Vol. 155: 193–198, 2023
https://doi.org/10.3354/dao03753

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published September 28

NOTE

Pseudacris regilla metamorphs acquire resistance to *Batrachochytrium dendrobatidis* after exposure to the killed fungus

Taegan A. McMahon[1,2,*], Caitlin L. Nordheim[2], Sarah E. Detmering[2], Pieter T. J. Johnson[3], Jason R. Rohr[4], David J. Civitello[5]

[1]Connecticut College, Department of Biology, New London, Connecticut 06320, USA
[2]University of Tampa, Department of Biology, Tampa, Florida 33606, USA
[3]University of Colorado, Ecology and Evolutionary Biology, Boulder, Colorado 80302, USA
[4]University of Notre Dame, Department of Biological Sciences, Notre Dame, Indiana 46556, USA
[5]Emory University, Department of Biology, Atlanta, Georgia 30322, USA

ABSTRACT: The pathogenic fungus *Batrachochytrium dendrobatidis* (*Bd*) is associated with drastic global amphibian declines. Prophylactic exposure to killed zoospores and the soluble chemicals they produce (*Bd* metabolites) can induce acquired resistance to *Bd* in adult Cuban treefrogs *Osteopilus septentrionalis*. Here, we exposed metamorphic frogs of a second species, the Pacific chorus frog *Pseudacris regilla*, to one of 2 prophylactic treatments prior to live *Bd* exposures: killed *Bd* zoospores with metabolites, killed zoospores alone, or a water control. Prior exposure to killed *Bd* zoospores with metabolites reduced *Bd* infection intensity in metamorphic Pacific chorus frogs by 60.4% compared to control frogs. Interestingly, *Bd* intensity in metamorphs previously exposed to killed zoospores alone did not differ in magnitude relative to the control metamorphs, nor to those treated with killed zoospores plus metabolites. Previous work indicated that *Bd* metabolites alone can induce acquired resistance in tadpoles, and so these findings together indicate that it is possible that the soluble *Bd* metabolites may contain immunomodulatory components that drive this resistance phenotype. Our results expand the generality of this prophylaxis work by identifying a second amphibian species (Pacific chorus frog) and an additional amphibian life stage (metamorphic frog) that can acquire resistance to *Bd* after metabolite exposure. This work increases hopes that a *Bd*-metabolite prophylaxis might be widely effective across amphibian species and life stages.

KEY WORDS: Chytrid · *Batrachochytrium dendrobatidis* · Prophylaxis · Pacific chorus frog · Acquired immunity · Wildlife vaccines · Killed zoospores

1. INTRODUCTION

The pathogenic fungus *Batrachochytrium dendrobatidis* (*Bd*) is associated with a panzootic disease and drastic global amphibian declines (Fisher et al. 2009, Scheele et al. 2019). In addition to the wide diversity of amphibian hosts that can be infected by *Bd* (Sauer et al. 2020), *Bd* also has non-amphibian reservoir hosts, such as crayfish (McMahon et al. 2013a, Brannelly et al. 2015, Oficialdegui et al. 2019). Not only does the presence of reservoir hosts (competent hosts that can maintain and transmit a patho-

*Corresponding author: taeganmcmahon@gmail.com

gen population) increase the likelihood of population-level harm to more susceptible hosts, such as amphibians (de Castro & Bolker 2005), but it decreases the likelihood of pathogen eradication.

Bd is globally distributed and unlikely to ever be eradicated, which means conservation strategies are needed that promote amphibian host resistance or tolerance to infection to facilitate amphibian coexistence with this pathogen (Venesky et al. 2014). For example, interventions that boost resistance to infection, such as prophylactic treatments or vaccines, could increase the persistence of amphibian populations even when pathogen eradication is infeasible (Barnett & Civitello 2020). There is evidence that amphibians can acquire resistance to *Bd* both naturally and in the laboratory (Shaw et al. 2010, McMahon et al. 2014, Nordheim et al. 2022). For example, field-collected Archey's frogs *Leiopelma archeyi* with self-cured *Bd* infections had reduced *Bd* loads upon a secondary infection (Shaw et al. 2010). Additionally, both tadpole and adult Cuban treefrogs *Osteopilus septentrionalis* exposed in the laboratory to treatments containing the soluble chemicals produced by *Bd* zoospores (*Bd* metabolites) had dramatically lower *Bd* loads when subsequently infected with live *Bd* (McMahon et al. 2014, Nordheim et al. 2022).

