

RESEARCH ARTICLE

Metabolites from the fungal pathogen *Batrachochytrium dendrobatidis* (Bd) reduce Bd load in Cuban treefrog tadpoles

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Funding information

National Institutes of Health, Grant/Award Number: 1R01GM135935-0 and KK 2022; National Science Foundation, Grant/Award Number: IOS-1754862, IOS-1754886 and IOS-1755002; University of Tampa

Handling Editor: Don Driscoll

Abstract

1. *Batrachochytrium dendrobatidis* (Bd) has been associated with massive amphibian population declines worldwide. Wildlife vaccination campaigns have proven effective for mitigating damage from other pathogens, and there is evidence that adult frogs can acquire resistance to Bd when exposed to killed Bd zoospores and the metabolites they produced.
2. Here, we investigated whether Cuban treefrogs tadpoles *Osteopilus septentrionalis* can gain protection from Bd through exposure to a prophylaxis treatment composed of killed zoospores or soluble Bd metabolites. We used a 2 × 2 factorial design, crossing the presence or absence of killed zoospores with the presence or absence of Bd metabolites. All hosts were subsequently exposed to live Bd to evaluate susceptibility.
3. Exposure to killed zoospores did not induce a protective response. However, tadpoles exposed to Bd metabolites had significantly lower Bd intensity and prevalence than tadpoles that were not exposed to metabolites.
4. The metabolites Bd produce pose no risk of Bd infection and therefore make an epidemiologically safe prophylaxis treatment, protecting tadpoles against Bd. This work provides a promising potential for protecting amphibians in the wild as a disease management strategy for controlling Bd-associated declines.

KEYWORDS

amphibian decline, chytrid, chytridiomycosis, host–parasite interactions, prophylaxis, vaccination, vaccine, wildlife vaccines

1 | INTRODUCTION

Amphibian populations have declined dramatically in the past five decades, with an estimated 48% of species rapidly declining and nearly 10% of amphibian species on the Critically Endangered list (Scheele et al., 2019; Stuart et al., 2004). *Batrachochytrium dendrobatidis* (Bd), the causative agent of chytridiomycosis (Berger et al., 1998),

is associated with many worldwide declines. Eradication of wildlife pathogens like Bd would be economically and ecologically infeasible (Cross et al., 2007), especially given that Bd has non-amphibian hosts (Brannelly et al., 2015; McMahon et al., 2013). Therefore, we need strategies focused on disease mitigation for protecting extant amphibian species.

Wildlife vaccination has proven effective for the management of other diseases (Cross et al., 2007; Freuling et al., 2013;

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Joseph et al., 2013; Mencher et al., 2004; Rocke et al., 2017; Tompkins et al., 2009), and has been used in human health and agricultural initiatives, and to protect endangered species (Cross et al., 2007). For instance, the utilization of the SAG2 rabies vaccine targeting wild carnivores resulted in the elimination of rabies from France, Italy, Switzerland and Estonia (Mähl et al., 2014). There is evidence that adult frogs can acquire resistance to Bd when exposed to killed Bd zoospores and the metabolites they produced (McMahon et al., 2014). Live Bd zoospores release many compounds, which we refer to here as Bd metabolites. While we do not know the entire make-up of the chemicals produced by Bd, we know that some of these chemicals are soluble, may assist in entering host tissue (Symonds et al., 2008) and can inhibit the proliferation of host lymphocytes (Rollins-Smith et al., 2015).

Despite extensive study of disease management and immunity in adult frogs (Rollins-Smith, 2017; see the reviews by Venesky et al., 2014), less work has been conducted on developing disease mitigation strategies for larval amphibians. This lack of research may stem from the assumption that tadpoles lack a robust immune system capable of adaptive immunity. Although juveniles and adult frogs are immunocompetent, the T and B cells in tadpoles are less diverse and have lower antibody affinity than the adult-produced cells (Hsu & Du Pasquier, 1984). It is difficult to predict whether tadpoles can acquire resistance to Bd in the same way adults can (McMahon et al., 2014; Sauer et al., 2020). Tadpoles often reach peak abundances that are several orders of magnitude higher than peak abundances for amphibian adults. If tadpoles can acquire resistance, then vaccination campaigns could impact a greater proportion of frog populations and consequently have a greater chance of inducing protective herd immunity.

