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NOTE

Metabolites produced by *Batrachochytrium dendrobatidis* alter development in tadpoles, but not growth or mortality

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ABSTRACT: The mass decline of amphibian populations poses a serious threat to global biodiversity and ecosystem stability. The pathogenic fungus *Batrachochytrium dendrobatidis* (*Bd*) has contributed to the extirpation and extinction of hundreds of amphibian species worldwide. *Bd* produces potentially damaging metabolites during the host infection process that may affect amphibian growth and development, even in the absence of infection. In this experiment, Cuban tree frog *Osteopilus septentrionalis* tadpoles and adults were exposed once to either artificial spring water (ASW) or *Bd* metabolites (n = 31 tadpoles per treatment and n = 20 and 30 adults per treatment, respectively). Tadpoles exposed to *Bd* metabolites alone developed faster than those exposed to ASW; however, there was no difference in tadpole length, weight change, or mortality between treatments. Despite the faster developmental speed, metabolite exposure did not reduce tadpole weight or length (compared at Gosner stages 27, 29, and 31). There was no effect of treatment on adult size or mortality. These results indicate that both tadpole and adult *O. septentrionalis* do not appear to be negatively impacted by exposure to non-infectious *Bd*-contaminated water. In fact, tadpoles developed faster when exposed to metabolites and were of equal size as those in their stage cohort, implying a potential long-term benefit if faster development allows them to leave *Bd*-infected waters sooner.

KEY WORDS: Chytrid fungus · *Batrachochytrium dendrobatidis* · Amphibian decline · Tadpole · Developmental plasticity · *Bd* metabolites

1. INTRODUCTION

Amphibians are the most threatened vertebrate taxon on the planet and have experienced massive global declines (Stuart et al. 2004). These declines pose a serious threat to biodiversity, ecosystem health, and stability worldwide. Despite the attention paid to this conservation crisis, many questions remain to be answered, e.g. the effects of indirect exposure to emerging pathogens, that may contribute to ecological models being developed to inform conservation management plans.

Batrachochytrium dendrobatidis (*Bd*), a unique pathogenic fungus, has contributed to hundreds of amphibian extirpation and extinction events globally (Stuart et al. 2004). *Bd* infects the keratinized tissue of both tadpoles and adults (Berger et al. 1998), but typically, tadpoles younger than Gosner stage 38 (Gosner 1960) carry lower infection loads compared to metamorphs and adults (McMahon & Rohr 2015). *Bd* does not usually cause mortality in tadpoles (Berger et al. 1998, Garner et al. 2009), but several papers have shown that *Bd* exposure or infections can alter tadpole behavior, growth, and development (Venesky et al. 2009, 2011, Romansic et al. 2011).

Bd zoospores produce metabolites, which presumably help them burrow into host tissue (Symonds

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et al. 2008). *Bd* produces several metabolites, including methylthioadenosine, tryptophan, and polyamine spermidine, which are known immunomodulators (Rollins-Smith et al. 2015, 2019), and appears to adapt metabolite production based on the local environment (Rollins-Smith et al. 2015). The identification of these metabolites is just the beginning; currently there is still a dearth of information about how these chemicals directly impact the host. We know that the impacts can be quite severe; for example, exposure to *Bd* metabolites damages crayfish gills, causing mortality even in the absence of infection (McMahon et al. 2013a). It is important to understand how these metabolites might impact other organisms exposed to *Bd* metabolites in the absence of *Bd* infection.

Here, we investigated how the metabolites produced by *Bd* (referring to the chemicals produced by *Bd* found in the filtered supernatant) affect 2 life stages of amphibians (tadpole and adult Cuban tree frogs *Osteopilus septentrionalis*) in the absence of infection. These 2 life stages have very different natural histories (e.g. terrestrial vs. aquatic), and therefore are likely to have different responses to *Bd*-metabolite exposure. Furthermore, tadpoles exposed to *Bd* metabolites but not infected with *Bd* itself may have altered development, which may affect their overall fitness.

2. MATERIALS AND METHODS

2.1. Tadpole exposure experiment

Cuban tree frog tadpoles were collected from 5 different ponds in Tampa, Florida, USA (at least 5 different clutches of tadpoles were used). They were housed individually in 500 ml of artificial spring water (ASW) in a 1 l plastic cup until the start of the experiment. Tadpoles were fed organic spinach ad libitum and were maintained at 22°C. This experiment was conducted in 2 temporal blocks (based on tadpole availability), and the same methods were used for both blocks.

