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Title Assessing the efficacy and feasibility of a prophylactic treatment for chytridiomycosis

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Assessing the efficacy and feasibility of a prophylactic treatment for chytridiomycosis

By

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Advisor: David J. Civitello, Ph.D.

An abstract of a dissertation submitted to the Faculty of the

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Abstract

Assessing the efficacy and feasibility of a prophylactic treatment for chytridiomycosis

By Katherine Mary Barnett

Chytridiomycosis is an infectious disease of amphibians caused by the fungal parasite *Batrachochytrium dendrobatidis* (Bd). The global distribution of Bd poses an imminent conservation threat as the introduction of Bd has led to mass mortality events in many species of frog, even resulting in 90 species' extinctions. Previous work found that exposure to non-infectious antigenic metabolites produced by Bd imperfectly immunizes frogs against the fungal parasite. Inducing acquired resistance (via vaccination or prophylaxis) is an impactful tool used for parasite elimination and eradication in public health, and vaccination in wildlife is increasingly applied for conservation and spillover disease prevention. For my dissertation, I combined laboratory experiments, disease modeling techniques, and a large-scale field manipulation experiment to determine the impacts of strain heterogeneity, host's pathogen exposure history, and partial protection on the effectiveness of Bd prophylaxis for conservation-motivated disease control. I found that protection provided by Bd metabolite prophylaxis was sensitive to ecological factors such as pathogen strain and the host's exposure history to Bd. Moreover, protection provided by Bd metabolites is partial, such that prophylactic treatment reduces infection intensities but does not completely block infections. Given this, I built a system-specific agent-based model to explore scenarios varying mode of prophylaxis protection, degree of treatment efficacy, and proportion of population treated. Lastly, I conducted a Before-After-Control-Impact field experiment to test the effectiveness of Bd metabolite prophylaxis when administered in natural populations. Unexpectedly, infection intensities significantly increased after Bd metabolite addition in field-treated frogs, as compared to frogs from ponds treated with a sham control. Model scenarios in which prophylaxis strongly boosts tolerance (i.e., a host's ability to survive high infection intensities), with no or minimal increase in resistance, are consistent with this field result. While tolerance is challenging to measure empirically, we suggest future studies measure the net transmission potential of treated versus untreated individuals to better project how partial protection at the individual level scales to key epidemiological outcomes on the population level. Overall, this dissertation rigorously evaluates the effectiveness of Bd metabolite prophylaxis under relevant ecological conditions, and the results caution its use to slow chytridiomycosis-induced biodiversity loss until further studies validate the mechanism behind observed increased infection intensities in field-treated frogs.

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Chapter 1: Introduction

Ecological and evolutionary challenges for wildlife vaccination

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ABSTRACT

Wildlife vaccination is of urgent interest to reduce disease-induced extinction and zoonotic spillover events. However, several challenges complicate its application to wildlife. For example, vaccines rarely provide perfect immunity. While some protection may seem better than none, imperfect vaccination can present epidemiological, ecological, and evolutionary challenges. While anti-infection and anti-transmission vaccines reduce parasite transmission, anti-disease vaccines may undermine herd immunity, select for increased virulence, or promote spillover. These imperfections interact with ecological and logistical constraints that are magnified in wildlife, such as poor control and substantial trait variation within and among species. Ultimately, we recommend approaches such as trait-based vaccination, modeling tools, and methods to assess community- and ecosystem-level vaccine safety to address these concerns and bolster wildlife vaccination campaigns.

The potential of wildlife vaccines

Vaccination, the process of exposing the immune system to an antigen to induce pathogen resistance, is a powerful tool for controlling disease. The benefits of vaccination are twofold: recipients are directly protected against infection and unvaccinated hosts are indirectly protected through **herd immunity** (**Glossary**), which reduces transmission and parasite-mediated harm to host populations [1]. Vaccination has been vastly successful for humans and livestock [2,3]. Successful vaccination campaigns against rabies in raccoons (*Procyon lotor*), red foxes (*Vulpes vulpes*), gray foxes (*Urocyon cinereoargenteus*), and coyotes (*Canis latrans*) suggest that vaccination efforts could be directed towards emerging infectious diseases (EIDs) that cause devastating host declines, e.g., amphibian chytridiomycosis, white nose syndrome, Tasmanian devil facial-tumor disease, and Ebola [4–10]. The success of vaccination in human and livestock populations, the pressing need for disease control tools in wildlife conservation, and the ever-increasing threat of zoonotic **spillover** events support a clear need to develop vaccination as an intervention tool for wildlife disease control. However, several outstanding challenges and questions remain before vaccination can emerge as a reliable tool for wildlife disease control. We argue that accounting for the limitations of imperfect vaccines, host and non-host ecology, and individual physiology in the development of vaccination campaigns is vital for harnessing the potential of wildlife vaccines successfully.

Objectives of wildlife vaccination

Biodiversity conservation and the prevention of pathogen spillover are two urgent concerns of wildlife disease control. Emerging diseases of wildlife threaten population and species persistence and contribute significantly to the ongoing loss of biodiversity [11].

Additionally, wildlife populations are **reservoir hosts** for many **zoonotic pathogens** such as rabies, Nipah virus, and coronaviruses that threaten the health of humans [12].

Controlling disease in wildlife reservoir populations can reduce spillover transmission, but complete prevention of spillover risk from a known pathogen requires elimination or eradication of a parasite within a reservoir host to prevent zoonotic transmission. Vaccines may be able to achieve this objective, but given the inherent antigenic specificity of all known vaccines, they will not prevent novel pathogen emergence. Theory underlying eradication often identifies a critical level of vaccine coverage, which drives the **effective reproductive ratio (R_{eff})** of a pathogen below the threshold value of one [1]. Combating rinderpest virus reintroduction during the eradication campaign exemplifies the intense effort needed for eradication [3].

In contrast, vaccination for conservation aims to maximize the persistence of host populations and communities by decreasing the risk of disease-induced extinction, rather than through achieving parasite elimination. Wildlife populations can generally withstand small-scale disease outbreaks, and so conservation-motivated vaccination does not always require pathogen eradication [13]. Thus, vaccination coverage required for conservation-motivated disease control tends to be lower than that required for spillover prevention. For example, modeling estimates suggest that maintaining low vaccination coverage, between 20-40%, will stave off rabies-induced extinction of Ethiopian wolves (*Canis simensis*)[13].

Vaccine efficacy and modes of imperfection

Despite their potential for controlling wildlife disease, vaccines rarely provide perfect immunity, which can compromise herd immunity or contribute to the evolution of increased

parasite virulence [14]. For example, a prototype vaccine partially protects amphibians from *Batrachochytrium dendrobatidis*; vaccination decreases, but does not eliminate, parasite proliferation [15]. In contrast, a theoretically perfect vaccine would provide permanent and complete resistance to infection for all recipients, but vaccines considered for wildlife often fall short of this definition [14]. Three broad aspects of vaccine imperfection are often discussed in the literature: waning, leaky, and partial immunity. However, "leaky" immunity is used inconsistently and imprecisely, generating confusion. One reason for this is that modeling frameworks, such as *Susceptible-Infected-Resistant* (SIR) compartment models can make it difficult to incorporate some types of vaccine imperfections. Therefore, we suggest a clarified categorization based on **waning**, **binary** and **partial immunity.** Importantly, these categories are not mutually exclusive, and we discuss the impacts of these varying levels of immunity on wildlife populations, vaccine efficacy, modeling frameworks.

Waning immunity

Waning describes the loss of resistance to infection over time. Individuals can vary in their waning rate, and immunity can be restored by subsequent exposures, i.e., "boosters". Vaccine-induced immunity often wanes faster than immunity generated from natural infection, which can leave vaccinated individuals at higher risk during recurrent or cyclical epidemics [16]. For example, Eastern Equine Encephalitis virus vaccination in sandhill (*Grus americana*) and whooping cranes (*Grus canadensis*) waned rapidly, requiring booster vaccination within 30 days [17]. Life history traits, immune boosting sources, and waning rate interact to determine vaccine utility [18]. Waning immunity is routinely and relatively easily incorporated into SIR compartment models by allowing resistant individuals to reenter the susceptible class.