In a previous study, adult frogs exposed to both killed zoospores and the metabolites they produced had reduced infection intensities and were five times more likely to survive live infection than unexposed frogs (McMahon et al., 2014). This work is a promising precursor to a wildlife vaccine for Bd, but critical knowledge gaps need to be filled before a safe and effective prophylaxis can be implemented in the field (see Grogan et al., 2018). For example, tadpoles outnumber adults during certain times of the year and so knowing if larval amphibians can acquire resistance to Bd is critical. Another gap is that it is unclear whether amphibian exposure to the killed zoospores or Bd metabolites confers Bd resistance. Previous work revealed that adult Cuban tree frogs acquired Bd resistance after exposure to killed zoospores (McMahon et al., 2014). However, this strategy could pose a risk of releasing live Bd into the field if the Bd culture is not completely killed. Previous work found that attenuated or low virulence Bd could protect amphibians against more virulent strains (Greener et al., 2020; Waddle et al., 2021); however, not all strain cross-combinations provide protection (Barnett et al., 2021). In addition, an ideal prophylactic treatment would not release any killed or attenuated Bd into the field. Thus, if Bd metabolites confer protection, they might represent a safer treatment than the release of killed zoospores. Importantly, we found that

exposure to Bd metabolites did not have deleterious impacts on tadpole growth or development (McMahon et al., 2019). The objectives of this study were to test whether Cuban treefrog *Osteopilus septentrionalis* tadpoles could acquire resistance to Bd and determine whether killed zoospores, Bd metabolites or both could confer resistance. Insights from this experiment have the potential to move conservation biology closer to the implementation of a Bd vaccine in the wild.

2 | MATERIALS AND METHODS

2.1 | Bd culture

Bd strain JEL 419 (isolated in Panamá) was cultured on 1% tryptone agar plates at 18°C for 2 weeks (Bd + plates). Bd + plates were flooded with artificial spring water (ASW; L. M. Cohen et al., 1980), which suspended the zoospores. The Bd + ASW was homogenized across all of the plates (Bd + stock = 1×10^5 zoospores/ml). To verify Bd + stock viability, 1 ml of Bd + stock was plated on 1% tryptone plates ($n = 3$ plates); all plates had verified growth after 8 days. An ASW control stock (Bd-free stock) was made by flooding Bd-free 1% tryptone plates following the same procedure.

2.2 | Treatment and ASW control preparation

We conducted a 2×2 factorial design crossing the presence and absence of killed Bd zoospores and Bd metabolites. The live Bd + stock was flash-frozen with liquid nitrogen to kill it. The resulting killed Bd + stock was inspected using a haemocytometer to verify the Bd was dead (see McMahon & Rohr, 2014 for methods). Additionally, 1 ml of the killed Bd + stock was plated on a 1% tryptone plate ($n = 3$ plates) and there was no growth after 8 days.

We filtered the killed Bd + stock through a 1.2- μ m filter (GE Whatman Laboratory Products) to prepare a Bd metabolite solution. We defined the Bd metabolite solution as the Bd-free filtrate that passed through the filter; this filtrate may be enriched with components of Bd and the chemicals it produced. A visual inspection of the Bd enriched filtrate on a haemocytometer was conducted to verify that no zoospores remained in this Bd metabolite treatment. We then washed the filters with the same amount of ASW that was passed through the filter initially to resuspend the killed zoospores. This process created a suspension of killed zoospores with the same concentration as the original solution but no metabolites. The ASW control treatment was created by filtering the Bd-free stock through a 1.2- μ m filter. We determined there was no difference for pH, copper (ppm), iron (ppm), nitrate (ppm), total alkalinity (ppm) or carbonate (ppm) between the Bd metabolite solution and the ASW. All treatments were maintained in a laboratory grade -20°C freezer until the day they were used. Individual treatment vials were defrosted each day, brought to experimental room temperature, and administered.

2.3 | Exposure trial

Bd-naïve Cuban treefrog tadpoles (Gosner stage 25; Gosner, 1960) were raised in the laboratory from field-collected eggs (collected in Hillsborough County, Tampa FL; the Cuban treefrogs used had no prior exposure to Bd and, therefore, gave us a clear view of their response to the treatments). The frogs were kept in tanks with ASW and fed fish food and organic spinach ad libitum (12:12 hr light: dark at 18°C; this temperature was chosen because it has been shown to yield ideal Bd growth on this species [Cohen et al., 2017], and this is a temperature the host species routinely experiences in the field). The tadpoles were considered Bd-naïve as the eggs were collected from an area with no known Bd infections despite extensive local screening (TA McMahon unpublished data) and a subset of tadpoles tested Bd-free at the start of the experiment. At the beginning of the experiment, tadpoles ($n = 20$ per treatment; sample size substantial enough to compensate for individual variation) were housed individually in 500ml of ASW and were randomly assigned and exposed to one of the four different treatments. Each tadpole was exposed to 1 ml of their respective treatment added directly to their tanks daily for 2 weeks. One day after the 2-week exposure period, all tadpole tanks were dosed with 1 ml of live Bd+stock (strain JEL 419: 1×10^5 zoospores/ml) and maintained for two more weeks. This time-frame was long enough for a strong Bd infection to develop but not long enough to see severe disease in the host, which should reduce the adverse effects the hosts experience. All containers received a 50% water change weekly. Mortality was tracked daily throughout the entire experiment. All of the tadpoles were euthanized, their mouthparts were harvested, and the Bd load for each individual was quantified with quantitative PCR (qPCR). This work was approved by the IACUC at the University of Tampa (IACUC #2018-2).

