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RESEARCH ARTICLE

Efficacy of Bd metabolite prophylaxis dose and duration on host defence against the deadly chytrid fungus *Batrachochytrium dendrobatidis*

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Abstract

1. *Batrachochytrium dendrobatidis* (Bd), an aquatic pathogenic fungus, is responsible for the decline of hundreds of amphibian species worldwide and negatively impacts biodiversity globally. Prophylactic exposure to the metabolites produced by Bd can provide protection for naïve tree frogs and reduce subsequent Bd infection intensity.
2. Here, we used a response surface design crossing Bd metabolite prophylaxis concentration and exposure duration to determine how these factors modulate prophylactic protection against Bd in Pacific chorus frog (*Pseudacris regilla*) tadpoles (5×5 surface design) and metamorphs (3×3 surface design). We exposed individuals every weekday to one of five Bd metabolite concentrations or a water control for 1–5 weeks, after which all animals were challenged with live Bd to evaluate their susceptibility.
3. Exposure to the Bd metabolite prophylaxis reduced Bd load and prevalence compared to the control for both the tadpoles and metamorphs. Increasing Bd metabolite prophylaxis concentration did not confer additional protection for either life stage, but increasing duration of exposure did benefit metamorphs by decreasing Bd prevalence but not Bd load.
4. On average, control tadpoles and metamorphs had 66.2% and 99.4% higher Bd loads, respectively, than tadpoles and metamorphs exposed to any Bd metabolite prophylaxis.
5. Additionally, Bd metabolite prophylaxis reduced Bd prevalence relative to controls in both tadpoles (20.5% vs. 56.3%, respectively) and metamorphs (21.9% vs. 87.5%, respectively).
6. *Synthesis and applications*: The efficacy of short-term exposures of relatively low concentrations of Bd metabolites at reducing Bd infections suggests that this approach has the potential to be scaled up to field use to aid in disease

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mitigation and conservation. Our results, combined with additional research on Bd metabolite prophylaxis for other amphibian species, suggest that this strategy may represent a broadly useful tool to protect at-risk amphibian populations.

KEYWORDS
acquired immunity, amphibian decline, chytridiomycosis, pathogen, prophylactic treatment, vaccination, vaccine, wildlife vaccines

1 | INTRODUCTION

Batrachochytrium dendrobatidis (Bd) is an aquatic pathogenic fungus that is responsible for catastrophic global declines of amphibians (Scheele et al., 2019). The drastic declines in amphibian populations have negatively impacted global biodiversity, which may have broad-reaching effects on ecosystem stability given amphibians' importance to food webs (Frauendorf et al., 2013). Research has focused on ways to mitigate declines, which includes studies showing that prophylactic treatments provide protection against severe Bd infections and can increase survivorship in amphibians (Barnett et al., 2021, 2023; McMahon et al., 2014; Nordheim et al., 2022).

One promising prophylactic treatment involves exposing amphibian hosts to the water-soluble metabolites that Bd zoospores readily release. The exact composition of these metabolites is unknown and likely varies among Bd strains. Some of these chemicals, like trypsin and chymotrypsin, may break down host tissue proteins (Symonds et al., 2008) and others, like methylthioadenosine and kynurenine, inhibit host lymphocyte production and survival (Rollins-Smith et al., 2015).

Despite these immunomodulatory qualities, exposure to these metabolites can provide partial protection against Bd and can reduce subsequent Bd infection intensity when administered before Bd infection (Barnett et al., 2021, 2023; McMahon et al., 2014; Nordheim et al., 2022). The metabolites contain no infectious materials and so field deployment of this treatment as a Bd metabolite prophylaxis should be relatively safe from an epidemiological perspective.

Prior work on Bd metabolite prophylaxis has used time intensive, high concentration dosing schemes (see McMahon et al., 2014; Nordheim et al., 2022), which are likely impractical for field application. In previous studies, amphibians were dosed with the prophylactic treatment every day or every other day for 2 weeks, and this exposure period was repeated up to four times with a 2-week break in between (Barnett et al., 2021; McMahon et al., 2014; Nordheim et al., 2022). In some cases, amphibians remained in this prophylactic exposure phase for several months (McMahon et al., 2014), which could be financially and logistically burdensome for researchers. This period may also exceed the development time for fast-growing amphibian species in natural systems (e.g. many toad and treefrog species). Such observations highlight the importance of identifying protocols that are appropriate for field application and take amphibian life stages into account.

