BIOLOGY LETTERS

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Research

Cite this article: Barnett KM, Detmering SE, McMahon TA, Civitello DJ. 2021 Asymmetric cross-strain protection for amphibians exposed to a fungal-metabolite prophylactic treatment. *Biol. Lett.* **17**: 20210207.
https://doi.org/10.1098/rsbl.2021.0207

Received: 14 April 2021
Accepted: 2 August 2021

Subject Areas:
ecology, health and disease and epidemiology

Keywords:
wildlife vaccination, amphibian decline, conservation, acquired resistance

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THE ROYAL SOCIETY PUBLISHING

Conservation biology

Asymmetric cross-strain protection for amphibians exposed to a fungal-metabolite prophylactic treatment

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Chytridiomycosis, an infectious disease of amphibians caused by the fungal pathogen *Batrachochytrium dendrobatidis* (Bd), poses an imminent conservation threat. The global spread of Bd has led to mass mortality events in many amphibian species, resulting in at least 90 species' extinctions to date. Exposure to Bd metabolites (i.e. non-infectious antigenic chemicals released by Bd) partially protects frogs during subsequent challenges with live Bd, suggesting its use as a prophylactic treatment and potential vaccine. However, we do not know whether Bd metabolite exposure protects against strains beyond the one used for treatment. To address this knowledge gap, we conducted a 3 × 2 experiment where we exposed adult Cuban treefrogs, *Osteopilus septentrionalis*, to one of three treatments (Bd metabolites from California-isolated strain JEL-270, Panamá-isolated strain JEL-419, or an artificial spring water control) and then challenged individuals with live Bd from either strain. We found that exposure to Bd metabolites from the California-isolated strain significantly reduced Bd loads of frogs challenged with the live Panamá-isolated strain, but no other treatments were found to confer protective effects. These findings demonstrate asymmetric cross-protection of a Bd metabolite prophylaxis and suggest that work investigating multiple, diverse strains is urgently needed.

1. Introduction

Pandemics and epidemics are increasing in frequency across taxonomic groups, and the high infection prevalence of these pathogens facilitates the emergence of novel pathogen strains [1–3]. Pathogen strains can differ in their ability to overcome host resistance mechanisms and can consequently influence the efficacy of disease control interventions [4]. Thus, successful disease management programmes must consider the strength of such interventions across pathogen strains.

The global emergence and spread of *Batrachochytrium dendrobatidis* (Bd) is a major driver of amphibian biodiversity loss [5]. Host death occurs by cardiac arrest when high Bd loads disrupt cutaneous osmoregulation and electrolyte balance [6]. Mass mortalities due to Bd have led to the decline of hundreds of frog populations and the extinction of at least 90 frog species to date [5]. Given the dire consequences of the Bd pandemic for global amphibian diversity, novel disease control methods are urgently needed.

Prophylactic treatments, like vaccines, could serve as a management intervention to stabilize amphibian populations endangered by Bd. Vaccination induces acquired resistance via non-pathogenic antigen exposure. Its success as a public health intervention stems from its population-level advantages. Vaccination can generate herd immunity, for example, which benefits both

vaccinated and unvaccinated hosts through interrupted pathogen transmission. Wildlife vaccination can prevent, reduce or eliminate disease outbreaks [7] and has been used to reduce the risk of disease-induced extinction in Ethiopian wolves, African wild dogs and prairie dogs [8–10].

Vaccinating amphibians could curtail Bd epidemics and prevent further Bd-induced biodiversity loss [11]. Amphibians can acquire resistance to Bd when exposed to killed Bd zoospores and metabolites (i.e. non-infectious antigenic chemicals produced by Bd) [11], a promising finding in the search for a vaccine against this deadly pathogen. Recent work using filtration to separate metabolites from killed zoospores demonstrated that exposure to Bd metabolites alone decreased Bd loads more upon subsequent live Bd challenge than exposure to killed Bd zoospores alone [12]. These findings indicated that Bd metabolites, a cell-free non-infectious treatment, can be used prophylactically to provide resistance against live Bd infection [12]. While Bd metabolites have prophylactic benefits, it remains unknown whether they confer resistance by stimulating the innate or adaptive immune system. Given this, we refer to Bd metabolites as a prophylactic treatment and we investigate its functional applications within the context of wildlife vaccination campaigns.