2.4 | Quantitative PCR

We followed the qPCR protocol described by Hyatt et al. (2007). Briefly, PrepMan Ultra (Applied Biosystems) was used to extract DNA from each mouthpart. The mouthpart tissue was placed in a cell disruptor (Disruptor Genie, Scientific Industries) and agitated for better extraction efficiency using 0.035 ± 0.05 g of zirconia/silica beads for 2.25 min. We screened all samples for inhibition using TaqMan Exogenous Internal Positive Control Reagents (Applied Biosystems). There was no evidence of inhibition.

2.5 | Statistical analysis

All statistical analyses were conducted in R statistical software (R: Development Core Team, 2020). Prior to analysing the data, we used model selection and AICc values to determine that using a zero-inflated model was the best fit for our data (see Appendix S1 in Supporting Information; Table S1). We simultaneously tested for differences among treatments in Bd prevalence and infection intensity using a

zero-inflated negative binomial generalized linear model (zi-glm). We crossed the factors, exposure killed Bd zoospores (Yes or No) and exposure to Bd metabolites (Yes or No), as predictors for both the 'zero-inflation' (prevalence) and 'conditional count' (intensity) components of the zi-glm (package: GLMMTMB, function: glmmTMB). We conducted planned pairwise comparisons among each treatment group to test for differences in prevalence and intensity with corrections for multiple comparisons (package: EMMEANS, functions: emmeans and pairs).

3 | RESULTS

Tadpoles exposed to treatments containing Bd metabolites were less likely to become infected (i.e. lower prevalence) when subsequently exposed to live Bd, relative to treatments that did not contain the metabolites (i.e. zero inflation component of zi-glm: $z = 3.43$, $p = 0.0006$; Figure 1A). Planned comparisons indicated

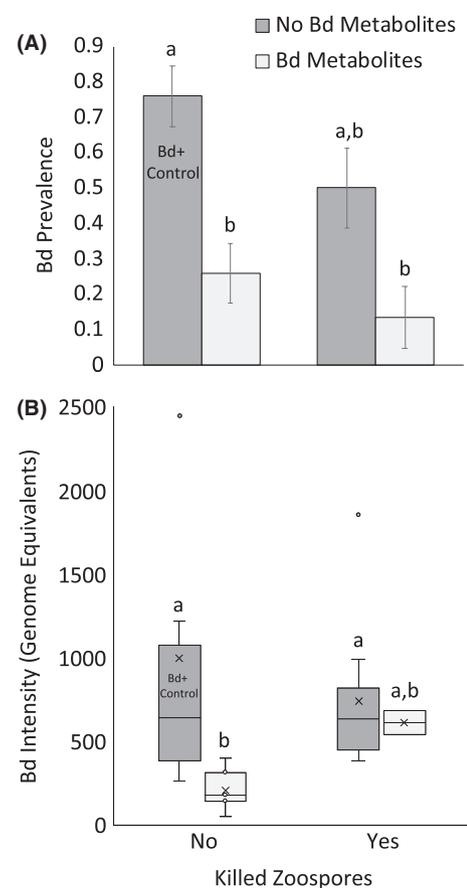


FIGURE 1 The effects of prophylactic treatments (2×2 design: Bd metabolites*killed Bd zoospores) on *Batrachochytrium dendrobatidis* (Bd) infection intensity in Cuban treefrog *Osteopilus septentrionalis* tadpoles. Shown are (A) Bd prevalence (means \pm SE) and (B) Bd intensity (genome equivalents) 2 weeks after exposure to live Bd. The top and bottom of the box represents the 75th and 25th percentiles, respectively, the line = median, the x = mean, the whiskers = the minimum and maximum values not included in the outliers, and the circle = the outliers. Letters signify significant differences based on estimated marginal means.

that relative to ASW control (no killed zoospores or Bd metabolites), exposure to just metabolites ($t = -3.43$, $p = 0.005$; Figure 1A) or both zoospores and metabolites ($t = -3.39$, $p = 0.006$; Figure 1A) reduced Bd prevalence. There was no interaction between killed zoospores and metabolites in regard to Bd prevalence ($z = -0.30$, $p = 0.762$).

Bd infection intensity was significantly lower in tadpoles exposed to Bd metabolites compared to the treatments without metabolites (conditional count component of *zi-glm*: $z = -5.51$, $p < 0.0001$; Figure 1B). There was a significant interaction between killed zoospores and metabolites ($z = 2.40$, $p = 0.016$) such that tadpoles receiving the metabolite-only treatment had significantly lower intensity than those receiving both components (Figure 1B). Planned comparisons indicated that exposure to metabolites-only reduced infection intensity ($t = 5.51$, $p < 0.0001$) and this effect was significant when comparing metabolites-only vs. killed zoospores-only ($t = 4.04$, $p = 0.0007$). There were no mortalities during this experiment and, therefore, no effect of treatment on mortality.