Binary immunity

Binary immunity occurs when vaccination does not induce immunity in all recipients [19]. This generates a binary outcome, wherein hosts are either resistant or susceptible, with no intermediate outcome. Binary outcomes of immunization have also been described as an "all-or-nothing qualitative response" [20]. For example, high rates of binary vaccine outcomes for the varicella vaccine in humans prompted the recommendation for a second dose within months of the first [21]. Differences in vaccine **immunogenicity**, **adjuvants,** vaccine storage, dosage, administration, host infection status, competence of the host's immune system, and host genetics can all shape binary immunity [19,22]. Random binary immunization outcomes are often incorporated into SIR models by effectively lowering vaccination coverage by the proportion of binary failure [23]. However, if certain host types are more prone to vaccine failure, then it might be critical to address how these different failure rates among different host class affect disease dynamics [24].

Partial immunity

In contrast to binary efficacy, which assumes a vaccine either succeeds in inducing an acquired immune response or fails, vaccines that provide partial immunity may not completely prevent infection, disease symptoms, or transmission in an immunized host. Partial immunity allows for vaccine efficacy to be measured on a proportional gradient from 0-1, rather than as a qualitative all-or-nothing response [25,26]. One critical complication is that partial immunity may impact a number of infection outcomes, such as resistance to infection, disease attributed to infection, and infectiousness [27]. The functional consequences of these changes are detailed

below. Partial immunity is less easily incorporated into SIR-type models and has therefore been relatively neglected compared to other modes of imperfection. Individual-based models (IBMs), which explicitly track individual traits and histories may be much better suited to investigate this vaccine imperfection.

Functional mechanisms and consequences of imperfect vaccines

Different resistance responses to imperfect vaccines have unique ecological and evolutionary consequences. Imperfect immunization can confer the following three phenotypic types of resistance responses: 1) anti-disease, 2) anti-infection, and 3) anti-transmission (**Figure 1**). These are also not mutually exclusive, and they can be assessed using either binary (qualitative) or partial (quantitative) metrics [26,28,29]. Because the majority of vaccines are imperfect, anticipating and addressing their potential deleterious consequences is a priority in determining vaccination feasibility in a wildlife context. For example, the **imperfect-vaccine hypothesis** postulates that partial immunity upon vaccination could drive the evolution of increased pathogen virulence, and the risk of vaccine-driven virulence evolution is dependent on the vaccination phenotype and efficacy [29].

Anti-disease vaccines

Anti-disease vaccines reduce virulence (i.e., increase **host tolerance**) without necessarily reducing the risk of infection or subsequent transmission. Therefore, these vaccines directly benefit recipients, but can counteract herd immunity if the infectious period is lengthened. Studies on Marek's disease in poultry and helminth and tuberculosis **coinfections** in African buffalo show that interventions which reduce the mortality of infected hosts, without decreasing

infection or transmission rates, increase parasite transmission in populations by extending the infectious period [29,30]. Despite this potential for increased transmission, anti-disease vaccines may still be effective for conservation if their net effect reduces total parasite-induced mortality or reproductive costs. A prototype anti-*Chlamydia pecorum* vaccine for koala (*Phascolarctos cinereus*) conservation offers potential as a therapeutic vaccine as it reduces disease in unexposed and infected koalas, with some reduction in infection incidence and loads [31]. However, anti-disease vaccines are unlikely to reduce spillover risk, precisely because they can promote transmission.

Evolutionarily, lengthening the infectious period through anti-disease vaccination is theorized to relax selection against high virulence [27,29]. This prediction, derived from the **transmission-virulence trade-off hypothesis**, arises because limiting host death allows for otherwise highly virulent genotypes to persist and even be favored by selection [29]. While experimental evidence explicitly demonstrating increased virulence driven by vaccination is lacking, a recent study on house finches (*Haemorhous mexicanus*) parasitized by the bacteria *Mycoplasma gallisepticum* demonstrated that an anti-disease phenotype conferred by a natural primary infection facilitated a two-fold increase in the fitness advantage of a high virulence strain during secondary infections [32]. However, anti-disease vaccines that vary in degree of protection among immunized individuals may be less risky for vaccine-driven virulence evolution, as variance in host protection will not uniformly favor the evolution of increased parasite virulence [27].

Anti-infection and anti-transmission vaccines

Vaccines that prevent or reduce parasite establishment in an immunized host are considered anti-infection vaccines. Anti-transmission vaccines, on the other hand, may permit infection but prevent or reduce onward transmission from the recipient. Both phenotypes contribute to herd immunity, and epidemiological models predict that parasite elimination can be achieved with high rates of coverage and efficacy [28]. Thus, both anti-infection and anti-transmission vaccines can be effective for spillover prevention and conservation. The *Mycobacterium bovis* bacille Calmette-Guérin (BCG) vaccine, used to prevent spillover of *M. bovis* into livestock, confers anti-infection resistance in Australian brushtail possums (*Trichosurus vulpecula*), and the transmission-reducing prototype *Batrachochytrium dendrobatidis* vaccine offers promise for use in amphibian conservation [15,33].

The evolutionary consequences of these vaccines depend crucially on the mode of imperfection. Binary anti-infection or anti-transmission vaccines do not favor virulence evolution and can, at times, even reduce selection for parasite virulence, by preventing coinfections for example [28,34]. Conversely, partial anti-infection or anti-transmission vaccines can select for increased virulence [25]. Partial anti-infection and anti-transmission phenotypes effectively increase the exposure dose required for establishment (i.e. infectious dose), which can select for increases in parasite reproduction rate [25,28]. Theory suggests that this type of anti-infection resistance favors virulence evolution by encouraging the increase in intrinsic parasite reproduction for successful infection establishment [25].