Here, we examine the efficacy of different exposure protocols to characterize the effects of concentration and duration of a Bd metabolite prophylaxis on the ability of tadpoles and metamorphs to resist subsequent challenges with Bd infection (described in Nordheim et al., 2022). We utilized a response surface design, crossing metabolite concentration with duration of exposure, to determine the efficacy of this potential Bd metabolite prophylaxis. Previous Bd metabolite prophylaxis work was conducted mainly on Cuban treefrogs (*Osteopilus septentrionalis*; see McMahon et al., 2014; Nordheim et al., 2022). With this work, we broaden the scope of our previous work by determining whether the Bd metabolite prophylaxis can provide protection against Bd for Pacific chorus frogs (*Pseudacris regilla*). This species has been identified as a key reservoir host driving the long-term persistence of Bd in amphibian metacommunities in northern California, USA (Wilber et al., 2020). Our long-term goal is to develop a prophylaxis or vaccine treatment protocol that is practical and effective at providing protection to multiple amphibian species under field settings. The work presented here helps inform and advance us towards developing this important conservation tool.

2 | MATERIALS AND METHODS

2.1 | Animal husbandry

2.1.1 | *Tadpoles*

Pacific chorus frog eggs collected from Blue Oak Ranch Reserve in California were shipped overnight to the University of Tampa in Tampa, Florida, and maintained in Bd-free artificial spring water (ASW; Cohen et al., 1980). Once hatched, all animals were maintained individually in 0.5 L plastic cups; the tadpoles (all tadpoles were Gosner stage 25; Gosner, 1960) were maintained in 200 mL of ASW and fed organic spinach ad libitum. To maintain water quality, faecal material and unconsumed spinach were removed daily with a sterile pipette, and ASW was added to maintain the original 200 mL level. This resulted in about a 20% water change every 3 days.

2.1.2 | *Metamorphs*

Approximately two-month-old metamorphs were collected from Blue Oak Ranch Reserve in California and shipped overnight to Connecticut College in New London, Connecticut. The metamorphs

were housed on ASW moistened paper towels and were fed calcium dusted crickets 3 times a week. Metamorphs were maintained individually in a Bd-free laboratory for a month prior to use in the experiment. We swabbed the entire abdomen and both hind limbs of each metamorph to verify that they were free of Bd infections. All metamorphs received a container and substrate change weekly. All animals were maintained at 20°C in a 12/12 light/dark cycle. All pacific chorus frogs were collected from California, USA, under the permit CA DFW S-193500003-20017-001. All laboratory work was conducted with approval by the institutional IACUCs (3×3: IACUC #2018-2; 5×5: ACUP 239).

2.2 | Bd culture and Bd metabolite prophylaxis development

The 5×5 tadpole and 3×3 metamorph experiments were run separately but utilized the following identical methodologies. Bd (California strain JEL 270) was grown on 1% tryptone agar plates for 10 days at 17°C. Plates were flooded with ASW for 3 min (work was conducted at 21°C), and the water from all plates were homogenized to create a single Bd+ stock solution. For the Bd metabolite prophylaxis, the Bd+ stock was filtered through a 1.2 μm filter (GE Whatman Laboratory Products, Springhill, PA) to remove the zoospores and zoosporangia. This yielded a Bd metabolite stock, which contained the soluble chemicals in the filtrate that were produced by the zoospores. This Bd metabolite stock was inspected visually on a haemocytometer to verify that there were no zoospores or zoosporangia remaining. To confirm the absence of live Bd, we plated 1 mL of this Bd metabolite stock on a 1% tryptone plate and ensured that there was no growth for 8 days ($n=3$).

ASW was used to serially dilute the Bd metabolite stock to the target dilutions for each treatment ($1\times10^2-1\times10^6$ zoospores-removed/mL; see Table S1 in Supporting Information). To reduce the number of times each treatment went through a freeze–thaw cycle, the amount needed for each treatment day was frozen in a separate tube, which were maintained in a laboratory grade freezer at −20°C until use. ASW was also frozen and stored identically to the Bd metabolite prophylaxis treatments to maintain a proper control. Each day of Bd metabolite prophylaxis exposure, the appropriate treatments were removed from the freezer and placed in a bath with room temperature water to thaw the metabolite to room temperature prior to use.

A second Bd+ stock was created just prior to use for the live infection part of both experiments (see below for specific concentrations used). Bd (California strain JEL 270) was grown on 1% tryptone agar plates for 10 days at 17°C and the ASW flooded on all of the plates was homogenized to create a single Bd+ stock solution for each experiment.

2.3 | 5× 5 tadpole Bd metabolite prophylaxis exposure protocol

Pacific chorus frog tadpoles ($n=6$–7 tadpoles/treatment and $n=16$ tadpoles/control) were exposed to 1 mL of their respective Bd metabolite prophylaxis treatment every weekday for 1 to 5 weeks using a standard response surface design (see Table S1; Inouye, 2001). Control tadpoles were exposed to 1 mL of ASW every weekday for 5 weeks. A response surface design was used because it is an efficient way of distinguishing among alternative models and enables tests for interactions among factors. To ensure that all tadpoles had the same experience throughout the experiment, tadpoles not dosed for the full 5-week duration with a Bd metabolite prophylaxis were dosed with ASW for the week(s) prior to their Bd metabolite prophylaxis treatment period. For example, tadpoles that were dosed with a Bd metabolite prophylaxis for 2 weeks were first dosed with ASW every weekday for 3 weeks then their Bd metabolite prophylaxis treatment for the last 2 weeks of the exposure period.

