response when comparing the crayfish exposure to live *Bd* and *Bd*-metabolites alone ($\chi^2_1 = 2.70$, $p = 0.101$), indicating that the metabolites may have been the causative agent responsible for the response. Additionally, oxygen consumption decreased with gill damage (tissue recession) in *Bd*-exposed individuals. We found that high doses of *Bd* cause outright mortality in crayfish, and we show here that sublethal *Bd*-induced inhibition of oxygen consumption could negatively impact crayfish in the field, possibly reducing their overall fitness. More research is needed to understand this understudied host–parasite system. It is essential that we incorporate the disease dynamics associated with *Bd* and crayfish in conservation disease models, as this is the only way to develop comprehensive community-based models.

KEY WORDS: Chytrid fungus · Metabolic rate · *Bd* metabolites · Gill damage · Alternative host · Ecophysiology · Oxygen consumption

1. INTRODUCTION

Batrachochytrium dendrobatidis (*Bd*) is a highly contagious, pathogenic fungus responsible for the extinction and extirpation of hundreds of amphibian species worldwide (Scheele et al. 2019). Given widespread catastrophic declines in amphibian populations, most research in this field has focused on the amphibian–*Bd* system. Amphibians, however, are not the only organisms that host *Bd*; freshwater crayfish, for example, are hosts that can support *Bd* growth in their gastrointestinal tracts (McMahon et al. 2013, Brannelly et al. 2015, Betancourt-Román et al. 2016, Oficialdegui et al. 2019), and they may even be reservoir hosts for the pathogen. We have only just begun investigating the crayfish–*Bd* system. Currently, we know that up to 20% of crayfish in the field in the USA may be *Bd*-positive, crayfish can maintain *Bd* long-term in the lab, and they can vector infectious zoospores to susceptible co-occurring tadpoles (McMahon et al. 2013, Brannelly et al. 2015). Additionally, in the field, crayfish can be exposed to *Bd* directly (contact with infectious zoospores) and indirectly (contact with the metabolites *Bd* produces and releases into the water), but more research is needed to understand the impacts of both direct and indirect exposure to *Bd* on the reservoir host itself.

Bd produces chemical metabolites that presumably allow the fungus to burrow into host tissue during infection (Symonds et al. 2008). While we do not know the exact composition of these metabolites, we know that some are immunomodulators and can degrade

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tissue (Rollins-Smith et al. 2015, 2019), and that they can alter tadpole development (McMahon et al. 2019). Exposure to either live *Bd* or *Bd*-metabolites alone caused gill damage (gill tissue recession) and, in many cases, rapid mortality in crayfish (McMahon et al. 2013). We might expect that gills damaged by exposure to an aqueous chemical (e.g. *Bd*-metabolites) result in reduced oxygen consumption since crayfish pass water across their gills and utilize diffusion for respiration. If extreme enough, this decreased oxygen consumption may lead to rapid mortality.

Crayfish are vitally important to freshwater ecosystems as they can act as ecosystem engineers. Crayfish can increase detrital breakdown rates (Schofield et al. 2001, Usio & Towsend 2001), alter sediment transport and accumulation (Statzner & Fievet 2000), and create new physical habitats for other dependent taxa, such as crayfish frogs, which are obligate burrow users (Heemeyer et al. 2012). Additionally, crayfish are a key element to many freshwater food webs, acting as both consumers (Lodge et al. 2000, Usio & Towsend 2001) and as prey (Wolff et al. 2015). Crayfish are also important economically, as they are globally traded as fish bait and food for human consumption; the total annual commercial harvest of crayfish exceeds 100 000 t (Huner 1988). Consequently, the global trade and movement of crayfish also make them potential vectors for the spread of Bd (Oficialdegui et al. 2019).

Here, we address how sublethal exposure to live *Bd* and *Bd*-metabolites affects *Procambarus alleni* gill tissue, mortality, and oxygen consumption. We explore the link between *Bd* exposure and crayfish oxygen consumption, which is a first step in determining how this devastating pathogen may be impacting this understudied host. *Bd* has non-amphibian hosts, and it is imperative to know more about how both direct and indirect exposure to *Bd* alters their physiology. Researchers are developing comprehensive disease models with the hope of finding a management plan that would ameliorate some of the damage associated with this panzootic. However, we must incorporate the impacts of *Bd* on non-amphibian hosts to develop robust and effective management strategies.