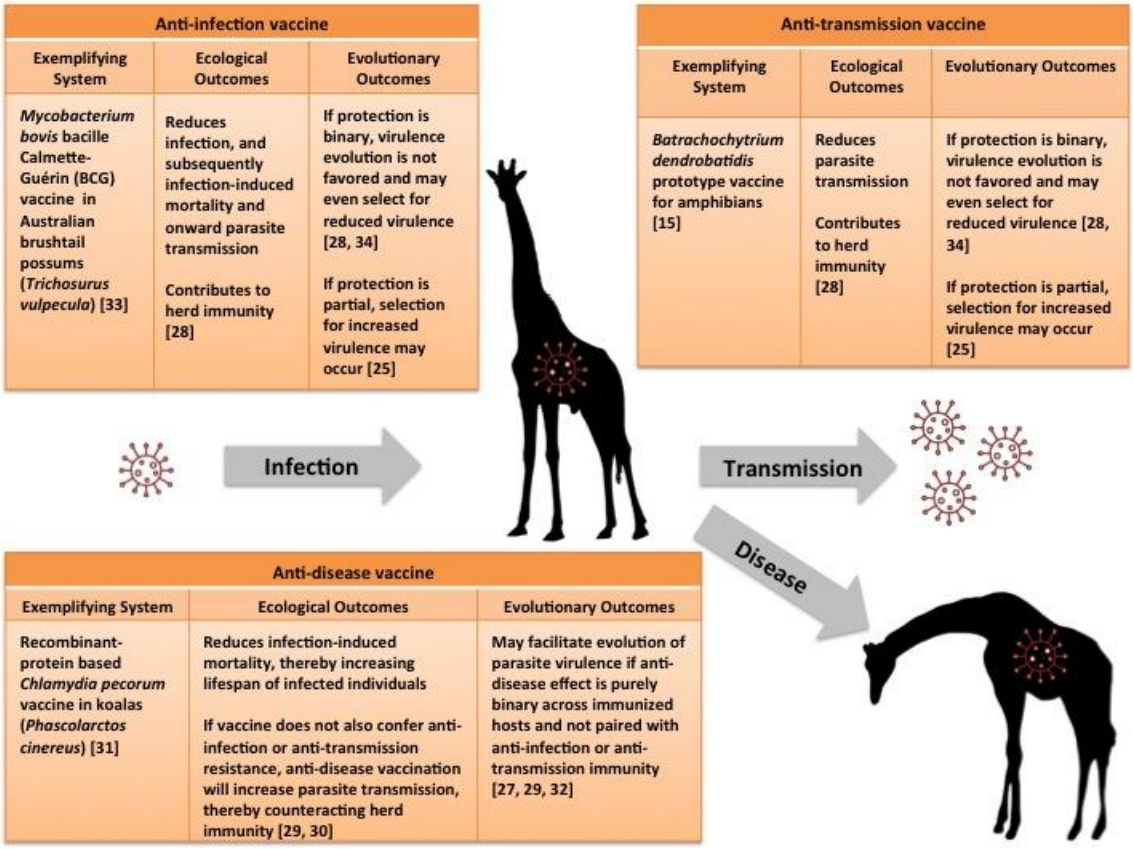

Figure 1. Imperfect vaccines can be categorized by the phenotypic resistance effects on vaccinated hosts, such as anti-infection, anti-disease, and anti-transmission. Each of these non-exclusive categories can influence epidemiology and pathogen evolution.

Ecological and logistical challenges of vaccination exacerbated in wildlife

Vaccines have strong potential to achieve disease control in wildlife. However, imperfect vaccines must also overcome physiological, behavioral, and ecological factors to succeed. Thus, complications arise from two primary factors: vaccine imperfections and vaccine administration. Lack of control and intraspecific, interspecific, and environmental heterogeneity are central sources of uncertainty in vaccine delivery, uptake, and response (**Box 1**). Vaccination success

hinges on high coverage of doses that induce a durable immune response without harming recipients [1]. In complex ecological communities, indirect deployment (i.e., oral baiting) campaigns risk simultaneously over- and under-dosing many organisms because wildlife can vary in 1) the amount of inoculum consumed or encountered and 2) their physiological response to a given dose.

Heterogeneity in host behavior, morphology, and habitat use all influence infection risk, and probability of vaccine exposure [35–37]. Assessing vaccine exposure in target and non-target wildlife can be done using biomarkers, such as fluorescent Rhodamine b [38]. Moreover, the immunological traits of most wildlife hosts remain poorly known, and even closely related species can exhibit marked variation in response to vaccination [39]. In vaccination campaigns using indirect deployment, assessing vaccine safety and impact on non-target hosts and non-hosts is a critical step to anticipating and preventing harmful unintended consequences on ecological communities and ecosystem functioning. **Dose-response profiles** are a useful and routine tool for assessing consequences of over- and under-dosing wildlife. Specifically, dose-response profiles can be useful for quantifying differences in dose-specific immune responses for distinct classes of hosts (e.g., species identity, developmental stage, age class, genotype). Additionally, the effect of vaccination on non-target wildlife can be evaluated by tracking community diversity metrics (e.g. abundance, richness, and evenness) and ecosystem function pre- and post-administration in both placebo and vaccinated environments [38]. Furthermore, **trait-based vaccination** may help to overcome issues related to patchy coverage and dosing.

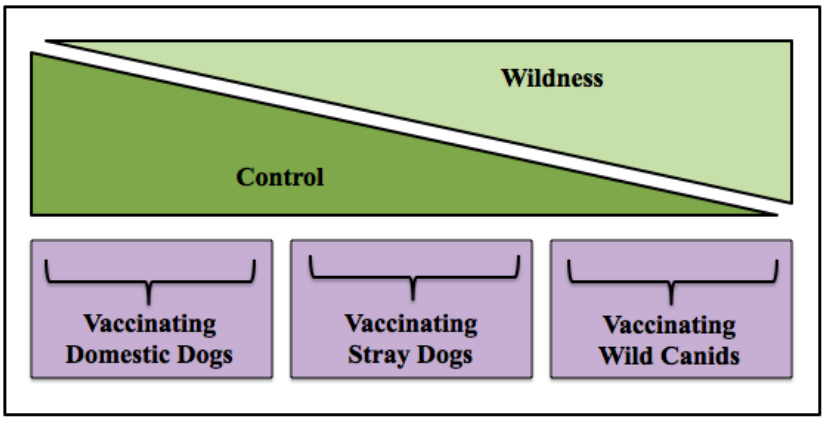

Figure 2. Rabies vaccination on a gradient of wildness.

Box 1. Canid rabies vaccination campaigns: limitations to control

Rabies vaccination of canids has been used to both prevent spillover transmission into human populations and protect endangered wildlife [51]. Rabies vaccination of domestic dogs, stray dogs, and wild canids demonstrates vaccination across a gradient of control and wildness (**Figure 2**). Globally, domestic dogs are the main source of rabies transmission to humans [52]. Consequently, owned dog vaccination is used to interrupt dog-to-human transmission and, largely due to the control afforded by ownership, has been successful in eliminating **enzootic** canine rabies in the U.S [53]. However, the unconstrained movement of stray dogs allows contact with wildlife, owned dogs, and humans, amplifying their importance in rabies transmission [54]. Difficulty catching stray dogs contributed to poor coverage, and hence failure, in a mass rabies vaccination campaign in Bangkok, Thailand [55]. Furthermore, high population growth, turnover, and translocation rates of stray dogs intensifies the challenge of achieving and maintaining vaccination coverage sufficient for herd immunity [54–56]. Combining vaccination with neutering can combat these challenges [57].

Vaccination of wildlife against rabies to prevent spillover into humans and domestic animals have also been hugely successful campaigns; locally eliminating rabies in red foxes and coyotes, while decreasing its prevalence in gray foxes [4–6]. This success is undoubtedly driven by the advent of oral bait vaccines, which can be distributed across large geographic scale [6]. Yet, although oral vaccination reduces the need for wildlife control via capture and handling and increases the geographic scale of administration, successful oral vaccination requires ecological knowledge of target and non-target foraging behaviors and home ranges for baiting, population turnover rates for estimating length of vaccination protection, and species-specific immunological responses [6,58,59]. Rabies vaccination has also been implemented as a conservation measure for endangered wild canids, such as the Ethiopian wolf (*Canis simensis*) and African wild dogs (*Lycaon pictus*) [56,60].

In these canid vaccination campaigns, control at the individual level, such as compliance, handling, and capture, prove most challenging. Thus, strategies that prioritize population-level measures, i.e., economic incentives through government support for owned dog vaccination, managing stray dog populations through neutering, and oral baiting of free-roaming and wild canids, significantly enhance vaccination success.

Trait-based vaccination

Which hosts should be prioritized for vaccination? Host factors such as age, immunity, behavior, and genetics all influence **host competence** [40]. These heterogeneous factors contribute significantly to disparities in parasite susceptibility and transmission between hosts, leading to relatively few individuals being responsible for most parasite transmission in a population [41]. This observation can be harnessed to tailor control methods using trait-based vaccination.

Random mixing is a fundamental assumption of classic vaccination and transmission models, but network analyses of wildlife show that traits such as territoriality or sociality often reveal non-random contacts, elevating the importance of accounting for contact and home range heterogeneity in vaccination [42,43]. Targeted vaccination of **superspreaders** has been continually proposed as a method to reduce required immunization coverage [44,45]. For example, targeted vaccination of socially-central chimpanzees, determined by detailed behavioral data or approximated using trait-based estimates, can significantly reduce the vaccination coverage threshold [44]. Incorporating contact networks into **transmissible vaccine** models, using an individual-based approach, could assess if behaviors associated with superspreading, such as gregariousness or boldness, increase vaccine transmission [46,47]. Alternatively, vaccination for conservation could target individuals that are disproportionately important to population growth or persistence [48].