While exposure to *Bd* metabolites has been shown to be protective for both tadpole and adult Cuban treefrogs (McMahon et al. 2014, Nordheim et al. 2022), it remains unknown whether metabolite exposure can protect metamorphic frogs. During metamorphosis, amphibians completely rebuild important body systems, e.g. the gastrointestinal tract (Schreiber et al. 2005). Although some immunological memory persists through metamorphosis (Barlow & Cohen 1983), the developing larvae suppress their immune system possibly to prevent the immune system from attacking and destroying their own developing tissue during development (Rollins-Smith et al. 1997). The metamorphic life stage of a frog is typically the most susceptible to *Bd* and has the highest odds of infection-induced mortality (Sauer et al. 2020). Protecting this particularly vulnerable yet abundant life stage could improve population persistence.

In addition to providing protection from *Bd* across amphibian life stages, induced acquired resistance must be feasible in several amphibian species for a prophylaxis or vaccine to be broadly effective. Host community composition strongly impacts disease dynamics (Johnson et al. 2013), because host species naturally vary in their disease susceptibility, resistance, and tolerance. Species that are pathogen tolerant, widespread, and highly abundant may have a disproportionately high impact on disease dynamics in a community (e.g. see Johnson et al. 2013). Pacific chorus frogs *Pseudacris regilla*, for example, have been identified as an important and widespread reservoir host for *Bd* that facilitate *Bd* persistence on the landscape and contribute to spillover onto co-occurring amphibian species, including some that are threatened (Reeder et al. 2012, Wilber et al. 2020). Therefore, inducing resistance in such an influential species could provide broader indirect benefits to the entire amphibian community.

Here, we investigated whether we can use a prophylaxis to protect the highly susceptible metamorphic life stage of amphibians against *Bd*, whether this prophylaxis can effectively protect an abundant and influential reservoir host of *Bd* (Pacific chorus frogs), and whether exposure to *Bd* metabolites are necessary for protection against *Bd*. Addressing these questions will help to broaden our knowledge about prophylactic treatments for *Bd* and refine a potentially important conservation management protocol.

2. MATERIALS AND METHODS

2.1. *Bd* culture

The *Bd* stock JEL 419 (isolated in Panama during a *Bd* outbreak; Brem & Lips 2008) was cultured on 1% tryptone agar plates for 10 d at 18°C (*Bd*+ plates). Artificial spring water (ASW; Cohen et al. 1980) was used to flood the *Bd*+ plates for 5 min. The *Bd* and ASW from all plates was homogenized into one *Bd*+ stock, which was diluted with ASW to 1.2×10^5 zoospores ml^{-1}, a *Bd* concentration chosen based on previous work (see Nordheim et al. 2022). We repeated this process twice: once to create the prophylactic treatments and again to create the *Bd*+ stock for the live *Bd* exposures.

2.2. Prophylactic treatments preparation

The Killed Zoospores with Metabolites (KZM) treatment was used to determine if Pacific chorus frogs could gain protection from the previously effective prophylaxis, and the Killed Zoospores Alone (KZA) treatment was used to determine if the zoospores alone could yield protection. We did not have a metabolites alone treatment because Nord-