2. MATERIALS AND METHODS

2.1. Animal husbandry

Adult *Procambarus alleni* were collected from 3 sites in Tampa, Hillsborough County, Florida, USA. All crayfish were housed individually in 500 ml of artificial spring water (ASW; Cohen et al. 1980) and were maintained at 23°C on a 12:12 h light:dark cycle. Crayfish were fed organic spinach ad libitum. Experiments lasted ≤6 d, so no water changes were needed.

2.2. *Bd* treatment preparation and exposure

We cultured *Bd* (Panamanian strain JEL 419) on 1% tryptone plates at 18°C for 7 d. Prior to crayfish inoculation, we prepared all *Bd* treatments from these stock cultures. We flooded the *Bd*-positive (*Bd*+) plates with ASW, suspending the zoospores for 15 min, and combined zoospores from all plates into one *Bd*+ stock. A hemocytometer was used to determine zoospore concentration, and ASW was used to dilute the *Bd*+ stock to the target concentration (high *Bd*: 1×10^6 zoospores ml^{-1} or low *Bd*: 1×10^4 zoospores ml^{-1}). The ASW control was created by flooding *Bd*-negative (*Bd*–) 1% tryptone plates with ASW. The *Bd*-metabolite treatment was created by filtering part of the low *Bd* stock through a 1.2 µm filter (GE Whatman Laboratory Products), removing the fungus and leaving behind only the metabolites the fungus produced. We plated 1 ml of this filtrate on 1% tryptone plates and monitored growth for 1 wk (n = 3 plates); there was no *Bd* growth. Animals were exposed to 3 ml of their respective treatments (high dose: 3×10^6 zoospores and low dose: 3×10^4 zoospores total) by pipetting the inoculate directly into their water (see individual experimental sections for duration details).

2.3. Crayfish mortality experiment

We wanted to track the effects of treatment on crayfish over multiple days, and therefore did not want to use a lethal treatment concentration. We conducted a mortality experiment to ensure the low *Bd* concentration selected could be used without inducing mortality. Crayfish were maintained individually in 500 ml of ASW and were exposed to a high *Bd*, low *Bd*, or ASW control treatments (n = 10, 10, and 7, respectively) once at the beginning of the experiment (the concentrations were selected based on the findings of McMahon et al. 2013). Mortality was monitored daily over 3 d. There was no mortality in the low *Bd* treatment group, and given that our goal was to track respiration over time, we used that treatment for all other experiments.

2.4. Overview of oxygen consumption measurements for all experiments

We used the following methodology to measure oxygen consumption for all 3 experiments (see the individual experimental sections for sample size, treatment, and experimental duration details). We used a repeated measures design and measured oxygen consumption (as a proxy for mass-specific metabolic rate; mg l^{-1} h^{-1} g^{-1}) in each crayfish before treatment exposure, as a control for individual variation, and then every 24 h after treatment exposure (see Chang & Lucy Hou 2005, Martins et al. 2011, Norin et al. 2016 for similar repeated measure design experiments). The animals were not fed 24 h prior to the beginning of the trials to ensure a fasted state (McFeeters et al. 2011). Crayfish were placed individually in a round, plastic respiration chamber (10.5 cm in diameter, 5.5 cm in height) filled with 236 ml of ASW at 23°C. A Vernier optical dissolved oxygen probe connected to a Labquest (Vernier) was inserted through an airtight port, and the respiration chamber was covered and left undisturbed for 10 min to ensure animals were rested (Carey et al. 2016, Bonachea 2021; both used this oxygen probe). After 10 min, oxygen levels in the container were recorded every 10 s for 30 min. Animals were weighed, and the rates of oxygen consumption were normalized to body mass, resulting in mass-specific metabolic rate (presented in mg l^{-1} h^{-1} g^{-1}).