Modeling wildlife vaccination

Susceptible-Infected-Resistant (SIR) models are the most common models used for predicting vaccination outcomes [27]. While valuable for modeling waning and binary modes of

imperfection, SIR models cannot capture the complexities of partial immunity, especially when spatial dynamics, social interactions or individual history are important [23,27,49]. Limitations of modeling partial immunity using ordinary differential equations (ODEs) can be overcome using individual-based models (IBMs), which are able to incorporate different host immune responses and space-based behaviors such as territoriality and migration [49]. For example, in the case of fox rabies control in Europe, IBM predictions recommended the use of a lower coverage vaccination strategy relative to an SIR model [50]. This lower coverage strategy was carried out successfully and saved considerable resources [49].While the simplicity and analytical tractability of ODE models can offer considerable advantages, we advocate for the increased consideration of IBMs in the study of wildlife disease because they can represent individual-level physiology, connect seamlessly with transmission networks or spatially-explicit movement models, and accommodate individual history and heterogeneity [49].

Concluding Remarks

Vaccines can advance biodiversity conservation and spillover control. However, vaccine imperfections can substantially compromise the achievement of herd immunity or promote the evolution of increased virulence, yet they are not always accounted for in theory, planning, or analysis of vaccine use in wildlife. Wildlife vaccination offers a frontier to explore advancing questions in eco-immunology, imperfect immunity, and disease control innovation. The biological factors shaping vaccination success, feasibility, and efficacy should be as central to decisions regarding wildlife vaccination as logistical limitations and financial resources (**Outstanding Questions**). Thorough empirical assessment of the vaccine-host-parasite biology

can both 1) prevent impractical vaccination campaigns and 2) ameliorate challenges regarding vaccine dose and coverage, saving time and limiting adverse outcomes.

Disentangling potential modes of imperfection is critical for predicting outcomes of vaccination. Incorporating these effects into models and experiments can predict otherwise counterintuitive deleterious outcomes, such as increased transmission caused by anti-disease resistance. We suggest that IBMs should be selected for vaccines conferring partial immunity or systems in which space-based behaviors drive disease dynamics. Additionally, vaccination outcomes should be simultaneously studied across ecological scales and evolutionary time. Imperfect vaccines impose subtle tension between individual- and population-level benefits, and deeper theoretical examination can help prevent the implementation of unfeasible or potentially harmful vaccines.

Furthermore, wild hosts and parasites are inherently heterogeneous and poorly controlled. Dose-response profiles and community diversity metrics should be used to account for heterogeneity when calculating safe and effective vaccine doses for wildlife individuals, populations, communities, and ecosystems. Trait-based vaccination approaches could prioritize hosts that disproportionately contribute to population persistence or parasite transmission thus minimizing coverage required for parasite eradication or host population viability. Ecological complexities and evolutionary consequences of imperfect immunity provide an abundance of challenges when vaccinating wildlife; but pursuing wildlife vaccination for use in conservation or spillover prevention is by no means foolish if informed by the system's underlying physiology and ecology.

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Glossary

Herd immunity: indirect protection of susceptible hosts by resistant hosts.

Spillover: transmission of parasites from a non-human host species to humans.

Reservoir host: a population of organisms that serve as an infection source for another host population.

Zoonotic pathogens: a parasite able to be transmitted from non-human animals to humans.

Effective reproductive ratio (R_{eff}): the number of secondary infections a primary infection contributes in a population with resistant individuals.

Parasite virulence: host death or pathology induced by infection.

Resistance phenotype: categories of incomplete immunity, including anti-disease immunity, anti-infection immunity, and anti-transmission immunity.

Immunogenicity: a vaccine's ability to induce an acquired immune response.

Adjuvants: vaccine additives to increase its immunogenicity.

Imperfect-vaccine hypothesis: theory suggesting that, depending on the phenotype of resistance, partial vaccination may select for increased parasite virulence.

Host tolerance: decreased mortality or pathology in response to infection.

Transmission-virulence trade-off hypothesis: hypothesis derived from the assumption that transmission rate and virulence are correlated, predicting that an intermediate level of virulence is favored by selection.

Coinfections: two or more parasite species simultaneously infecting the same host**.**

Dose-response profiles: quantifying an organism's physiological response to varying doses of vaccine.

Trait-based vaccination: vaccine distribution prioritizing individuals with specific characteristics.

Host competence: the relative ability of a host to become infected by and transmit a parasite.

Superspreader: an individual that disproportionately contributes to parasite transmission within a given population.

Transmissible vaccine: vaccines that autonomously spread from treated to untreated individuals.

Enzootic: a pathogen endemic in non-human animals.

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Chapter 2: Asymmetric cross-strain protection for amphibians exposed to a fungal-metabolite prophylactic treatment

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Abstract

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 3x2 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 suggests that work investigating multiple, diverse strains is urgently needed.

Introduction

Pandemics and epidemics are increasing in frequency across taxonomic groups and the high infection prevalence of these pathogens facilitate 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 programs 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 noninfectious 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 a 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.

Methods

Frog Husbandry

We collected adult Bd-naïve 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 1L plastic deli cups with paper towels dampened with 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 with compliance with IACUC at the University of Tampa.

Bd Culture and Bd 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 artificial spring water (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 3mL of a single strain for a total of 4-5 plates per strain and maintained them at 18ºC for two weeks. We flooded the plates (4-5 plates per strain) with ASW for ~3 minutes 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). 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 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 in reference to this pre-filtration concentration. We maintained aliquots of the Bd metabolite filtrate in a laboratory grade -20ºC freezer and thawed the necessary volume to room temperature for each dosing event.

Study Design

We used a 3x2 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 thirteen days, we dosed each frog daily with 1 mL of their respective prophylactic treatment dispensed on their dorsal surface. After the thirteen 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) 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). 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.

Molecular detection of Bd

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 in the sample. Given that strains have different genome equivalents (GE) per zoospore (18) and we wanted to compare the Bd loads across strains, we standardized the zoospore quantities according to the number of genome equivalents per zoospore (Panamá: 19.22 GE/zoospore and California: 253.1 GE/zoospore). Importantly, the results we present are in zoospores, not genome equivalents.

Data Analysis

We conducted all statistical analyses in R statistical software, version 4.0.3 (19). We used the Cox Proportional-Hazards Model (package: KMsurv, function: coxph) with prophylactic treatment crossed with live Bd strain as predictors to assess mortality (20). 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 (21). 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 $\alpha = 0.0083$).

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 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 x 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**).

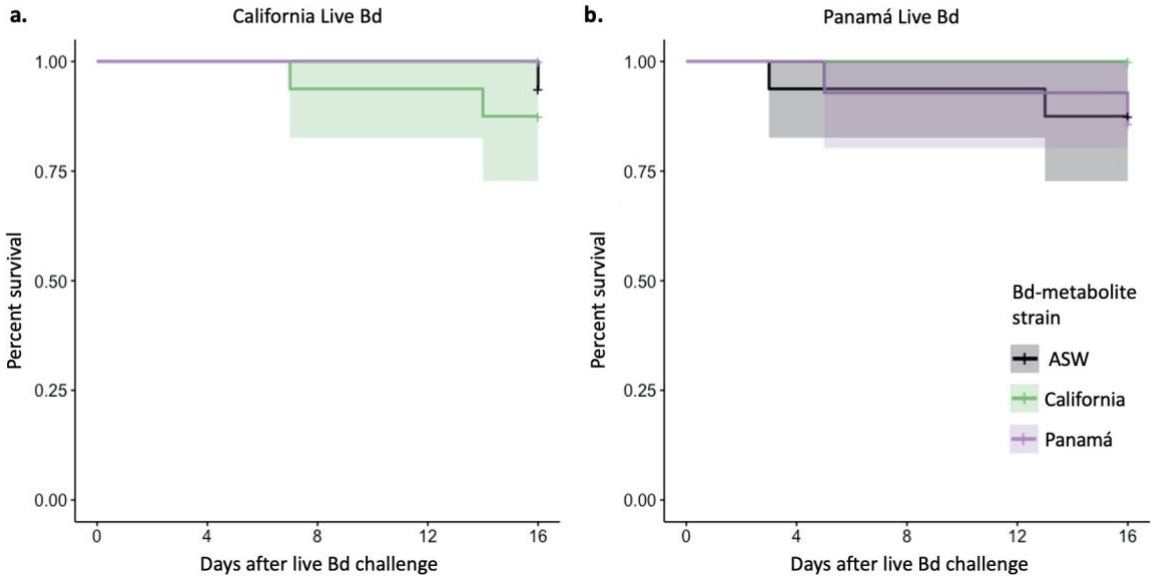

Figure 1. Percent survival following live Bd challenge for frogs exposed to Bd metabolites from one of three prophylactic treatments: Bd metabolites from a California-isolated strain (green), Bd metabolites from a Panamá-isolated strain (purple), and artificial spring water (ASW) - control (black). Following 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 percent survival and the bands represent the 95% CI.