After 5 weeks of Bd metabolite prophylaxis or control exposures, all tadpoles were exposed to 1 mL of live Bd (1.1×10^6 zoospores/mL). Following exposure to live Bd, tadpoles were maintained at 18°C for 2 weeks in 1 L plastic cups with 700 mL ASW. A 50% water change was conducted at the midpoint of this 2-week period. Tadpoles were then euthanized with an overdose of MS222, weighed, Gosner staged (Gosner stage ranged from 25 to 44), and screened for Bd. Given that Bd transitions from the mouthparts to keratinized skin in tadpoles around Gosner stage 38 (McMahon & Rohr, 2015), we sampled both the skin and mouthparts at the end of this 2-week period. We swabbed all tadpoles 10 times from mouth to tip of tail and stored these swabs in a laboratory grade −20°C freezer. The mouthparts were dissected from the tadpoles using sterile technique and stored in 70% ethanol. The amount of Bd on swabs and mouthparts was determined using quantitative PCR (qPCR; see below for details), and the Bd values reported are total Bd load, representing the sum of the swab and mouthpart DNA.

2.4 | 3× 3 metamorph Bd metabolite prophylaxis exposure protocol

We used the same basic design as the tadpole experiment (see Section 2.3 for details) except that we only had three levels of concentration and duration of Bd metabolite prophylaxis instead of five of each (Table S1) and the experiment was conducted on Pacific chorus frog metamorphs rather than tadpoles ($n=10$ for Bd metabolite prophylaxis treatments and $n=20$ for the control). After 3 weeks of Bd metabolite prophylaxis or control exposures, all metamorphs were exposed to 1 mL of live Bd (1.5×10^5 zoospores/mL; Bd concentration was chosen based on previous work; McMahon et al., 2023). Following exposure to live Bd, metamorphs were maintained individually at 18°C for 2 weeks. Their feeding and container change schedules were maintained as described in the *Animal Husbandry* section above. We swabbed the entire abdomen and both hind limbs of each metamorph 10 times to evaluate Bd infections. Swabs were stored in a laboratory grade −20°C freezer.

2.5 | Quantitative PCR (qPCR)

Bd load for each individual was quantified by evaluating genome equivalents (GE) using qPCR. We followed the protocol described by Hyatt et al. (2007). In brief, we used PrepMan Ultra (Applied Biosystems) to extract DNA from both the tadpole mouthparts and swabs. The tadpole mouthparts received an additional step to increase extraction efficiency, in which each mouthpart was placed in a tissue disruptor (Disruptor Genie, Scientific Industries) and was agitated with 0.035 ± 0.005 g of zirconia/silica beads for a total of 2.25 min. We screened all samples for inhibition using TaqMan Exogenous Internal Positive Control Reagents (Applied Biosystems) and there was no evidence of inhibition.

2.6 | Statistical analysis

All statistical analyses were conducted in R statistical software version 4.2.2 (R Development Core Team, 2020). The 5 × 5 tadpole and 3 × 3 metamorph experiments were run independently and so were analysed separately (see Table S2 for model details). We used the following analyses for both experiments. Throughout these analyses, we designated the ASW control as having a 0-week exposure period because the control animals were never exposed to any Bd metabolite prophylaxis (see the supplemental results for analyses where ASW control was coded as 5-week exposure for the tadpoles).

To understand the impact of the different Bd metabolite prophylaxis regimens (i.e. log concentration × duration) on final Bd load (measured as abundance), and to compare all the Bd metabolite prophylaxis treatments to the control, we used a negative binomial generalized linear model (package: glmmTMB, function: glmmTMB; family: nbinom2; Brooks et al., 2017; function: summary). We used model selection (function: extractAIC) to determine if Gosner stage (Bd metabolite prophylaxis can alter tadpole development (see McMahon et al., 2019); examined for the 5 × 5 experiment only) or final mass (an individual's size may impact overall infection load; examined for the 3 × 3 experiment only) were cofactors that improved the fit of the models. Gosner stage was used as a covariate for the 5 × 5 tadpole experiment and no covariates were used for the 3 × 3 metamorph experiment (see Table S3). To determine if there was a difference among the Bd metabolite prophylaxis treatments in terms of impact on Bd load, we removed the controls and re-ran the same negative binomial generalized linear model (package: glmmTMB, function: glmmTMB; family: nbinom2; function: summary). Additionally, we ran a negative binomial generalized linear model (package: glmmTMB, function: glmmTMB; family: nbinom2; function: summary) to evaluate if there was an effect of Bd metabolite prophylaxis exposure in general on Bd load. For this analysis, we worked within each experiment and grouped all individuals that were exposed to any Bd metabolite prophylaxis together and compared them to the control animals.