Each tadpole (Gosner stage 25–29; Gosner 1960) was transferred to 40 ml of ASW and was exposed once to 3 ml of treatment inoculate (*Bd* metabolites in ASW, or just ASW; see Section 2.3 for treatment descriptions; n = 31 total tadpoles per treatment). Tadpoles were randomly assigned to treatments, and there were no differences among treatments in tadpole stage or size at the start of the experiment (stage: $\chi^2_1 = 3.04$, p = 0.08; length: $\chi^2_1 = 0.17$, p = 0.68; mass: $\chi^2_1 = 1.11$, p = 0.29). The tanks were not redosed after the first exposure, and a 30% water change was conducted weekly. To track growth, we recorded length (mouth to tip of tail) and body mass, and to track development, we recorded Gosner stage (to have a standardized comparison of development). These metrics were monitored weekly for 5 wk, and mortality was recorded daily.

2.2. Adult exposure experiment

Adult Cuban tree frogs were collected from Hillsborough County, Florida, and were housed individually in 1 l plastic cups. All frogs were fed vitamin- and calcium-dusted crickets ad libitum, and bedding and containers were changed weekly. Frogs were randomly assigned to treatments. There were no differences in frog mass at the start of the experiment ($\chi^2_1 = 0.66$, p = 0.42), and they were considered young adults that were not fully grown.

Each frog was dosed with 3 ml of treatment inoculate (*Bd* metabolites in ASW, or just ASW; see Section 2.3 for treatment descriptions; n = 30 or 20 frogs per treatment, respectively), directly on their dorsal side, and the extra liquid was allowed to pool in the bottom of their container. Frogs were exposed to the treatment inoculate every 2 d for 5 wk (we followed the inoculation techniques described by McMahon et al. 2014). The animals were maintained at 22°C and were weighed at the beginning and end of the experiment.

2.3. Bd culture and inoculation

The *Bd* inoculate was prepared by growing *Bd* (California strain: JEL 270) on 1% tryptone agar plates for 10 d at 22°C. Each plate was flooded with ASW to suspend the zoospores, and the suspensions from all plates were homogenized. Initial *Bd* stock concentration was determined using a hemocytometer and was then diluted with ASW to reach a final concentration of 9.6×10^6 zoospores ml^{-1}. The *Bd* metabolite inoculate was created by filtering the initial *Bd* stock through a 1.2 µm filter (GE Whatman Laboratory Products) to remove all of the infectious *Bd* from the solution, leaving behind only the metabolites produced by *Bd*. A hemocytometer was used to visually verify that there was no remaining *Bd* in the *Bd* metabolite inoculate. The ASW control treatment was created by flooding a *Bd*-free 1% tryptone plate with ASW.

The study animals were not exposed to live *Bd* because this would not have yielded new information in the field, and because exposing adult frogs to live

Bd for 5 wk would result in high mortality (Raffel et al. 2013). Instead, the inoculations aimed to determine whether the *Bd* metabolites themselves impacted the amphibians.

2.4. Statistical analysis

All statistical analyses were conducted in R statistical software (R Development Core Team 2013). For both experiments, we tested for the effect of treatment on survival using a Cox-proportional hazards regression (package: 'survival;' function: 'coxph'). For the adults, a general linear model (package: 'stats;' function: 'glm') was used to determine if there was an effect of treatment on growth. For the tadpole experiment, there was no effect of block on any of the treatments with a general linear model ($p > 0.05$ for all). Therefore, both temporal blocks were analyzed and presented together for all other statistical tests. A general linear model was used (package: 'stats;' function: 'glm') to determine if there was an effect of treatment on tadpole growth (length and weight change) or development (stage change), and a general linear model (package: 'stats;' function: 'glm') was used to determine if there was an effect of treatment on length and weight at Gosner stages 27, 29, and 31. These stages were chosen because they occurred before any individuals began to metamorphose and because the sample size at each of these stages was appropriate for sufficient statistical power.