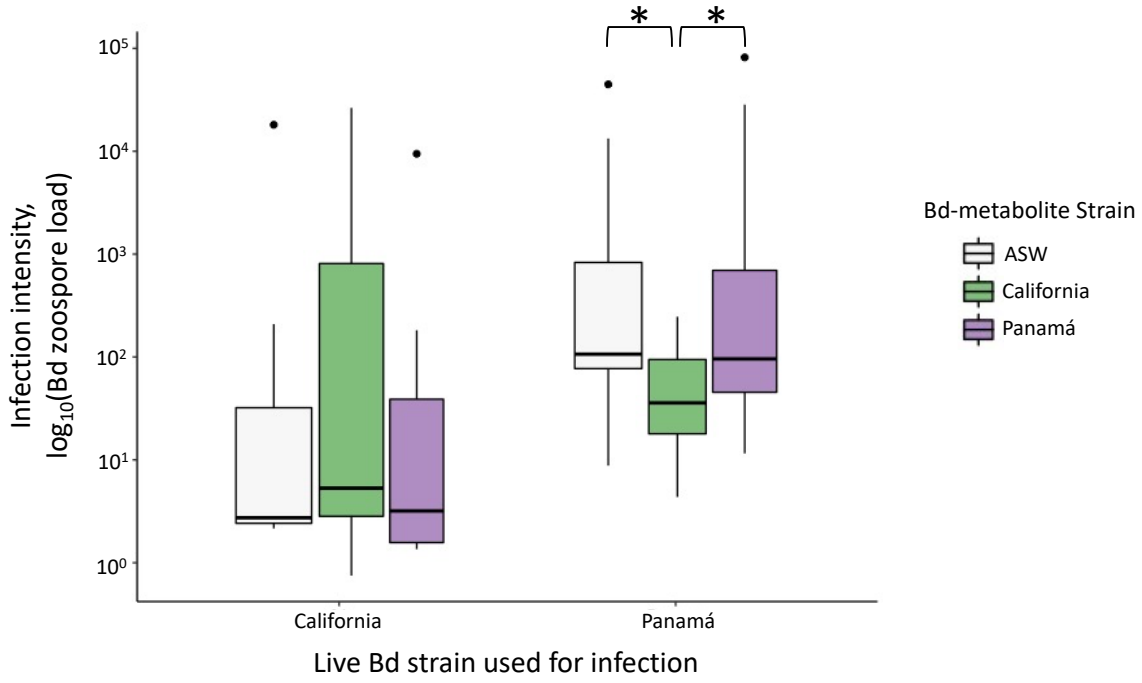

Figure 2. Infection intensity (i.e., zoospore load of infected individuals) for frogs exposed to Bd metabolites from one of three prophylactic treatments (Bd metabolites from a California-isolated strain, Bd metabolites from a Panamá-isolated strain, and ASW-control) and subsequently challenged with 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.

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 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, spermidine) are immunosuppressive (22,23). These factors can suppress immunity by decreasing lymphocyte functioning and proliferation and inducing apoptosis (22,24). 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 (22,23).

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 (25), 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.

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Chapter 3: Fungal metabolites provide pre-exposure protection but no post-exposure benefit or harm against *Batrachochytrium dendrobatidis*

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Data accessibility: Data and analysis code are available via the Github: https://github.com/kmbarn4/pre-vs-post-exposure

Abstract

Disease control tools are needed to mitigate the impact of the fungal pathogen *Batrachochytrium dendrobatidis* (Bd) on amphibian biodiversity loss. In previous experiments, Bd metabolites (i.e., non-infectious chemicals released by Bd) have been shown to induce partial resistance to Bd when administered prior to live pathogen exposure, and therefore have potential as an intervention strategy to curb Bd outbreaks. In the wild, however, amphibians inhabiting Bd-endemic ecosystems may have already been exposed to or infected with Bd before metabolite administration. It is therefore critical to evaluate the efficacy and safety of Bd metabolites applied post-exposure to live Bd. We tested whether Bd metabolites administered post-exposure would induce resistance, exacerbate infections, or have no effect. The results confirmed that Bd metabolites applied before pathogen exposure significantly reduced infection intensity, but Bd metabolites applied after pathogen exposure neither protected against nor exacerbated infections. These results reveal the importance of timing Bd metabolite application early in the transmission season for Bd endemic ecosystems, and emphasize that Bd metabolite prophylaxis may be a useful tool in captive-reintroduction campaigns where Bd threatens the success of re-establishing endangered amphibian populations.

Introduction

Emerging infectious diseases of wildlife such as chytridiomycosis, Tasmanian devil facial tumor disease, white nose syndrome, and hemorrhagic septicemia are increasingly linked to biodiversity loss (Smith et al. 2009; Fisher et al. 2012; Fereidouni et al. 2019; Scheele et al.

2019). Wildlife populations that have already declined due to habitat destruction, invasive species, pollution and climate change are especially vulnerable to disease-induced extinction (Smith et al. 2009; McCallum 2012; Fereidouni et al. 2019). Additionally, novel pathogens may threaten otherwise stable populations (McCallum 2012). Disease control interventions are needed to prevent further biodiversity loss and promote the conservation of many wildlife taxa.

Chytridiomycosis is a disease threatening amphibian biodiversity that is caused by the aquatic fungal pathogen *Batrachochytrium dendrobatidis* (Bd; Scheele et al. 2019). This pathogen is a host generalist, infecting amphibians and invertebrates, and has spread globally in recent decades (McMahon et al. 2013; Scheele et al. 2019). The contribution of chytridiomycosis to biodiversity loss is huge, with a connection to at least 90 amphibian species extinctions and the decline of hundreds more (Scheele et al. 2019). There is a pressing need to mitigate Bd-induced declines, and many methods to control Bd (e.g., antifungal treatments, microbiome augmentation, and vaccination) are being explored (McMahon et al. 2014; Knapp et al. 2021; Waddle et al. 2021).

Prophylactic treatments, such as vaccines, enable vulnerable populations to better withstand disease outbreaks and are promising tools to prevent disease-induced extinctions (Barnett and Civitello 2020). Vaccination has been implemented to protect prairie dog populations (Tripp et al. 2017), and has been recently proposed for Amur tigers (Gilbert et al. 2020) and little brown bats (Rocke et al. 2019; Gilbert et al. 2020). Environmentally distributed vaccines (e.g., oral vaccine baits) are very useful for increasing vaccination coverage in wildlife, given that parenteral vaccines require a catch-vaccinate-release or darting strategy, which may be challenging or impractical (Undurraga et al. 2020). However, environmentally distributed vaccines require the assessment of additional ecological factors, such as host exposure history, to

optimize intervention success and ensure that vaccines are safe for target populations, ecological communities, and ecosystems (Barnett and Civitello 2020). Timing vaccine administration according to host life history traits may increase population coverage, and is especially impactful for hosts, such as amphibians, with short life spans and seasonal population fluctuations (Schreiner et al. 2020). In a scenario where vaccination has no effect on previously exposed hosts, administering vaccines at or immediately after the end of a birth pulse may increase vaccination coverage compared to vaccinating later in the season, when the endemic pathogen has had more time to infect the newly-born susceptible hosts (Schreiner et al. 2020).