We used a generalized linear model (package: stats, function: glm; function: summary) to evaluate if there was a difference in impact of the Bd metabolite prophylaxis treatments (i.e. log concentration × duration) on prevalence. Additionally, to determine if there was an effect of Bd metabolite prophylaxis exposure in general on prevalence, within each experiment we grouped all individuals that were exposed to any Bd metabolite prophylaxis together and compared them to the control animals (the response variable was still individual infection status) using a generalized linear model (package: stats, function: glm; family: binomial; function: summary).

Prior work has shown that extremely high concentrations of Bd metabolites can harm nontarget organisms (McMahon et al., 2013; Nordheim et al., 2021) and that metabolite exposure can alter amphibian growth and development (McMahon et al., 2019). To test for potential deleterious effects of the Bd metabolite prophylaxis on growth, development, and survival, generalized linear models (package: stats, function: glm; function: summary) were run to determine if log concentration × duration impacted tadpole Gosner stage (Gosner stage models were only run with the tadpole data) and tadpole and metamorph final mass (final mass was run in addition to Gosner stage in tadpoles because it can be independent of developmental stage). A Cox Proportional-Hazards Model (package: KMsurv, function: coxph; function: summary) was used to determine if there was an impact of log concentration × duration on mortality. Figure 1 was created in R (package: ggplot2, function: ggplot; Wickham, 2016) and the other figures were created in Excel (version 16.78.3).

3 | RESULTS

3.1 | 5 × 5 tadpole experiment

When examining the entire data set, exposure to greater metabolite concentrations significantly reduced Bd load ($z = -2.08$, $p = 0.038$; Figure 1), but there was no significant effect of the duration, Gosner stage or the concentration × duration interaction on Bd load ($z = -1.50$, $p = 0.135$, $z = 0.108$, $p = 0.914$, $z = 1.13$, $p = 0.258$, respectively). When analysing only the tadpoles that received the Bd metabolite prophylaxis, that is, the controls were removed from this analysis, there was no effect of log concentration, duration, Gosner stage or log concentration × duration ($z = 3.50$, $p = 0.852$, $z = 0.078$, $p = 0.781$, $z = 0.424$, $p = 0.515$, or $z = 0.063$, $p = 0.803$, respectively; Figure 2). These findings indicate that significance was driven by the difference between controls and any level of Bd metabolite prophylaxis exposure. When comparing tadpoles that received any Bd metabolite prophylaxis to the control animals there was a reduction in Bd load but no effect of Gosner stage ($z = -2.95$, $p = 0.003$, $z = 0.280$, $p = 0.780$, respectively; Figure 2). There was no effect of log Bd metabolite prophylaxis log concentration, duration or log concentration × duration ($z = -1.89$, $p = 0.059$, $z = -0.449$, $p = 0.653$, $z = 0.240$, $p = 0.811$, respectively;