3. RESULTS

Tadpoles exposed to *Bd* metabolites developed faster than those exposed to ASW ($\chi^2_1 = 18.15$, $p < 0.001$; Fig. 1); however on Day 28, there was no difference in average tadpole stage between the treatments ($\chi^2_1 = 0.56$, $p = 0.45$). Additionally, there was no difference in tadpole length or weight within each stage (for stages 27, 29, and 31, respectively; length: $\chi^2_1 = 0.97$, $p = 0.32$, $\chi^2_1 = 0.316$, $p = 0.58$, $\chi^2_1 = 0.43$, $p = 0.51$; weight: $\chi^2_1 = 0.61$, $p = 0.43$, $\chi^2_1 = 0.2.4$, $p = 0.12$, $\chi^2_1 = 0.14$, $p = 0.71$). There was also no difference in tadpole length change, weight change, or mortality between treatments (length or weight change: $\chi^2_1 = 0.29$, $p = 0.58$, $\chi^2_1 = 0.68$, $p = 0.41$, respectively). The only factor that influenced tadpole stage on Day 28 was tadpole length (treatment: $\chi^2_1 = 1.08$, $p = 0.30$, length: $\chi^2_1 = 10.89$, $p = 0.001$, and treatment × length: $\chi^2_1 = 1.94$, $p = 0.16$); generally, larger tadpoles were later-stage tadpoles (Fig. 2).

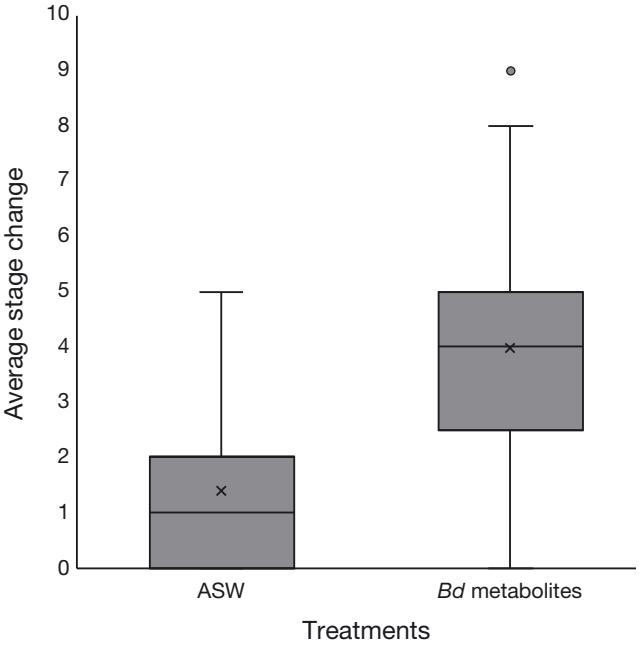

Fig. 1. Cuban tree frog *Osteopilus septentrionalis* tadpoles exposed to artificial spring water (ASW) developed significantly slower than those exposed to *Batrachochytrium dendrobatidis* (*Bd*) metabolites ($\chi^2_1 = 18.15$, $p < 0.001$). Average stage change is the average number of Gosner stages through which each tadpole transitioned during the experimental period. ×: mean; bar: median; box: interquartile range (IQR); whiskers: min./max. values <1.5× IQR below/above box, respectively; dot: outlier

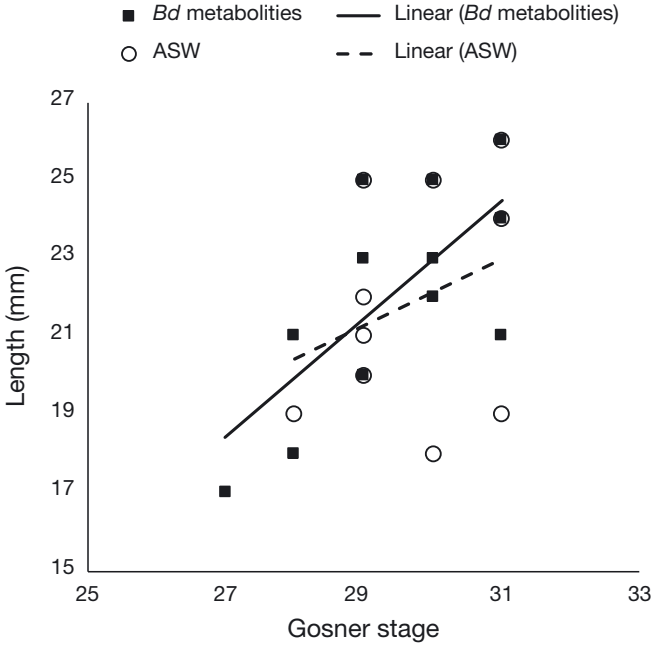

Fig. 2. Cuban tree frog *Osteopilus septentrionalis* tadpole length at each Gosner stage (ASW alone, versus exposure to *Batrachochytrium dendrobatidis* (*Bd*) metabolites). There was no significant difference between treatments.