Wildlife vaccination success is subject to the complexities of wildlife and parasite ecology [13], and there remain outstanding questions regarding the efficacy and feasibility of Bd metabolites as a method to control Bd outbreaks. Given the high genetic diversity [14] and global distribution of Bd [5], it is important to determine whether Bd strains vary in strength or breadth (i.e. cross-protection) of resistance. Evaluating strain variation in efficacy and cross-protection is critical for the development and deployment of a prophylactic treatment, like a vaccine, to combat amphibian declines.

Here, as the first test of cross-strain protection, we experimentally assess strain specificity in the efficacy (quantified as reduced pathogen prevalence and intensity) of Bd metabolite prophylactic treatments using a comparison of strains isolated from Panamá and California. We anticipated strain-based differences in infection prevalence, intensity and virulence because the Panamá strain was isolated during an epidemic amphibian mortality event [15], while the California strain was isolated from a stable and tolerant amphibian population. We predicted same-strain treatments (i.e. exposure to Bd metabolites of the same strain as that used for the live Bd challenge) to have the strongest protective effect, and cross-strain treatments (i.e. exposure to Bd metabolites of a different strain than that used for the live Bd challenge) to be less effective. Ultimately, strong cross-strain protection would increase the feasibility of large-scale Bd vaccination campaigns, while narrow protection would suggest that vaccination strains might need to be tailored to individual populations or regions.

2. Methods

(a) Frog husbandry

We collected adult Bd-naive Cuban treefrogs (*Osteopilus septentrionalis)* from Hillsborough County, Tampa, FL and maintained them at 18°C in a 12 : 12 light : dark photoperiod during the entire experiment. This temperature is ideal for Bd growth [16] and does not appear to cause the frogs distress. We fed the frogs calcium-dusted, vitamin-enriched crickets and maintained them in 1 l plastic deli cups with paper towels dampened with artificial spring water (ASW). We conducted weekly container changes, checked mortality daily, and any dead animal was swabbed for Bd immediately (see *Molecular detection of Bd* for details). The work was approved by and conducted in compliance with IACUC at the University of Tampa.

(b) *Batrachochytrium dendrobatidis* culture and *Batrachochytrium dendrobatidis* metabolite treatment preparation

We used the same methodology as Nordheim *et al*. to produce the stock Bd culture and Bd metabolite treatments (for detailed methods see [8]). We used strains isolated from California (JEL 270) and Panamá (JEL 419) for both Bd metabolite treatments and live challenges and ASW [11] as the control treatment. To increase readability, we refer to the strains by their collection location (California or Panamá), but we are not suggesting that these strains are necessarily broadly representative of these regions. We cultured Bd strains separately in 1% tryptone broth. We then inoculated 1% tryptone agar plates (60 mm diameter) with 3 ml of a single strain for a total of 4–5 plates per strain and maintained them at 18°C for two weeks. We flooded the plates (4–5 plates per strain) with ASW for approximately 3 min to suspend the zoospores and zoosporangia and homogenized the liquid across all plates to create a Bd+ stock for each strain. We detected no difference in zoospore production between strains (two-sample *t*-test on zoospore concentration; $n = 4$/strain, $p = 0.71$). We then standardized these concentrations to (9×10^5 zoospores ml^{-1}). To produce the Bd metabolite treatment for each strain, we filtered the Bd+ stock liquid through a 1.2 µm filter (GE Whatman Laboratory Products) to remove zoospores and zoosporangia. We conducted a visual inspection with a light microscope to verify no zoospores or zoosporangia remained in the Bd metabolite treatment. Additionally, a 1 ml aliquot of the Bd metabolite treatment from each stock was plated on 1% tryptone plates to verify there was no growth over an 8-day period ($n = 3$/strain; there was no growth). We refer to the concentration of this filtrate as 9×10^5 zoospores removed ml^{-1} in reference to this pre-filtration concentration. We maintained aliquots of the Bd metabolite filtrate in a laboratory-grade −20°C freezer and thawed the necessary volume to room temperature for each dosing event.