2.5. Experimental design control

To determine if there was an effect of experimental procedure itself on crayfish oxygen consumption over time, we ran a control experiment where we tracked oxygen consumption using the exact same equipment described above. We used the same experimental procedure and tracked oxygen consumption over 6 d in crayfish exposed to ASW (n = 10). We could not run the control and exposure experiments simultaneously due to equipment limitations. Additionally, we screened these crayfish for gill recession (see Section 2.6). We found no effect of the experimental conditions on oxygen consumption over the 6 d period (see Section 3.1). However, we did find individual variation amongst the control crayfish, and therefore, to control for this individual variation, we used the repeated measures design. With this design, we controlled for individual variation among crayfish within each of our statistical analyses and did not include additional untreated crayfish in each experiment.

2.6. Live *Bd* oxygen consumption experiment

To determine if there was an effect of live *Bd* exposure on oxygen consumption over time and whether that was correlated with gill damage, we used a repeated measures design and dosed crayfish (n = 10) with the live low *Bd* stock and tracked oxygen consumption over 6 d (following the above methods). We assessed gill damage at the end of that 6 d exposure period following the methodology described by McMahon et al. (2013). Briefly, crayfish were pithed and frozen prior to the extraction of their gills, a process shown to not further damage the gills (McMahon et al. 2013). Gills from each crayfish were removed and photographed using a compound light microscope. Double-blind measurements were taken using ImageJ (Schneider et al. 2012) to determine the distance from the tip of the external gill cuticle to the hemolymph and gill tissue (n = 5 randomly selected gills per crayfish and 3 measurements per gill). We removed large sections of the gills to ensure that the removal process did not impact the section of the gills we measured.

2.7. *Bd*-metabolite oxygen consumption experiment

To determine if the response seen in the previous experiment (Section 2.6) was due to exposure to live *Bd* or just *Bd*-metabolites, we conducted a follow-up experiment in which we examined the impact of *Bd*-metabolites alone on oxygen consumption 24 h after exposure. Using the same repeated measures design, we dosed crayfish (n = 15) with the *Bd*-metabolite stock and recorded their oxygen consumption before exposure and 24 h after exposure.

2.8. Statistical analysis

All statistical analyses were conducted in R statistical software (R Development Core Team 2020), and the statistics for each experiment were analyzed separately, except where specified. We used a Cox-proportional hazards regression (package: 'survival', function: 'coxph') to determine if there was an effect of treatment on survival. We used a general linear model (package: 'stats', function: 'glm') to determine if there was an effect of experimental proce-

dure in the absence of any exposure treatment (see Section 2.5) on oxygen consumption over time. For all other analyses, we used a linear mixed-effects model (package: 'nlme', function: 'lme'), and we controlled for individual variation by nesting the pre-exposure oxygen consumption of each individual within the model. We used this linear mixed-effects nested design to determine if there was an effect of time after treatment exposure on oxygen consumption, and to determine if there was an acute response to exposure by testing if there was an effect of treatment (low *Bd* and *Bd*-metabolites) on the change in oxygen consumption between pre-exposure and 24 h post-exposure. Additionally, for both the control and live *Bd* oxygen consumption experiments, we used the same linear mixed-effects nested design described above to determine whether there was an effect of gill recession on oxygen consumption.

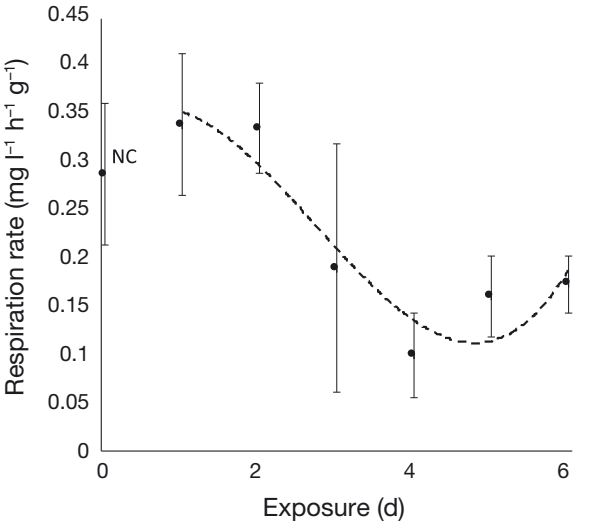

Fig. 1. Effect of exposure to live *Batrachochytrium dendrobatidis* on respiration rate of crayfish. Dashed line is a third-order polynomial fit: $R^2 = 0.879$. NC (negative control; n = 7) is the mean respiration rate of all crayfish pre-exposure. Data are mean ± SE