4 | DISCUSSION

Here, we demonstrated that prophylactic exposure to Bd metabolites can offer protection to tadpoles against Bd infection in terms of disease intensity and prevalence. Given that adults can also acquire resistance to Bd (McMahon et al., 2014), inducing disease resistance across amphibian life stages would greatly increase the chances of this prophylactic treatment being developed into a successful wildlife vaccine, especially if similar results are found in additional amphibian species.

We found that the effective component of the prophylactic treatment was the Bd metabolites, not the killed zoospores themselves. Indeed, there was no difference in Bd intensity between the control tadpoles and those exposed to the killed Bd zoospores alone, signifying that the killed zoospores do not induce a protective response for tadpoles. The Bd metabolites treatment, which contains no cellular components larger than the 1.2 μm filter or infectious agents, has a lower risk of releasing live Bd than a treatment that uses killed or attenuated Bd. Importantly, exposure to this prophylactic metabolite treatment could protect individuals in the wild from this devastating fungal pathogen, and currently, there are very few prophylaxis or vaccine treatments available for fungal pathogens (but see Rocke et al., 2019). Additionally, this potential field treatment could be an incredibly powerful conservation tool if herd immunity could be induced in wild populations; though more research is needed to determine if this is possible.

Reducing Bd loads in the aquatic tadpole stage is particularly advantageous because it would allow managers to dose a waterbody directly, exposing multiple individuals of different life stages and species all at once. Protecting the aquatic life stage would enable us to protect individuals in species with terrestrial adult stages, which would otherwise be difficult to do on a wide scale. Protecting

terrestrial adults would require catching and exposing individuals, which is logistically challenging and time-consuming. While more research is needed to determine how long this protective response lasts, and the mechanism for it, reducing the Bd loads in tadpoles in a population may lead to lower pathogen prevalence and lower population-wide mortality, therefore, allowing more individuals to reach the breeding stage. Moreover, in addition to increasing individual resistance to the pathogen, inducing protection across life stages may increase population-scale resistance, further reducing the prevalence and intensity of the pathogen itself in the environment (Miller et al., 2006).

The Bd metabolite prophylaxis is easy to administer, can be produced by local researchers and conservation groups, and is effective without containing infectious agents. We can induce protection in Cuban treefrog tadpoles. If we can do the same in other species, then we can potentially use this preliminary work to develop a wildlife vaccination campaign, which could be used as an effective disease management tool to control Bd-associated declines. Further work is needed to identify the chemical compounds within the Bd metabolites that confer resistance and determine whether the protection wanes with time. Additionally, before any potential prophylaxis can be used in the field, its non-target effects would need to be explored. A wildlife vaccine campaign could be an effective tool in protecting at-risk amphibian populations from this highly destructive pathogen.

AUTHORS' CONTRIBUTIONS

T.A.M., D.J.C., P.T.J.J. and J.R.R. designed the experiments, C.L.N., S.E.D. and T.A.M. conducted the experiments, T.A.M. and D.J.C. conducted the statistical analyses; C.L.N. and T.A.M. wrote the paper, T.A.M., P.T.J.J. and D.J.C. provided funding and all authors provided editorial advice.

ACKNOWLEDGEMENTS

We would like to thank Dr. K. Dobrinski, A. Garcia and B. Soompholphakdy for their assistance with this work. We would also like to thank Dr. J. Longcore for providing the Bd strains. This work was supported by the National Science Foundation (T.A.M.: IOS-1754862; D.J.C.: IOS-1755002; P.T.J.J.: IOS-1754886), the National Institutes of Health (T.A.M.: 1R01GM135935-0, KK 2022) and the University of Tampa (T.A.M.: Faculty Development Dana and Delo Grants).

CONFLICT OF INTEREST

None of the authors have a conflict of interest to declare. Jason Rohr is an Associate Editor of Journal of Applied Ecology, but took no part in the peer review and decision-making processes for this paper.

DATA AVAILABILITY STATEMENT

Data available via the Dryad Digital Repository <https://doi.org/10.5061/dryad.p2ngf1vt4> (Nordheim et al., 2022).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Nordheim, C. L., Detmering, S. E., Civitello, D. J., Johnson, P. T. J., Rohr, J. R., & McMahon, T. A. (2022). Metabolites from the fungal pathogen *Batrachochytrium dendrobatidis* (bd) reduce Bd load in Cuban treefrog tadpoles. *Journal of Applied Ecology*, 59, 2398–2403. <https://doi.org/10.1111/1365-2664.14242>