Mounting evidence shows that frogs can acquire resistance to Bd following any of these treatments: a live Bd exposure and clearance regime using itraconazole or temperatures outside the thermal tolerance of Bd; killed Bd zoospores with Bd metabolites (i.e., water-soluble non-infectious chemicals released by Bd); and Bd metabolites alone (McMahon et al. 2014; Barnett et al. 2021; Waddle et al. 2021; Nordheim et al. 2022), suggesting that Bd vaccination may be effective. Direct comparisons of killed Bd zoospores alone, killed Bd zoospores with Bd metabolites, and Bd metabolites alone have indicated that prophylactic exposure to Bd metabolites may drive equal or better resistance responses than killed Bd zoospores alone (Nordheim et al. 2022). Moreover, Bd metabolites have been found to be effective at inducing resistance across amphibian life stages (tadpoles and adults) and in at least two frog species (Cuban treefrog, *Osteopilus septentrionalis,* and Pacific chorus frog, *Pseudacris regilla*); however, importantly, Bd strain may impact treatment efficacy (Barnett et al. 2021; Nordheim et al. 2022).

For disease control and conservation, success would be maximized if Bd metabolites induced resistance regardless of exposure history and could be used as both a pre-exposure (i.e.,

Bd metabolites applied before frogs have been exposed to live Bd) prophylaxis and post-exposure (i.e., Bd metabolites applied after frogs had been exposed to live Bd) treatment. Previous controlled laboratory studies have shown that prophylactic exposure to Bd metabolites provided protection against subsequent Bd challenge in frogs, but these studies only tested the prophylaxis on Bd-naïve animals (Barnett et al. 2021; Nordheim et al. 2022). In the wild, amphibians inhabiting Bd-endemic ecosystems may have already been infected with Bd prior to the time of prophylaxis administration. It is possible that Bd metabolites applied post-exposure might exacerbate infections by increasing Bd infection probability or intensity, given that some metabolites released by Bd have immunosuppressive properties and are hypothesized to aid zoospore infection establishment (Rollins-Smith et al. 2019). If this were the case, it could be detrimental to broadly administer a Bd metabolite treatment to a Bd-endemic system. Thus, evaluating the effect of a post-exposure Bd metabolite treatment is crucial for optimizing treatment efficacy and assessing the safety of a Bd metabolite treatment for use in the wild.

We tested whether Bd metabolites administered pre-exposure or post-exposure to live Bd would induce resistance, exacerbate infections, or have no effect.

Materials and Methods

Egg collection and tadpole husbandry

Pacific chorus frogs (*P. regilla;* listed as least concern according to the IUCN Red List (Hammerson and Santos-Barrera 2004)) tadpoles, are a well-studied reservoir of Bd (Reeder et al. 2012). We collected Pacific chorus frog (*P. regilla*) egg clutches from Alameda County, California, US, under permit CA DFW S-193500003-20017-001 and sent to New London, Connecticut, US. All laboratory procedures were approved by the Connecticut College

Institutional Care and Use Committee, under protocol #236. *Batrachochytrium dendrobatidis* is endemic in Alameda County; however, collected eggs were presumed to be Bd-free, because Bd appears not to be associated with amphibian eggs (Bancroft et al. 2011), being found only on keratinized tissues, which eggs lack (Marantelli et al. 2004). Tadpoles were maintained together in low densities (150 tadpoles in a 38 L container) until they reached Gosner stage 25, when they were separated into individual 500 mL plastic containers with 200 mL of artificial spring water (ASW; Cohen et al. 1980). Throughout the entire experiment, tadpoles were maintained in a natural light regime (10:14 h light:dark photoperiod) at 19 C, a temperature well within the thermal tolerance range for both tadpoles of this species and Bd (Brattstrom 1963; Cohen et al. 2017). We fed the tadpoles fish flakes that are high in plant-based protein every second day. We conducted daily mortality checks and removed fecal matter from containers every 3 d.

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

We produced stock Bd culture and Bd metabolite treatments as previously described (McMahon et al. 2019). We used Bd isolate JEL 270 (isolated from California) for both the Bd metabolite treatments and live challenges). In brief, we cultured Bd in 1% tryptone broth and then inoculated 1% tryptone agar plates with 3 mL of the Bd isolate. Plates were maintained at 19 C for 2 wk, after which we flooded the plates with ASW for approximately 3 min to suspend the zoospores and zoosporangia, then homogenized the liquid across all plates to create a Bd-positive (Bd+) stock consisting of ASW, Bd, and Bd metabolites. We determined the concentration of zoospores in the Bd+ stock by analyzing a 10 µL aliquot on a hemocytometer and averaged the number of zoospores from the four field of view quadrats, methods standard in the field. We then diluted the concentration to 400 zoospores/mL with ASW (we refer to the concentration of this Bd metabolite filtrate as 400 zoospores-removed/mL in reference to this

pre-filtration concentration). This diluted Bd+ stock was filtered through a 1.2 μm filter (GE Whatman Laboratory Products) to remove zoospores and zoosporangia, creating the Bd metabolite treatment. We verified that no zoospores or zoosporangia remained in the Bd metabolite treatment using the same light microscopy approach that was used for calculating the concentration of zoospores in the Bd+ stock explained above. All Bd metabolite aliquots were maintained in a laboratory grade -20 C freezer and the amount needed for each day was brought to room temperature before each dosing event.

Study design

We conducted a 24-d infection experiment with three treatments, pre-exposure prophylaxis, post-exposure prophylaxis, and an ASW control. The experiment began with 30 tadpoles per treatment; all tadpoles that died did so before the live Bd challenge and were excluded from analysis. Every second day from the start of the experiment until the day of live Bd exposure (i.e., days 1, 3, 5, 7, 9, and 11), tadpoles in the pre-exposure treatment were dosed topically with 1 mL Bd metabolites (the solution was diluted into the 200 mL of ASW in the tadpole's housing container, for a final treatment dose of 2 zoospores-removed per mL) and tadpoles in the other treatments were dosed with 1 mL ASW. On day 12, we challenged all tadpoles with 1 mL of live Bd (4×10^5 zoospores/mL), which was diluted into the 200 mL housing containers for a final exposure dose of 2,000 live zoospores per mL. To reduce water fouling, a minimal water change was performed to remove fecal matter on the day following live Bd challenge. Starting on day 13, on every second day (i.e., days 13, 15, 17, 19, and 21), tadpoles in the post-exposure treatment were dosed topically with 1 mL Bd metabolites as described for pre-exposure treatment, while tadpoles in the other treatments received 1 mL ASW.

Molecular detection of *Batrachochytrium dendrobatidis* (Bd)

On the 24th day of the experiment, all tadpoles were euthanized with an overdose of MS 222 (10 g/L of ASW) buffered with sodium bicarbonate as needed to maintain a neutral pH (Leary et al. 2020), and mouthparts were dissected for molecular detection of Bd. We quantified the Bd load in number of genome equivalents (GE) from each tadpole using quantitative PCR (qPCR, see (Boyle et al. 2004)) with plasmid standards designed to target Bd/Bsal (Pisces Molecular). We screened for, and confirmed lack of, inhibition in every sample using TaqMan Exogenous Internal Control Reagents (Applied Biosystems, Foster City, California USA).