(c) Study design

We used a 3×2 factorial design with three prophylactic treatments (California strain metabolites, Panamá strain metabolites or an ASW control) and two Bd strains (California strain and Panamá strain) for the live pathogen challenge. The sample size per treatment ranged from 13 to 17 frogs ($n = 89$ frogs). Based on a generalized linear model of log-transformed initial masses, there were no significant differences (all $p > 0.1$) in mean mass of frogs between treatment groups. For the first 13 days, we dosed each frog daily with 1 ml of their respective prophylactic treatment dispensed on their dorsal surface. After the 13 days of prophylactic exposures, we exposed half of the frogs in each prophylactic treatment to 1 ml of live Bd (9×10^5 zoospores ml^{-1}) from either the California or Panamá strain. We obtained live Bd inoculum as above, and again detected no difference in zoospore production between strains (two-sample *t*-test on zoospore concentration; $n = 4$/strain, $p = 0.86$) prior to standardization at (9×10^5 zoospores ml^{-1}). We maintained the frogs for 16 days, after which they were swabbed 10 times from hip to toe on their left hind limb. These swabs were used for molecular detection of Bd.

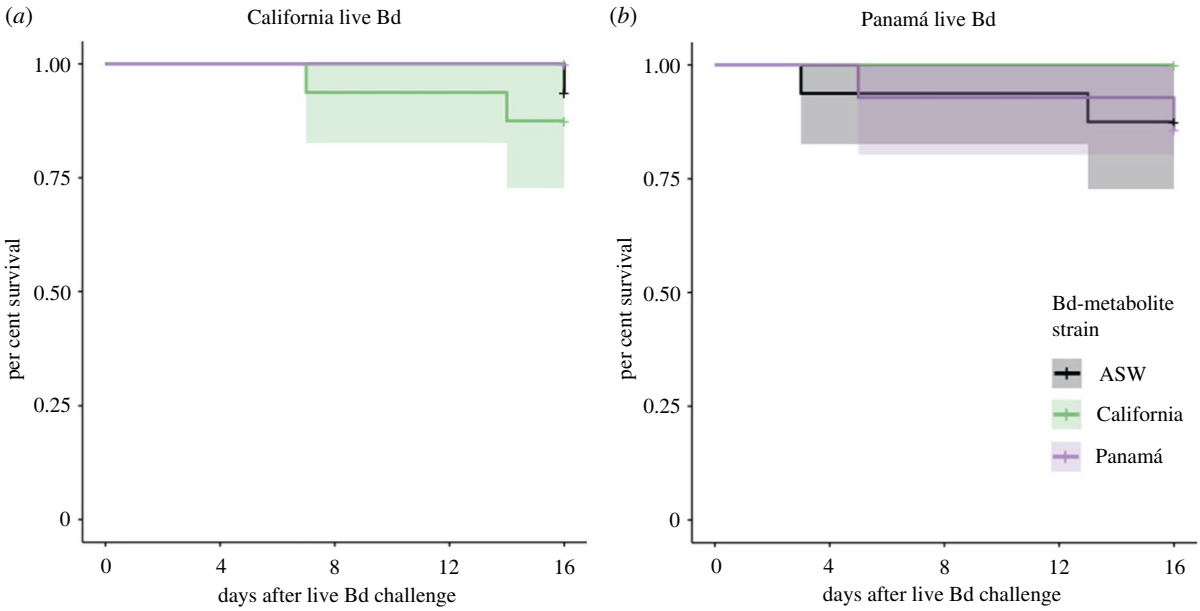

Figure 1. Per cent survival following live Bd challenge for frogs exposed to Bd metabolites from one of three prophylactic treatments: Bd metabolites from a California-isolated strain (green), Bd metabolites from a Panamá-isolated strain (purple) and ASW control (black). Following metabolite exposure, frogs were challenged with either (*a*) the California-isolated strain or (*b*) the Panamá-isolated strain. Survival was high throughout the experiment, and there were no differences in mortality among treatments. The lines indicate the per cent survival and the bands represent the 95% CI.