3. RESULTS

3.1. Experimental design control

There was no effect of experimental procedure itself on crayfish respiration over time ($\chi^2_1 = 2.64$, p = 0.104), and there was no relationship between gill recession and respiration in crayfish exposed to ASW ($\chi^2_1 = 0.17$, p = 0.68).

3.2. Crayfish mortality experiment

There was an effect of *Bd* concentration on crayfish mortality ($\chi^2_1 = 14.0$, p = 0.001). The high *Bd* treatment caused 60% mortality, but there was no mortality in the low *Bd* and ASW treatments. In fact, the low *Bd* and ASW treatments did not cause mortality in any of the following experiments.

3.3. Live *Bd* and *Bd*-metabolite oxygen consumption experiments

Crayfish exposed to both live *Bd* and *Bd*-metabolites had higher oxygen consumption 24 h after exposure than they did prior to exposure (low *Bd*: $\chi^2_1 = 5.50$, p = 0.019; *Bd*-metabolites: $\chi^2_1 = 7.08$, p = 0.008; Figs. 1 & 2). Further, the magnitude of increase in oxygen consumption 24 h after exposure was similar between live *Bd* and *Bd*-metabolite exposed crayfish ($\chi^2_1 = 2.70$, p = 0.101; Fig. 2). The effect sizes for the

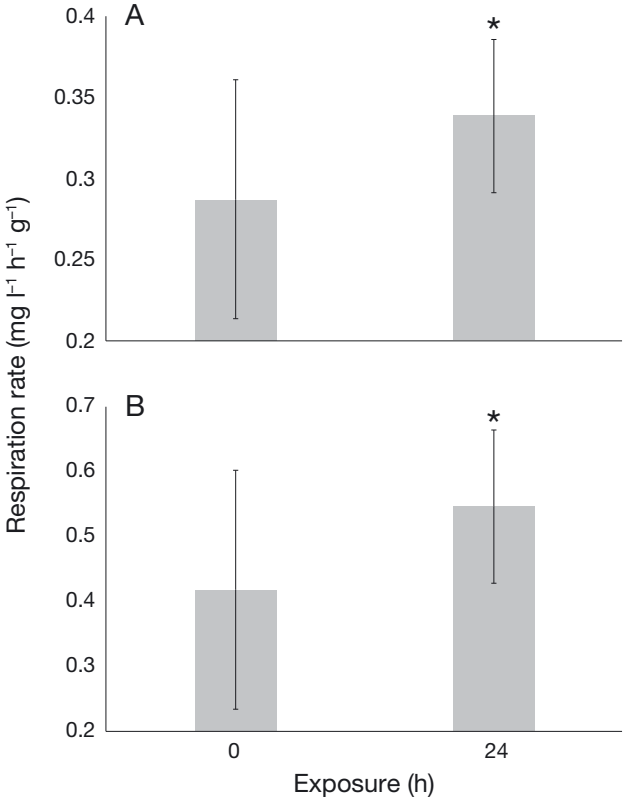

Fig. 2. Crayfish exposed to (A) live *Batrachochytrium dendrobatidis* (*Bd*) and (B) *Bd*-metabolites alone had increased oxygen consumption, but there was no difference in the magnitude of increase between the 2 treatments ($\chi^2_1 = 2.70$, p = 0.101). Data are mean ± SE; *significance (p < 0.05); n = 10 and 15 for live *Bd* and *Bd*-metabolites, respectively

live *Bd* and *Bd*-metabolites comparing pre- and 24 h post-exposures were 0.35 and 0.39, respectively. After this initial increase, the oxygen consumption decreased over the 6 d following live *Bd* exposure (χ^2_1 = 6.39, p = 0.012; Fig. 1).