Data analysis

We conducted all statistical analyses in R statistical software, version 4.0.3 (R Core Team 2020). We verified the proportional hazards assumption (P= 0.58, package: survival, function: *cox.zph)* and used the Cox proportional-hazards model (package: KMsurv, function: coxph) with treatment as the predictor to assess mortality. We used a binomial generalized linear model (GLM) on binary infection status to assess treatment effects on probability of Bd infection (package: glmmTMB, function: glmmTMB) and we calculated confidence intervals for the probability of infection using the Wilson Score interval (Brown et al. 2001). In both cases, we used likelihood ratio tests (package: stats, function: anova) to evaluate significance. We found no effect of treatment on probability of infection, therefore, we tested for differences in infection intensity using a zero-inflated negative binomial generalized linear model (package: glmmTMB, function: glmmTMB) using treatment as the predictor for infection intensity. Given the similarity in probability of infection among treatments, we fit a common intercept for the zero-inflation component of the model. Furthermore, we conducted pairwise *post-hoc* tests by re-running the zero-inflated negative binomial glmmTMB models across all treatment combinations and using

Bonferroni corrections for multiple testing (corrected α=0.017). We extracted the mean infection intensity estimates for each treatment from the model using the emmeans package (package: emmeans, function: emmeans).

Results

Overall, 94% of tadpoles survived the entire experiment. There was no significant difference in mortality (Cox-proportional-hazards model: *P*=0.8) or probability of Bd infection across treatments (binomial GLM): *P*=0.42; Fig. 1). Using the zero-inflated models we found that tadpoles treated with the pre-exposure Bd metabolite treatment exhibited a 97% reduction in infection intensities compared to the control treatment (GLM *P*=0.003) and a 98% reduction in infection intensities compared to the post-exposure treatment (GLM *P*=0.002; Fig. 2). Additionally, we found no effect of the post-exposure Bd metabolite prophylactic treatment on infection intensity compared to the control group (*P*=0.77).

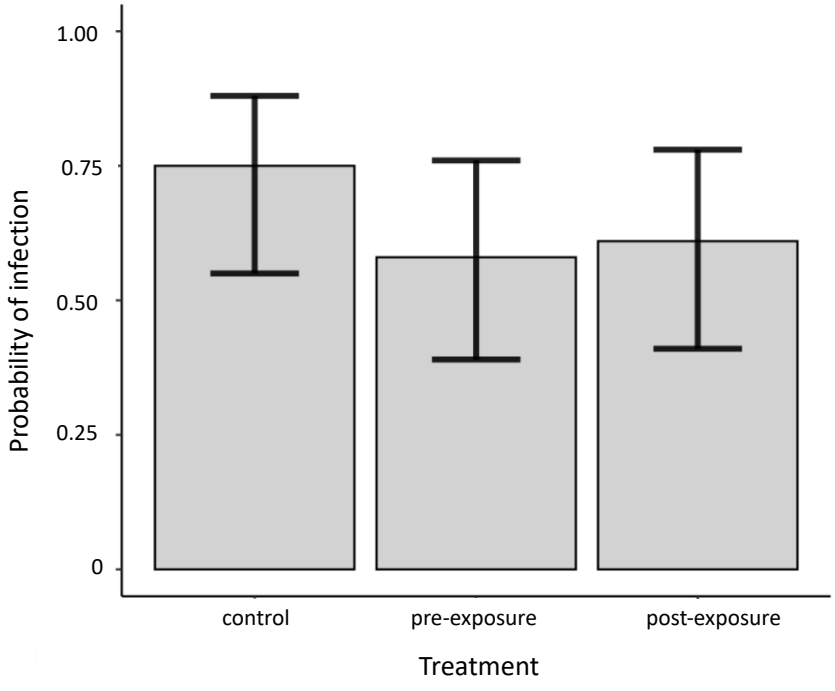

Figure 1. Probability of *Batrachochytrium dendrobatidis* (Bd) infection for Pacific chorus frog (*Pseudacris regilla*) tadpoles treated with: a) control treatment: Artificial Spring Water (ASW) before and after a live Bd challenge; b) pre-exposure treatment: Bd metabolites prior to a live Bd challenge; or c) post-exposure treatment: Bd metabolites after a live Bd challenge. There was no difference in probability of infection among treatments. The bars on the plot represent 95% confidence intervals.

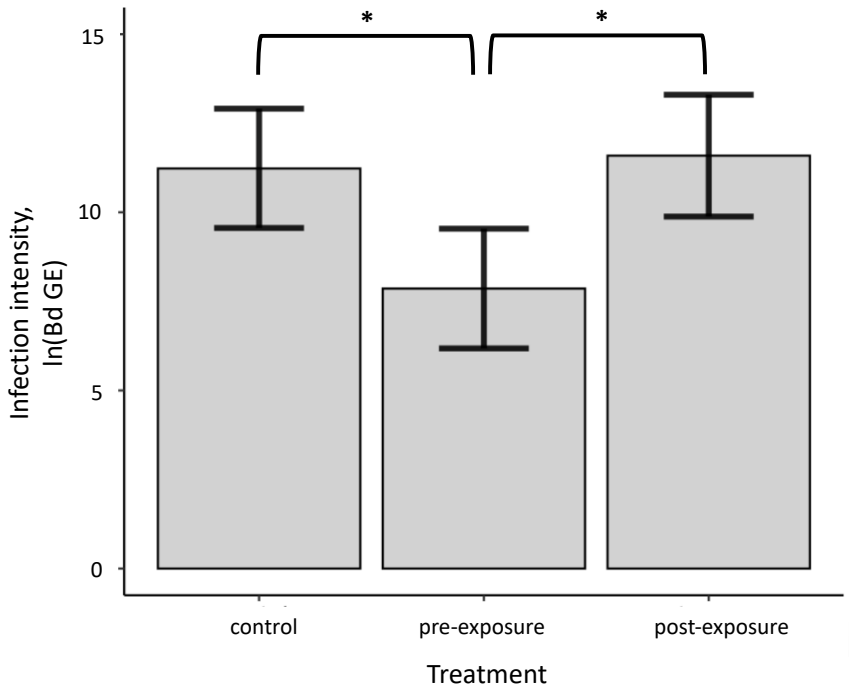

Figure 2. Estimated mean infection intensity (i.e. *Batrachochytrium dendrobatidis* (Bd) genome equivalents of infected individuals) for Pacific chorus frog (*Pseudacris regilla*) tadpoles treated with: a) control treatment: Artificial Spring Water (ASW) before and after a live Bd challenge; b) pre-exposure treatment: Bd metabolites prior to a live Bd challenge; or c) post-exposure treatment: Bd metabolites after a live Bd challenge. Tadpoles in the pre-exposure treatment had significantly lower mean infection intensities than tadpoles in the control and post-exposure

treatments (* denotes a significant difference). The bars on the plot represent 95% confidence intervals.

Discussion

We found that Bd metabolites were effective as a pre-exposure prophylaxis but did not reduce or increase Bd loads when applied post-exposure. Given that Bd metabolites do not induce resistance when applied post-exposure, our results indicate that a Bd metabolite intervention should occur early in the transmission season, before a considerable amount of the population has already been exposed to Bd. This work highlights that timing of prophylaxis exposure is an important factor for optimizing disease control interventions, especially when the pathogen is endemic (Schreiner et al. 2020). These empirical data are the first step to understanding the importance of prophylaxis timing for Bd metabolite prophylaxis; in the future, modeling various timing scenarios for Bd metabolite administration would be useful to determine optimal intervention strategies.

Although some Bd metabolites have been thought to facilitate infection establishment (Rollins-Smith et al. 2019), our experiment found that Bd metabolites did not increase probability of infection or infection intensity in tadpoles when applied post-exposure. Protection regardless of exposure history would be ideal from a management perspective, but our findings do suggest that field administration of the treatment is unlikely to be detrimental to hosts that are already infected.

Our findings also suggest that Bd metabolite prophylaxis may be beneficial as a proactive measure to curb Bd epidemics and reduce the ability of Bd to expand into new populations. Given that Bd-induced mortality is associated with high infection loads (Voyles et al. 2009), by

reducing infection intensity, Bd metabolite pre-exposure treatment may also decrease Bd-induced mortality. For the benefit of animal welfare, our experiment ended in a shorter timeframe than Bd-induced mortality is expected to occur, but a future study should directly assess the impact of Bd metabolite prophylaxis on infection-induced mortality. Furthermore, Bd metabolite prophylaxis reduces onward transmission by decreasing zoospore loads; transmission modeling studies should investigate if, under certain conditions, this effect is sufficient enough to generate herd immunity. Environmental persistence of Bd has been a barrier to successful reintroduction of endangered amphibians susceptible to chytridiomycosis (Hammond et al. 2021); Bd metabolite prophylaxis might serve as a powerful tool to remedy this challenge. For example, Bd metabolites could be used to treat captive-bred, Bd-naïve amphibians prior to their release into Bd endemic systems, providing the reintroduced amphibians with some protection against Bd to facilitate their successful establishment.