heim et al. (2022) found that metabolites alone was an effective prophylaxis. The *Bd*+ stock was flash frozen with liquid nitrogen to kill the zoospores, which was used as the KZM treatment. A portion of this killed *Bd*+ stock was then passed through a 1.2 µm filter (GE Whatman) to remove the zoospores. The filter with the killed zoospores was then washed with the same amount of ASW that was removed from the killed *Bd*+ stock in order to reconcentrate the killed *Bd* zoospores. This KZA treatment contained killed *Bd* zoospores in ASW and had no *Bd* metabolites. We verified the killed zoospore concentration using a hemocytometer. Additionally, we verified that the *Bd* was dead for both the KZM and the KZA treatments in 2 ways. First, we screened for live and dead zoospores using trypan blue staining and microscopy (McMahon & Rohr 2014); there were no live zoospores present. Then we plated 1 ml on a 1% tryptone plate (n = 3 plates treatment^{-1}) and tracked *Bd* growth for 8 d; there was no *Bd* growth.

The ASW Control treatment (ASWC) was created using the same techniques as the *Bd*+ plates, but no *Bd* was added. We flooded *Bd*-free 1% tryptone agar plates for 5 min, and the ASW from these plates was homogenized to create one *Bd* negative (*Bd*–) stock.

All treatments were divided into separate vials, containing the amount needed for one day's administration. They were stored frozen until the day they were used, which reduced the number of times the treatments went through the freeze/thaw cycle. On the day of application, they were brought to experimental room temperature prior to administration.

2.3. Animal husbandry

Pacific chorus frog *Pseudacris regilla* eggs were field collected (San Francisco Bay area, CA) and shipped overnight to University of Tampa, Tampa, FL. They were housed together in ASW (12:12 h light:dark cycle at 20°C) until they hatched, and then they were housed individually in 400 ml of ASW (0.5 l plastic deli cups) and fed fish food and organic spinach ad libitum (12:12 h light:dark at 20°C). As tadpoles metamorphosed into metamorphic frogs (Gosner stage 46; Gosner 1960), they were transferred to individual lidded 0.5 l plastic deli cups with ASW-moistened paper towels. Metamorphs were swabbed thoroughly (5 times each down both hind limbs, covering their ventral surface and covering their dorsal surface) to verify that they were not *Bd*+. We used quantitative PCR (qPCR) to detect *Bd* (see Section 2.5), and all metamorphs were *Bd*-free. Throughout the experiment, frogs were monitored daily for mortality, they were fed calcium dusted crickets ad libitum, and containers were changed weekly.

2.4. Metamorphic exposure experiment

Frogs were randomly assigned to their treatment (KZM, KZA, and ASWC). During the prophylactic exposure period, each metamorph (n = 15 treatment^{-1}) was exposed to their respective prophylaxis daily for 14 d. For each metamorph, 1 ml the prophylaxis was pipetted directly on their dorsal surface, and the excess liquid was allowed to collect on the moist paper towels in the enclosure. After the prophylactic exposure period, all metamorphs were exposed individually to 1 ml of live *Bd* (1.2×10^5 zoospores ml^{-1}). They were maintained for 2 wk post exposure, and then each individual was swabbed for qPCR processing on the right hind limb from hip to toe 10 times with a sterile cotton swab.

2.5. Quantitative PCR

We used PrepMan Ultra (Applied Biosystems) to extract DNA from each swab and then followed the protocol described by Hyatt et al. (2007) to process the qPCR samples. We screened for inhibition in all samples using TaqMan Exogenous Internal Positive Control Reagents (Applied Biosystems) and found no evidence of inhibition.

2.6. Statistical analysis

All statistical analyses were performed using R v.4.0.3 (R Core Team 2020). A Cox proportional-hazards model was used to determine whether there was an effect of treatment on mortality (package: KM surv; function: coxph). A generalized linear model with a zero-inflated negative binomial distribution was used to simultaneously determine if there was an effect of treatment on prevalence, i.e. zero-inflation, and infection intensity (package: glmmTMB; function: glmmTMB; ziformula = ~ treatment; this function examines both prevalence and intensity simultaneously); animal mass was used as a covariate. An estimated marginal means test was run to make pairwise comparisons among treatments (package: emmeans; functions: emmeans and pairs).