3.4. Crayfish gill damage from the live *Bd* oxygen consumption experiment

There was a negative relationship between the percent change in oxygen consumption (calculated by comparing the within individual pre-*Bd* exposure values to the 6 d post-*Bd* exposure values) and gill recession in crayfish after being exposed to the low *Bd* treatment for 6 d (χ^2_1 = 7.47, p = 0.006; Figs. 3 & 4). In other words, the higher the gill recession, the higher the reduction in oxygen consumption in the 6 d post *Bd*-exposure measurements.

4. DISCUSSION

Crayfish gills allow for the diffusion of oxygen, carbon dioxide, electrolytes, and ammonia waste across the surface of the gill cuticle, which are all vital functions for survival and homeostasis (Dickson et al. 1991). We found that gill tissue receded from the gill cuticle in animals exposed to *Bd* (Fig. 4) and that this *Bd* exposure-induced gill damage inhibits oxygen consumption over time, even at sublethal concentrations. It is likely that the gill tissue recession is the causative process leading to the reduced oxygen consumption in crayfish exposed to Bd (Fig. 3). Indeed, these findings help explain the link between *Bd*- and *Bd*-metabolite-induced gill damage and reduced survival found previously in crayfish (McMahon et al. 2013). The changes in gill structure, described as recessed gills (Fig. 4), would reduce oxygen diffusion and therefore biosynthesis of ATP via aerobic respiration. Furthermore, a reduced ability to respire may explain the rapid mortality seen in the crayfish exposed to high *Bd* (see Section 3.2) and in previously published crayfish–*Bd* work (McMahon et al. 2013).

Crayfish exposed to *Bd* and *Bd*-metabolites alone experienced increased oxygen consumption 24 h

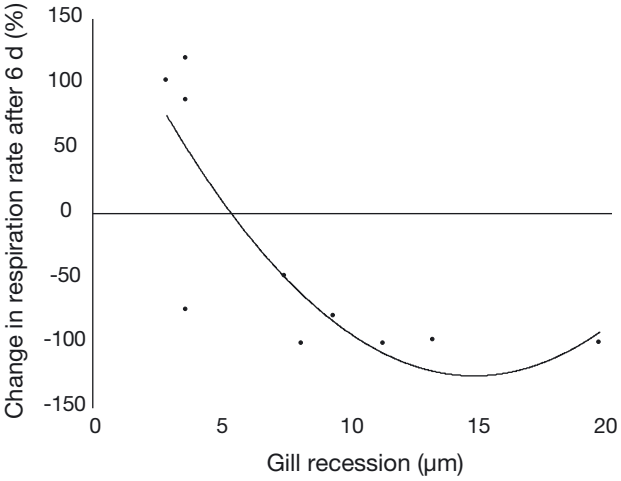

Fig. 3. Negative relationship between the percent change in respiration rate (mg l^{-1} h^{-1} g^{-1}) pre-exposure and 6 d post-exposure, and gill recession in crayfish (n = 10) after being exposed to a sublethal dose of live *Batrachochytrium dendrobatidis* for 6 d (χ^2_1 = 7.47, p = 0.006). Each dot represents an individual crayfish. Solid line is the polynomial fit trendline: R^2 = 0.684