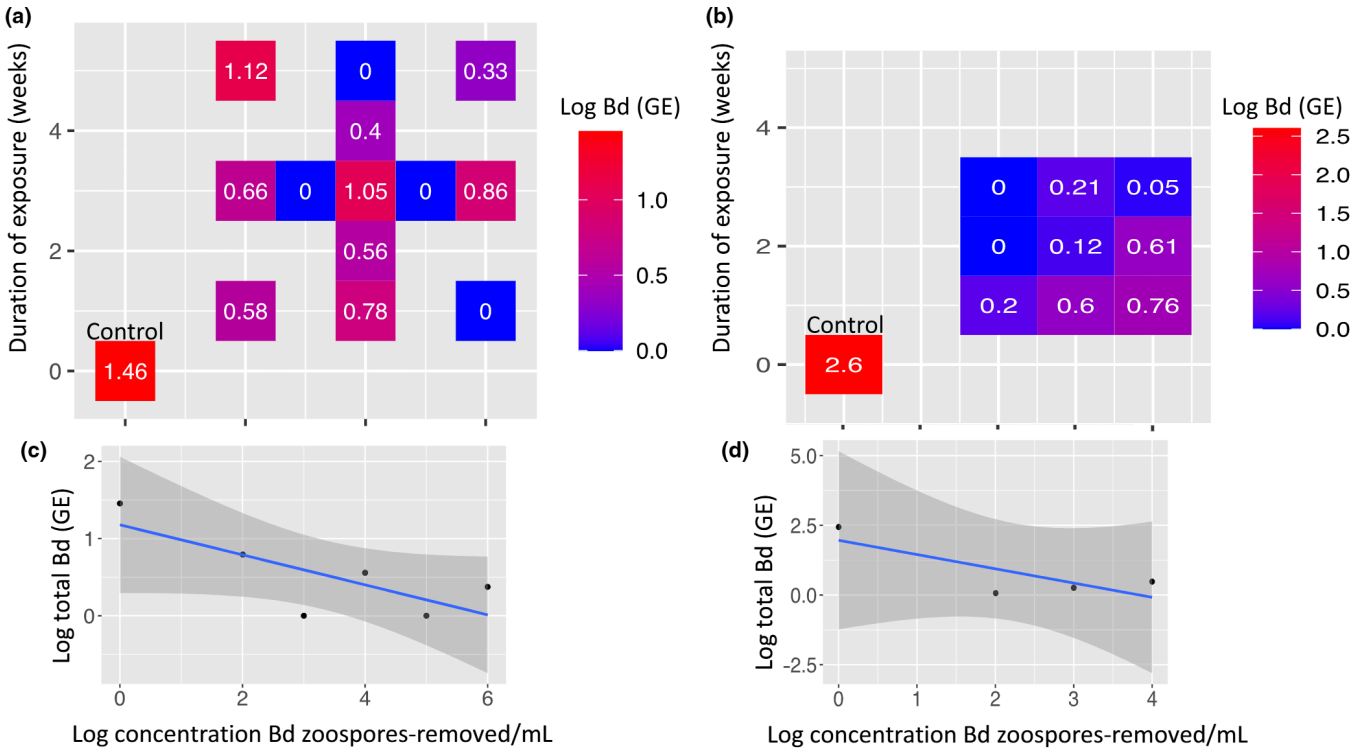

FIGURE 1 The average final *Batrachochytrium dendrobatidis* (Bd) load (log Genome Equivalents) in *Pseudacris regilla* tadpoles (a) and metamorphs (b) exposed to a Bd metabolite prophylaxis or ASW control. There was a negative correlation with Bd metabolite concentration and Bd load for both tadpoles (c) and metamorphs (d). Data for (c and d) are shown with all duration points grouped together to show effect of concentration on Bd load. Control tadpoles were exposed to an ASW control and were not exposed to the Bd metabolite prophylaxis. Shown are means and 95% CI.