3. RESULTS

There was no effect of treatment on mortality (χ^2_1 = 4.28, p = 0.118), and mortality was low for all treatments (13, 6, and 13% for Killed Zoospores with Metabolites (KZM), Killed Zoospores Alone (KZA) and ASW Control (ASWC), respectively). Metamorphs exposed to the KZM prophylaxis had reduced *Bd* intensity compared to those exposed to the ASWC (t = 2.91, p = 0.016; Fig. 1) but not compared to those exposed to the KZA treatment (t = 1.34, p = 0.391). There was no difference in *Bd* intensity between the ASWC and the KZA treatment groups (t = 1.89, p = 0.155). There was no effect of treatment on *Bd* prevalence (ASWC: 60% prevalence, z = −0.769, p = 0.442; KZA: 75% prevalence, z = −1.28, p = 0.201; and KZM: 53% prevalence, z = 0.368, p = 0.713).

4. DISCUSSION

We found that a prophylactic treatment containing killed zoospores and *Bd* metabolites together reduced *Bd* infection intensities in metamorphic Pacific chorus frogs by 60.4% compared to controls. Interestingly, *Bd* infection intensities in the Killed Zoospores Alone (KZA) treatment were not significantly different compared to the Killed Zoospores with Metabolites (KZM) or control treatment. While we cannot say that the KZM yielded more protection than the KZA from this study, evidence from previous work found that the prophylactic treatments that contained *Bd* metabolites yielded the strongest protection for tadpoles and adults (McMahon et al. 2014, Nordheim et al. 2022). All the available evidence suggests that the soluble *Bd* metabolites contain *Bd*-resistance inducing component(s) and can be used as an effective prophylaxis treatment.

There was no effect of treatment on *Bd* prevalence. While this *Bd* metabolite prophylaxis has been found to reduce *Bd* intensity repeatedly, some studies found that it reduced prevalence while others did not (McMahon et al. 2014, Barnett et al. 2021, Nordheim et al. 2022). It is possible the differences we have seen on prevalence are due to species differences or a lack of statistical power to detect effects on *Bd* prevalence in some of these studies.

For a wildlife prophylaxis to be effective at the population level, it would need to substantially contribute to herd immunity by reducing infection or transmission among a large proportion of the population or community (Barnett & Civitello 2020). Given that the loads measured represent zoospores, the propagules of transmission, such reductions are likely to represent reduced transmission rates as well. Indeed, *Bd* load positively influences the zoospore pool, which in turn positively influences the transmission parameter (Briggs et al. 2010). Our results add evidence for induced resistance to *Bd* in metamorphic frogs to existing demonstrations in tadpoles (Nordheim et al. 2022) and adults (McMahon et al. 2014). The ability to increase resistance in all life stages of amphibian hosts substantially increases the potential for population and community-wide protection and persistence. The findings from this study are particularly important because, along with tadpoles, metamorphs are found in high abundance in comparison to adults. Moreover, metamorphs are the most susceptible life stage to *Bd* for most amphibian taxa (Sauer et al. 2020; but see Bradley et al. 2019). Providing protection for this especially vulnerable life stage could have strong population-level benefits.

The fact that this prophylactic treatment reduces *Bd* load in 2 species, Cuban treefrogs (McMahon et al. 2014, Barnett et al. 2021, Nordheim et al. 2022) and Pacific chorus frogs, has considerable implications for *Bd* disease mitigation strategies. For instance, the Pacific chorus frog is an important maintenance host for *Bd* when co-occurring