(d) Molecular detection of *Batrachochytrium dendrobatidis*

We quantified the Bd load from each frog using quantitative PCR (qPCR; see [14]) with plasmid standards designed to target Bd from Pisces Molecular. The qPCR methods we used yielded the number of genome equivalents (GE) in the sample. Given that strains have different GE per zoospore [17] and we wanted to compare the Bd loads across strains, we standardized the zoospore quantities according to the number of GE per zoospore (Panamá: 19.22GE/zoospore and California: 253.1GE/zoospore). Importantly, the results we present are in zoospores, not GE.

(e) Data analysis

We conducted all statistical analyses in R statistical software, v. 4.0.3 [18]. We used the Cox proportional-hazards model (package: KMsurv, function: coxph) with prophylactic treatment crossed with live Bd strain as predictors to assess mortality [19]. A binomial generalized linear model on binary infection status indicated that prevalence did not differ among the treatments. Therefore, we tested for differences in infection intensity using a zero-inflated negative binomial generalized linear model (package: glmmTMB, function: glmmTMB) using prophylactic treatment crossed with live Bd strain as predictors for infection intensity. Given the similarity in prevalence among treatments, we fit a common intercept for the zero-inflation component of the model [20]. We also conducted pairwise *post hoc* tests to compare each of the three prophylactic treatments within each level of the live Bd strain by re-running the glmmTMB zero-inflated negative binomial models isolating pairs of treatments and using Bonferroni corrections for multiple testing (corrected $\alpha = 0.0083$).

3. Results

Overall, 89% of frogs survived the length of the experiment and neither Bd metabolite strain nor live challenge strain affected mortality (figure 1). Zoospore loads (rounded to the nearest integer) ranged from 1 to 81 726. While there was no significant difference in mortality or prevalence, we found a significant interaction between prophylactic treatment (Bd metabolite strain) and live Bd challenge strain on infection intensity in the zero-inflated model (prophylactic treatment × live Bd interaction; $B = -5.22$, $z = -3.38$, $p = 0.001$). The pairwise contrasts indicated that frogs exposed to Bd metabolites of the California strain and then exposed to the live Panamá strain had lower Bd loads than frogs exposed to Bd metabolites of the Panamá strain ($B = 5.53$, $z = 5.44$, $p < 0.0001$) and the ASW treatment ($B = -4.66$, $z = -4.91$, $p < 0.0001$, figure 2).

4. Discussion

Here, we demonstrate asymmetric cross-strain protection of a Bd metabolite prophylactic treatment, which contradicts the hypothesis that same-strain treatments would be more effective due to antigenic similarity. Indeed, we found that the California-strain Bd metabolite treatment was more effective than the same-strain treatment against the live Panamá strain, whereas we detected no protective effects against infections with the California strain. Thus, cross-strain protection may not be a generalizable outcome to mismatched treatments. While we did not detect a significant acquired resistance response in same-strain treatments, previous experiments have found these effects using killed Bd zoospores and metabolites [11] and Bd metabolites alone [12]. We suspect low infection intensities in the control treatment limited our statistical power to detect previously observed same-strain protection, but it is also possible that same-strain treatment efficacy is dependent on strain or host life stage. Additionally, low infection intensities in the control treatment may have limited our ability to detect an effect of cross-strain protection in frogs exposed to Bd metabolites of the Panamá strain and then challenged with the live California strain. Furthermore, while we hypothesized differences in strain virulence between the two live Bd strains used, we were not able to fully evaluate the impact of strain virulence

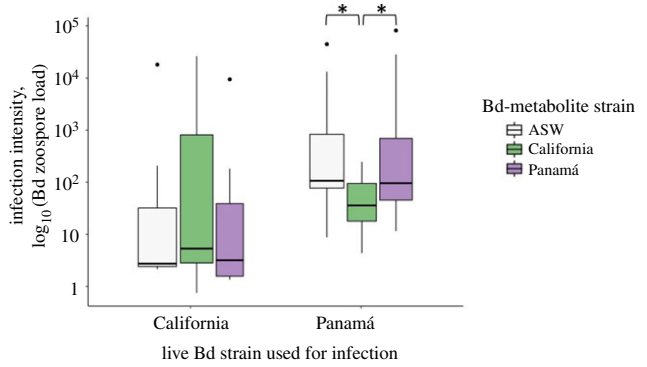

Figure 2. Infection intensity (i.e. zoospore load of infected individuals) for frogs exposed to Bd metabolites from one of three prophylactic treatments (Bd metabolites from a California-isolated strain, Bd metabolites from a Panamá-isolated strain and ASW control) and subsequently challenged with one of the two live Bd strains (California-isolated or Panamá-isolated). Frogs treated with Bd metabolites from the California-isolated strain and challenged with the live Panamá-isolated strain had significantly lower Bd zoospore loads than frogs treated with Bd metabolites from the Panamá-isolated strain and frogs treated with the ASW control. The dots above the boxplot whiskers represent observations that extend more than 1.5 times beyond the interquartile range.