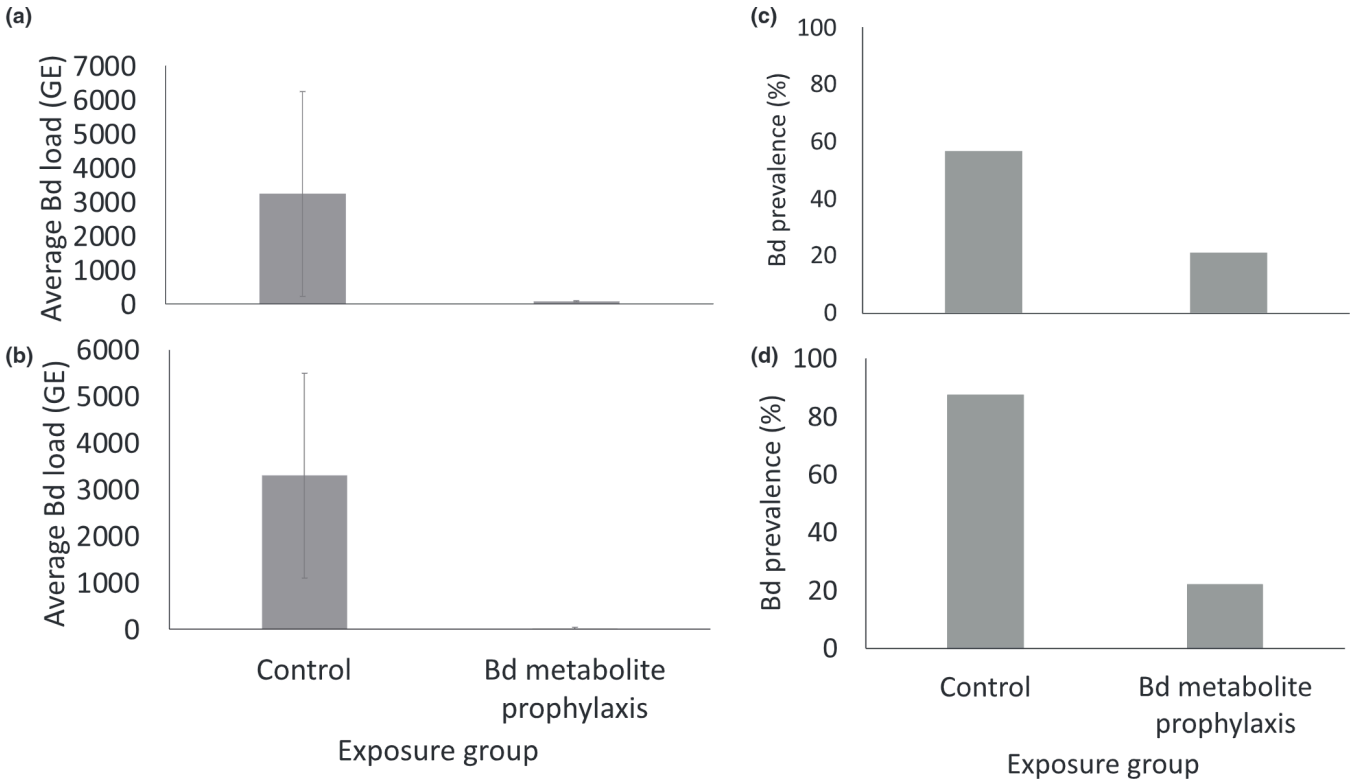

FIGURE 2 *Pseudacris regilla* exposed to a *Batrachochytrium dendrobatidis* (Bd) metabolite prophylaxis (all Bd metabolite prophylaxis treatments were grouped together) had lower Bd load (tadpoles [a] and metamorphs [b]) and Bd prevalence (tadpoles [c] and metamorphs [d]) than animals exposed to an ASW control. For tadpoles $n = 16$ and 87, and for metamorphs $n = 20$ and 90, controls and Bd metabolite prophylaxis, respectively for both.

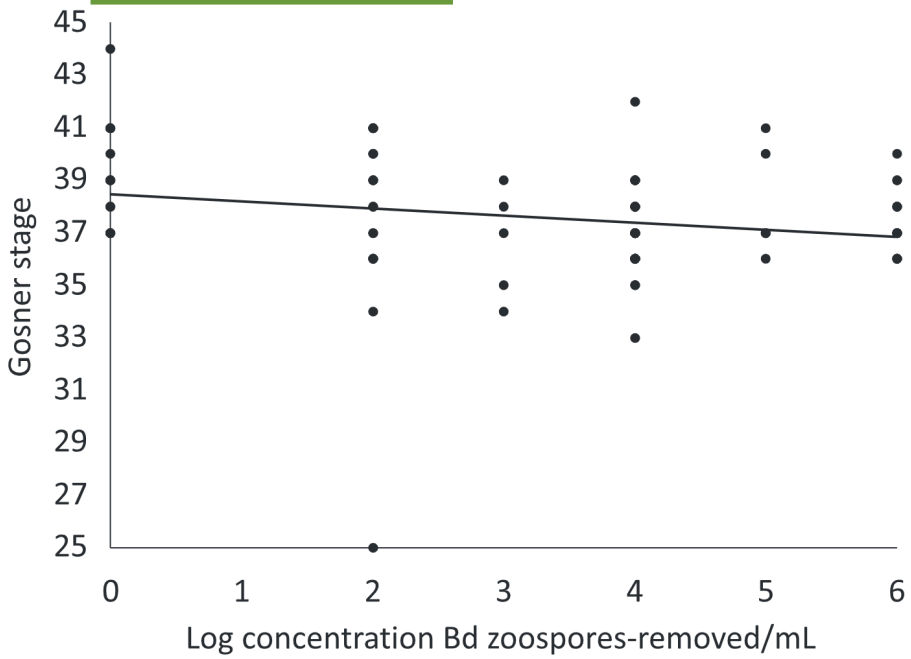

FIGURE 3 There was a negative correlation between log concentration of Bd (*Batrachochytrium dendrobatidis*) metabolites (Bd zoospores-removed/mL) and Gosner stage of *Pseudacris regilla* tadpoles exposed to a Bd metabolite prophylaxis. All control animals were exposed to an artificial spring water control and were not exposed to the Bd metabolite prophylaxis.

Figure S1) on prevalence. Bd metabolite prophylaxis exposure reduced Bd prevalence when comparing tadpoles that received any Bd metabolite prophylaxis to the control animals ($z=-2.80$, $p=0.005$; prevalence for control: 56.3% and Bd metabolite prophylaxis: 20.5%; Figure 2).

We examined the effect of prophylactic exposure on host development, growth and survival and we found that there was an effect of log concentration but not duration, or log concentration × duration on Gosner stage ($t=-2.21$, $p=0.03$, $t=-1.79$, $p=0.078$, $t=1.57$, $p=0.12$, respectively; Figure 3; see Section 2.6 for reasoning). There was no effect of log concentration and duration, or log concentration × duration on final mass ($t=0.741$, $p=0.460$, $t=0.345$, $p=0.731$, $t=1.16$, $p=0.249$, respectively). There was no effect of log concentration, duration or log concentration × duration on mortality ($z=-0.294$, $p=0.769$, $z=-0.068$, $p=0.946$, and $z=1.140$, $p=0.254$, respectively; Table S4).