There was no effect of treatment on adult growth or mortality ($\chi^2_1 = 0.11$, p = 0.74, $\chi^2_1 = 0.05$, p = 0.83, respectively). On average, the adults in both the *Bd* metabolite and ASW treatments gained weight during the experiment (mean ± SEM: 8.6 ± 2.71% and 7.3 ± 3.05% mass change, respectively).

4. DISCUSSION

Given the aquatic and semi-aquatic nature of many amphibians, exposure to the metabolites of *Bd* may be inevitable, as an individual's entire body may be in contact with the water. Despite the damaging effects the live *Bd* and metabolites had on crayfish gills (McMahon et al. 2013a), there was no effect of metabolites on mortality of Cuban tree frog adults or tadpoles (see McMahon et al. 2013b for similar mortality findings) or on their growth. These findings are promising, considering that *Bd* metabolites were a component of the inoculation used to induce acquired resistance to *Bd* (McMahon et al. 2014). Exposure of adults to the noninfectious fungal vaccine (e.g. dead *Bd* or *Bd* metabolites; McMahon et al. 2014) could become an important conservation tool in combating amphibian declines and facilitating reintroductions.

Here, we found that tadpoles exposed to *Bd* metabolites developed faster but were the same size when compared to others within the same stage cohort exposed to ASW. In fact, when we compared tadpole sizes (weight and length) within developmental stages, there was no effect of treatment. Because these metabolite-exposed tadpoles developed faster but had equal growth, it is possible that exposure without infection may actually benefit the tadpoles as it cues them to leave the water faster.

Tadpoles are able to use chemical cues in the water to evade danger (e.g. predators or deleterious chemicals; e.g. Kiesecker et al. 1996, Takahara et al. 2012). Tadpoles display developmental plasticity and can increase their developmental speed, despite lack of additional resources, when developing in an unsuitable or stressful environment (e.g. Newman 1988, Bridges 2002, Rohr et al. 2004). By shortening the time to metamorphosis, tadpoles can escape adverse environments (e.g. the presence of a predator, detrimental chemicals, or reduced water). The *Bd*-metabolite-exposed tadpoles may develop faster in an attempt to escape the pathogen, as the metabolites are used during pathogen infection and may be deleterious to the tadpoles (Rollins-Smith et al. 2015, 2019). If the metabolite-exposed tadpoles metamorphosed at a reduced size and with fewer energy reserves, they would be less successful than their more robust counterparts who developed at a normal pace (Goater et al. 1993, Goater 1994, Scott et al. 2007). Instead, they developed faster but were the same size as their stage cohort. This increase in developmental speed may be advantageous, allowing tadpoles to move out of the contagion-filled water faster, potentially reducing their infection risk.

These findings may explain exposure effects noted in previous experiments without actual fungal infection. For example, Pacific tree frog *Pseudacris regilla* tadpoles were exposed to *Bd*, but did not become infected; despite this lack of actual infection, they displayed accelerated development as observed in our experiment (Romansic et al. 2011). Additionally, the metabolites that *Bd* produces are likely used during infection, possibly to actively burrow through host tissue. Given that these metabolites can cause direct damage to the host without infection (Brutyn et al. 2012, Fites et al. 2013, McMahon et al. 2013a, Rollins-Smith et al. 2015), exposure to the chemicals may be a strong enough cue to induce an increased developmental speed.

Bd metabolites may function as a cue for the tadpoles so they can avoid disease-causing zoospores. Previous work has shown amphibians can learn to avoid *Bd* (McMahon et al. 2014); therefore, tadpoles might increase developmental speed to escape these adverse conditions. Consequently, given that there was no mortality or reduced growth, the fitness implications are much less severe compared to actual *Bd* infection. Exposure to these metabolites may help tadpoles learn to identify and avoid the fungus prior to infection.

5. CONCLUSIONS

Our results indicate that the metabolites produced by *Bd* did not affect mortality or growth for either tadpole or adult Cuban tree frogs. These findings are encouraging because conservation efforts may use the *Bd* metabolites themselves to induce acquired resistance in amphibians (McMahon et al. 2014). Tadpoles developing in *Bd*-contaminated water may use cues to develop faster and leave the water even when they are not directly exposed to the pathogenic fungus. Ecological models should take this rapid development into account when considering the impact of *Bd* exposure on tadpoles and amphibian populations. Metabolite-exposed tadpoles are not likely to be less biologically fit than unexposed tadpoles, because they developed faster and were of equal size

to their stage cohort. Additionally, they may have reduced infection risk, as they are able to escape the contagion-filled water sooner. If studies aim to have a robust understanding of the effect of *Bd* on amphibian populations, it is essential that we consider the impacts of *Bd* metabolites.

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