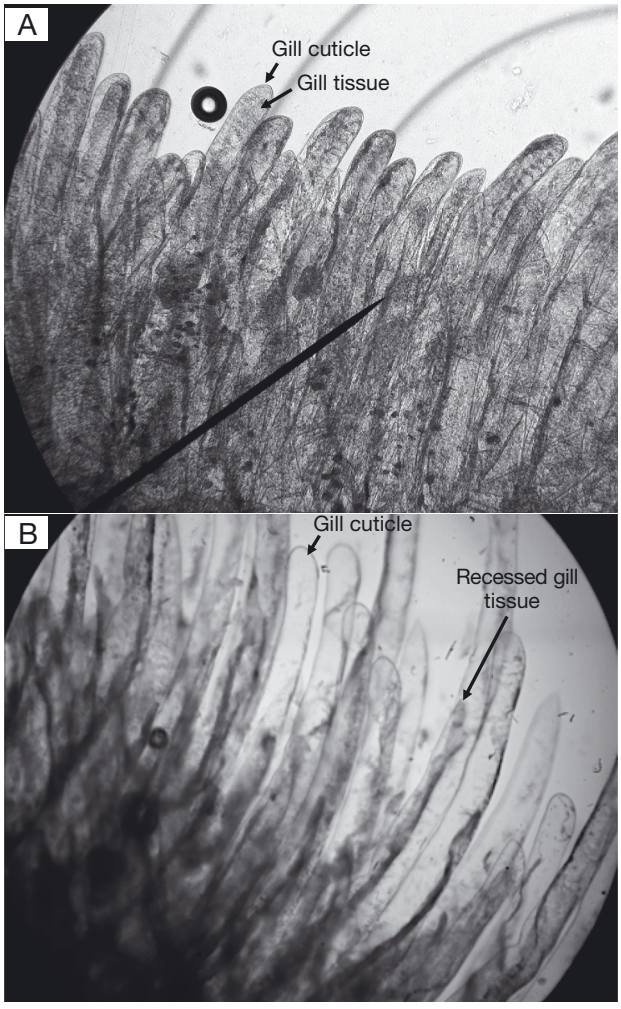

Fig. 4. Light compound microscope image of crayfish gills exposed to (A) only artificial spring water or (B) a low concentration (1×10^4 zoospores ml^{-1}) of live *Batrachochytrium dendrobatidis* for 6 d. The gill tissue seen here has recessed from the gill cuticle, which is associated with reduced metabolic rate

after exposure (Figs. 1 & 2). Given that we found a similar response in crayfish exposed to both treatments, it is possible that this initial response is due to exposure to *Bd*-metabolites, which is the commonality between the treatments (Fig. 2). Importantly, we did not confirm *Bd* infection and cannot say definitively whether the physiological responses are due to an active *Bd* infection, exposure to live *Bd*, or exposure to the metabolites *Bd* produces. However, these data strongly imply that the *Bd*-metabolites are the biologically active components responsible for the negative effects on host anatomy and physiology. Indeed, McMahon et al. (2013) also found that crayfish exposed to *Bd*-metabolites in the absence of the live zoospores had gill recession as well.

The increase in oxygen consumption seen 24 h after *Bd* or *Bd*-metabolite exposure is potentially indicative of a physiological stress response. Robust immune responses are supported by an increased metabolism in vertebrates (Ganeshan & Chawla 2014), and immunological work on crayfish has revealed functional similarities between crayfish immune factors and the vertebrate complement system (summarized by Cerenius & Söderhäll 2018). It is therefore plausible that the acute increase in oxygen consumption seen in response to *Bd* or *Bd*-metabolite exposure (Fig. 1) reflects a mounted immunological response. However, this stress response may be insufficient and may not prevent infection or reduce damage from *Bd* or *Bd*-metabolite exposure, which may lead to a rapidly declining metabolic rate (as seen in Fig. 1). Further, extended exposure to *Bd* or *Bd*-metabolites can yield tissue damage (see Figs. 3 & 4 and McMahon et al. 2013). The negative correlation between gill recession and the final measurement of oxygen consumption (Fig. 3) may have demonstrated a reduction in respiratory efficiency. This would indicate that *Bd*-exposed animals were unable to sustain the increased energetic demand required for a prolonged immunological defense.

Chytrid disease models have traditionally focused on the *Bd*–amphibian host system because amphibians have seen the most obvious declines. Crayfish are exposed to and infected with *Bd* in the wild (McMahon et al. 2013, Brannelly et al. 2015, Oficialdegui et al. 2019), and *Bd* can cause mortality in crayfish. Unfortunately, many crayfish populations are invasive, and therefore, not well monitored, which means we do not have strong field data on their populations and may have missed important field dynamics. It is essential that the dynamics of this host–parasite (crayfish–*Bd*) system are better understood and accounted for to create effective and robust predictive disease models. In order to obtain a holistic understanding of the impacts of *Bd*, arguably one of the most devastating pathogens on earth in terms of the number of global extinctions and extirpations (Scheele et al. 2019), more research is needed on the impacts of *Bd* on non-amphibian hosts and carriers.

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