3.2 | 3 × 3 metamorph experiment

Bd metabolite prophylaxis log concentration and duration of exposure reduced Bd load and there was a log concentration × duration interaction on Bd load ($z=--3.36$, $p<0.001$, $z=-4.47$, $p<0.0001$, $z=3.52$, $p<0.001$, respectively; Figure 1). When analysing only the metamorphs that received the Bd metabolite prophylaxis, that is, the controls were removed from this analysis, there was no effect of log concentration, duration, or log concentration × duration on Bd load ($z=0.128$, $p=0.898$, $z=-1.15$, $p=0.249$, $z=0.971$, $p=0.332$, respectively; Figure 2). These findings indicate again that the significance described above was driven by the difference between controls and any level of Bd metabolite prophylaxis exposure. There was no effect of log concentration on prevalence but there was an effect of duration and log concentration × duration on prevalence ($z=-1.27$, $p=0.206$, $z=-3.343$, $p<0.001$, $z=2.536$, $p=0.011$, respectively; Figure S1). Bd metabolite prophylaxis exposure reduced Bd prevalence when comparing metamorphs that received any Bd metabolite prophylaxis to the control animals ($z=-3.99$, $p<0.0001$; prevalence for control: 87.5% and Bd metabolite prophylaxis: 21.9%; Figure 2). There was no effect of log concentration, duration, or log concentration × duration on final metamorph mass ($t=-0.123$, $p=0.902$, $t=-0.094$, $p=0.925$, and $t=-0.313$, $p=0.755$, respectively) or mortality ($z=1.201$, $p=0.230$, $z=0.016$, $p=0.987$, and $z=-0.701$, $p=0.483$, respectively).

4 | DISCUSSION

Bd is arguably one of the deadliest organisms on earth as it has been linked to hundreds of species declines and extinctions (Scheele et al., 2019). Previous work found that Cuban treefrog tadpoles can gain protection from Bd after a Bd metabolite prophylaxis treatment (Barnett et al., 2021; Nordheim et al., 2022). Our long-term goal is to use this work to help develop a prophylactic that could be used to protect amphibian populations in the field. Indeed, wildlife vaccination has been used effectively to help protect several wildlife species (e.g. Ethiopian wolves and prairie dogs; Randall et al., 2006; Rocke et al., 2017; Tripp et al., 2017). Previously, we found that both the Bd strain used to create the Bd metabolites and the timing of Bd metabolite prophylaxis exposure were crucial to yield effective protection against Bd (Barnett et al., 2021, 2023). Here, we further refine our understanding of how to deliver prophylactic protection to amphibians efficaciously. This laboratory work is crucial to develop effective field tools to mitigate the current amphibian conservation crisis.

Here, we show that the tested Bd metabolite prophylaxis can be used in a laboratory to effectively reduce Bd loads in a second

species of amphibians, Pacific chorus frogs. Thus, we now have evidence that the Bd metabolite prophylaxis confers some protection to Bd in two amphibian species, Cuban treefrogs and Pacific chorus frogs, and across multiple life stages (Barnett et al., 2021, 2023; Nordheim et al., 2022). For both tadpoles and metamorphs, the Bd metabolite prophylaxis may help reduce Bd infection occurrence; we found that animals exposed to the Bd metabolite prophylaxis had reduced prevalence compared to those exposed to the control. Additionally, for both life stages, exposure to the Bd metabolite prophylaxis reduced Bd load. While there was an effect of Bd metabolite prophylaxis concentration when comparing to the control treatment, there was no difference in Bd load when comparing within the Bd metabolite prophylaxis treatment groups alone. This indicates that the effect was driven by the difference in load between the controls and any treatment group receiving Bd metabolite prophylaxis, regardless of concentration. We also found a significant reduction in Bd load when comparing all the Bd metabolite prophylaxis treatments lumped together to the controls in both experiments. Exposure to the Bd metabolite prophylaxis reduced infection intensity by over 65% in both the tadpoles and metamorphs. If a metabolite-based vaccine can effectively protect the majority of susceptible individuals in a community, it would have a strong chance of achieving herd immunity (Anderson, 1992; Fine et al., 2011) and offering population-level protection.

Realistically, to implement treatments in the field (please note, more research is needed before we can suggest wide spread field exposures; see Barnett et al., 2021, 2023), exposing tadpoles to high concentrations of Bd metabolites for long durations of time would be extremely difficult. Fortunately, we found that animals exposed every weekday for 1 week to the lowest concentration of Bd metabolites tested (100 zoospores-removed/mL) gained some protection against Bd. The efficacy of these low concentrations should make the application of a field vaccine more practical.