because we ended the experiment 16 days after exposure to live Bd in accordance with IACUC. We found high survival overall and no significant difference in mortality among treatments, which was not unexpected given that infection-induced mortality does not typically begin that soon after Bd exposure in this species.

While our study demonstrates asymmetric cross-protection, it does not explicitly implicate a mechanism. However, contextualizing our findings with recent research on Bd metabolites points to a new hypothesis regarding strain variation in efficacy of a Bd metabolite prophylaxis. Our observation of asymmetric cross-protection might be a result of differences in strain virulence and immunosuppression. Some of the metabolites Bd produces (e.g. methylthioadenosine, tryptophan and spermidine) are immunosuppressive [21,22]. These factors can suppress immunity by decreasing lymphocyte functioning and proliferation and inducing apoptosis [21,23]. Given that our Bd metabolite treatments are composed of all of the soluble chemicals Bd produces, the Bd metabolites we used to induce acquired resistance also presumably contain these immunosuppressive factors [21,22].

Differences in treatment efficacy among Bd strain combinations could be attributable to differences in either the properties or relative concentrations of resistance-inducing components or immunosuppressive factors. If immunosuppressive factors are correlated with virulence, or even contribute to higher virulence, then we hypothesize that Bd metabolites from higher virulence strains will be less effective or ineffective prophylaxis treatments. Indeed, the Bd strains we used likely differed in virulence [24], which may have influenced our findings. The Panamá strain was isolated during an amphibian die-off event [15] and is thought to be a highly virulent strain, whereas the California strain is thought to be endemic and less virulent because it was isolated in a stable population. We speculate that the same-strain Panamá treatment may have been ineffective if Panamá metabolites contain a large concentration of virulence or immunosuppressive factors. Broad comparative tests are needed at the physiological level to identify immune-inducing and immunosuppressing compounds contained within Bd metabolite profiles, and at the organismal level to evaluate this hypothesized correlation.

In order for a prophylactic treatment or vaccine to be feasibly implemented at large scales to reduce Bd-induced amphibian declines, we need a strong understanding of the ecological heterogeneities, such as differences driven by Bd strain and host species, that impact its efficacy. Our findings provide evidence that strain specificity can influence the effectiveness of inducing acquired resistance against Bd and thus these results contribute to the development of feasible large-scale vaccination campaigns for amphibians. Comprehensive comparative studies of strain specific acquired immunity, paired with metabolomic profiling of each strain, could identify the specific active compounds responsible for potent and broad resistance to Bd and therefore strengthen conservation efforts for hundreds of amphibian species.

Ethics. All procedures were approved by the University of Tampa IACUC, under protocol no. 2018-2.

Data accessibility. Data and analysis code are available via the Dryad Digital Repository: https://doi.org/10.5061/dryad.qjq2bvqgd [25].

Authors' Contribution. K.M.B., T.A.M. and D.J.C. conceived the experiments. K.M.B., S.E.D. and T.A.M. conducted the experiments. K.M.B. and D.J.C. conducted the analyses and wrote the manuscript. T.A.M. and D.J.C. provided funding. All authors edited and approved the final manuscript and agree to be held accountable for the content of our findings.

Competing interests. We declare we have no competing interests.

Funding. Funds were provided by grants from the National Science Foundation (D.J.C.: IOS-1755002, T.A.M.: IOS-1754862, K.M.B.: GRFP DGE-193797) and the National Institutes of Health (T.A.M.: 1R01GM135935-0, KK 2022).

Acknowledgements. We thank members of the Civitello Lab for their valuable feedback on the manuscript and J. Longcore for providing the Bd strains. We also acknowledge the lives of the frogs used in this study, without which this study could not have been conducted.

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