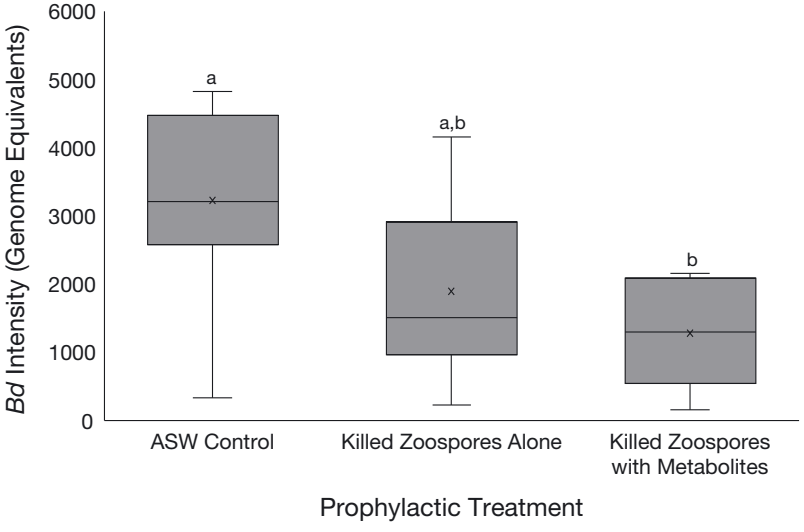

Fig. 1. Effects of prophylactic treatments on *Batrachochytrium dendrobatidis* (*Bd*) infection intensity in Pacific chorus frog *Pseudacris regilla* metamorphs. Top and bottom of the box: 75th and 25th percentiles, respectively; horizontal line: median; x: mean; whiskers: minimum and maximum values. Letters signify significant differences based on estimated marginal means

with other amphibians (Wilber et al. 2020). Thus, the ability to reduce the pathogen load in a reservoir host like the Pacific chorus frog could dramatically reduce the infection risk of co-occurring species, including those that are threatened by *Bd* or other stressors. In fact, Pacific chorus frogs were found to contribute to the maintenance of *Bd* in 91% of metacommunities observed in the San Francisco Bay area study system (Wilber et al. 2020). Thus, lowering infection intensity in this maintenance host may reduce *Bd* disease risk in the field.

Prophylactic treatments may be a promising path towards protecting amphibians from *Bd* related extinctions. Previous microbiome-related prophylaxis research found direct exposure to prophylactic treatments containing the betaproteobacterium *Janthinobacterium lividum* reduced *Bd* infections and increased survival (Becker et al. 2009). We now highlight, using a similar direct exposure method, that the *Bd* metabolite-containing prophylactic treatments described here are also protective across amphibian life stages and species. This work is especially promising given that other management research has utilized antifungal chemicals as a post exposure treatment and found mixed results (McMahon et al. 2013b). Additionally, the use of these often-immunomodulatory chemicals may actually cause harm to the ecosystem rather than help (Rohr et al. 2017). Importantly, the potential to use the same treatment for all life stages and multiple species will make the prophylaxis treatment logistically simpler and less expensive to implement in the field. More research is needed to determine whether the protective effects of this treatment will wane with time, which components of the *Bd* metabolites yield the strongest effects, how effective the treatment is on other amphibian species, and whether there are effects on non-target organisms. This *Bd* metabolite prophylaxis is likely producible at a large scale, though local *Bd* strain should be taken into consideration (see Barnett et al. 2021) and therefore has the potential to be used on a large scale in the lab and field. It is crucial that continued refinement of this prophylaxis treatment and investigation of these unanswered questions occur prior to large-scale field implementation. With those important cautions in mind, this prophylactic treatment has the potential to protect a wide range of amphibians and may possibly be used as an effective disease management tool to help control this devastating wildlife disease.

Acknowledgements. We thank A. Garcia and B. Soomphol-phakdy for their assistance. Funds were provided by the National Science Foundation (T.A.M.: IOS-1754862, D.J.C.: IOS-1755002, P.T.J.J.: IOS-1754886 and DEB-1754171, and J.R.R. DEB-2017785 and IOS-1754868), the National Institutes of Health (T.A.M. and P.T.J.J.: 1R01GM135935), US Department of Agriculture (J.R.R.: 2021-38420-34065), David and Lucile Packard Foundation (P.T.J.J.) and the University of Tampa (T.A.M.: Faculty Development Grants).

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Editorial responsibility: Douglas Woodhams, Boston, Massachusetts, USA
Reviewed by: 2 anonymous referees

Submitted: April 28, 2023
Accepted: August 4, 2023
Proofs received from author(s): September 18, 2023