In both tadpoles and metamorphs, we found that there was a negative correlation between Bd metabolite concentration exposure and subsequent Bd load after challenge infections; and importantly, even exposure to the lowest concentration provided protection against Bd. We also found that exposure to the same Bd metabolite prophylaxis altered the host microbiome increasing the Bd-inhibitory taxa (Siomko et al., 2023). Indeed, there was a positive correlation between Bd metabolite concentration and duration and this protective anti-Bd microbiome (Siomko et al., 2023). Host microbiome modulation may have a strong impact on host-pathogen resistance (Woodhams et al., 2020), which may in turn impact prophylaxis efficacy.

Duration of prophylactic exposure did not have an effect on Bd load for tadpoles, though it did for metamorphs. Importantly for both life stages, we found that the shortest duration of exposure tested (1 week of exposures) was sufficient to yield a protective response and therefore, we suggest use of this exposure duration to minimize personnel effort and the possibility of any deleterious impacts on non-targeted taxa. There was no effect of treatment on mortality in the metamorphs or tadpoles. Additionally, many species of tadpoles, including the species studied here, are ephemeral and do not spend more than a few weeks in a body of water, making a shorter duration of exposure more feasible in the field.

Before widely distributing a Bd vaccine, it must first be shown to be safe and effective. To this end, there was no effect of treatment on tadpole mass, but there was a negative correlation between log concentration and Gosner stage in the tadpoles exposed to the Bd metabolite prophylaxis. While tadpole development may be slower, tadpoles of a lower Gosner stage were equal in mass to the later stage tadpoles and so they may be larger comparatively when they reach metamorphosis. More robust tadpoles may have larger energy reserves at metamorphosis and may be more successful than less robust counterparts (Goater, 1994; Goater et al., 1993; Scott et al., 2007). We previously found that exposure to the highest Bd metabolite prophylaxis tested in this study did not have deleterious impacts on the development of Cuban treefrog tadpoles (McMahon et al., 2019).

Researchers have been working to develop effective mitigation protocols to help alleviate the adverse impact of Bd on amphibian populations (e.g. see Bletz et al., 2013; Muletz et al., 2012; Waddle et al., 2021). The potential vaccine discussed here does not yield complete resistance, but short-term exposure to low concentrations of Bd metabolites did provide protection by reducing infection loads. Given that Bd metabolites have now been shown to yield protection across amphibian life stages and in multiple species, researchers should move forward in testing whether Bd metabolite prophylaxis can proactively protect amphibian populations in the field from Bd outbreaks, particularly for at-risk populations and species of conservation concern. While any large-scale field exposure would be logistically intensive, the preparation of the Bd metabolite prophylaxis does not require expensive equipment, and so field implementation would not likely be too cost prohibitive. This work should facilitate both conservation and restoration efforts targeted at amphibians (Rohr et al., 2018).

AUTHOR CONTRIBUTIONS

Taegan A. McMahon, David J. Civitello, Pieter T. J. Johnson, and Jason R. Rohr designed experiments, Taegan A. McMahon, Alexis M. Garcia, Aiza J. Malinias, Manuela Mosalve, Liam C. Muldro, and Edin O. Sisson conducted experiments, Taegan A. McMahon conducted statistical analyses and wrote the paper, and all authors provided funding and editorial advice.

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CONFLICT OF INTEREST STATEMENT

Jason R. 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. All other authors have no conflict of interest to declare.

DATA AVAILABILITY STATEMENT

Data available via the Dryad Digital Repository https://doi.org/10.5061/dryad.9zw3r22qn (McMahon et al., 2024).

STATEMENT OF INCLUSION

Our experimental group is comprised of a diverse group of researchers, including individuals from different countries, ethnicities, and racial and gender identities. About 67% of the coauthors identify with one or more underrepresented groups in academia and 56% of the coauthors are undergraduate researchers.

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SUPPORTING INFORMATION

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

Figure S1. (A) There was no effect of log concentration, duration of exposure, or log concentration-by-duration on prevalence in *Pseudacris regilla* tadpoles exposed to a Bd (*Batrachochytrium dendrobatidis*) metabolite prophylaxis. (B) There was no effect of log concentration on prevalence but there was an effect of duration and log concentration-by-duration on prevalence.

Table S1. Experimental design and sample sizes for the tadpole (5 × 5) and metamorph (3 × 3) experiments.

Table S2. Final statistical analyses used to determine the efficacy of Bd metabolite prophylaxis exposure on *Pseudacris regilla* tadpoles (5 × 5) and metamorphs (3 × 3).

Table S3. Model selection was used to determine the best fit model examining if log concentration × duration impacted Bd load.

Table S4. The percent mortality of *Pseudacris regilla* tadpoles (5 × 5) and metamorphs (3 × 3) exposed to each treatment.

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