Our experiment lasted only 11 d after live Bd exposure because previous studies (Barnett et al. 2021) have shown that Bd resistance can develop within a short timeframe and we were looking to conserve resources and mitigate animal suffering. However, it is possible that there could be a lag period in mounting the immune response that exceeds 11 d (e.g., it takes adult *Xenopus laevis* 1 mo to clear *Ranavirus* (FV3) infections; Gantress et al. 2003) and that Bd metabolites applied post-exposure might facilitate faster clearance of Bd in a delayed response that we were unable to detect. Additionally, the impact of combined Bd metabolite pre- and post-exposure treatment remains unknown, and it is possible that a post-exposure Bd metabolite treatment might boost the resistance response in tadpoles that had already received a pre-exposure Bd metabolite treatment. More work is needed to investigate these possibilities.

Immune defenses may vary greatly based on life stage in amphibians, due to reorganization of the immune system during metamorphosis (Gantress et al. 2003; Humphries et al. 2022). Although Bd metabolites have been effective at inducing resistance when applied pre-Bd exposure in both tadpoles (Nordheim et al. 2022) and adults (Barnett et al. 2021), it is possible that adult frogs or frogs undergoing metamorphosis may respond differently than tadpoles to post-exposure Bd metabolite treatment. Although metamorphs are more likely to succumb to Bd-induced mortality than are tadpoles (Rachowicz al. 2006), metamorphs' immune systems are more mature than that of tadpoles, exemplified by their increased expression of MHC classes I and II and presence of antimicrobial peptides (Humphries et al. 2022). Given the maturity of their immune system, metamorphs and adult frogs may be able to acquire resistance via post-exposure treatment with Bd metabolites even though this study found post-exposure prophylaxis was ineffective in tadpoles.

There are now three published studies (this study, Barnett et al. 2021, and Nordheim et al. 2022) showing that Bd metabolites are effective at significantly reducing Bd infection intensity when applied pre-exposure. The consistent reproducibility of this result indicates that Bd metabolite prophylaxis may be a useful tool against Bd-induced biodiversity declines. To be effective, Bd metabolite prophylaxis in Bd-endemic ecosystems should be applied early in the transmission season or in conjunction with influxes of new susceptible hosts, whether reproduction pulses or reintroductions. Further work needed includes evaluation of the safety of Bd metabolites to non-target wildlife; testing of the efficacy of Bd metabolite prophylaxis in a field setting; and investigation of the potential for Bd metabolite prophylaxis to work synergistically with other Bd mitigation strategies.

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Chapter 4: Prophylactic treatment for *Batrachochytrium dendrobatidis* increases amphibian infection loads in natural populations: A possible consequence of increased host survival

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O.M., J.C., J.B., B.H., W.M., T.M., and C.N. conducted the fieldwork. T.A.M., A.D.S., H.N.B., K.M.B., S.E.D., and B.A.F. conducted the lab experiments, processed samples, and prepared the treatments. K.M.B. and D.J.C. conducted the analyses and wrote the manuscript. D.J.C., T.A.M., P.T.J.J., J.R.R., and K.M.B. provided funding.

Data accessibility: Data and analysis code are available via the Github: https://github.com/kmbarn4/bd_vaccine_abm_and_field_trial

Abstract

Disease control tools for the aquatic fungal pathogen *Batrachochytrium dendrobatidis* (Bd) are urgently required for amphibian conservation. Several laboratory experiments have demonstrated that prophylactic exposure to metabolites produced by Bd significantly reduces infection loads in amphibians subsequently challenged with live Bd. Because Bd metabolites are non-infectious and applied topically, this treatment can be administered directly to waterbodies, holding promise as a feasible conservation tool. To test the impact of this treatment when administered to natural populations, we conducted a Before-After-Control-Impact experiment wherein we applied low-levels of Bd metabolites or a sham control treatment to ponds in California and returned to quantify Bd prevalence and infection intensity in metamorphosing Pacific chorus frogs (*Pseudacris regilla*). We compared these data with baseline data from non-intervention years and found that Bd infection intensity significantly increased after ponds were treated with Bd metabolites. While these findings were unexpected, simulations from an agent-based model of this system suggest this result can occur if the prophylactic treatment greatly increases tolerance (i.e. increases a host's ability to withstand high infection burdens). Though

enhanced tolerance is advantageous for individuals, it can be problematic at the population-level if longer infection durations increase onward transmission, thereby increasing risk of infection to untreated sympatric amphibians. In search of a control tool for chytridiomycosis, these findings underline the importance of accounting for how different mechanisms of individual-level partial protection can generate population-level outcomes that paradoxically undermine conservation objectives.

Significance statement

Wildlife vaccination is increasingly explored as a strategy to mitigate disease-induced biodiversity losses, though many vaccines available for wildlife diseases provide only limited protection. Here, we use both an eco-epidemiological model and field manipulation experiment to assess the effectiveness of an imperfect prophylactic treatment (akin to a prototype vaccine) for chytridiomycosis, a disease implicated in the massive decline of amphibian biodiversity worldwide. We unexpectedly found that prophylaxis addition increased pathogen loads in natural populations and model results suggest this may be the result of enhanced tolerance. This study signifies the importance of accounting for differences in the transmission potential of treated versus untreated hosts when designing conservation-motivated disease control campaigns and cautions the use of this prophylaxis for amphibian conservation.

Introduction

Wildlife vaccination is a promising conservation tool to mitigate the risk of disease-induced biodiversity loss (1–3) and a powerful public health intervention for the prevention of disease spillover to humans and livestock (4, 5). The strength of vaccination lies in its ability to disrupt transmission. Often, protection generated for vaccinated individuals indirectly protects unvaccinated individuals, a mechanism termed "herd immunity" (6). Additionally, the feasibility of wildlife vaccination has increased in recent years due to the growing availability of environmentally distributed vaccines, such as oral vaccine baits (7).

Ideally, vaccines provide "perfect" (or sterilizing) protection, wherein all vaccinated individuals have lifelong resistance against infection. When vaccination provides perfect protection, epidemiological models predict increasing protection for populations as the proportion of the immunized population (i.e. vaccination coverage) increases (6). However, in practice, many vaccines fall short of perfection and instead provide only partial reductions in infection establishment, infection load, or disease severity which often wane in efficacy over time (8, 9). Partially protective vaccine campaigns can also confer population-level benefits, but may also backfire under certain circumstances. Specifically, imperfect immunity that boosts tolerance (i.e. reduces infection-induced mortality; also known as anti-disease immunity) can lead to greater pathogen transmission by extending the duration of infectiousness, and it may favor the selection of hypervirulent strains (10–13). However, the adverse consequences of tolerance-boosting vaccines are mediated by vaccine coverage and the degree to which vaccination provides resistance through anti-infection (i.e., reduction in infection establishment), anti-growth (i.e., decrease in within-host pathogen replication or increase in pathogen clearance), and anti-transmission (i.e., reduction in pathogen shedding) mechanisms (8, 13).

Vaccine efficacy can also be impacted by environmental factors infrequently accounted for in laboratory experiments, such as pathogen variants, environmental conditions, and pathogen exposure doses (14–17). As a result, estimates of vaccine efficacy based solely on laboratory studies may not be consistent with the effectiveness of the treatment when administered in real-world conditions. Given the complexity of eco-immunological interactions, an integrated approach of laboratory experiments, mechanistic ecological and epidemiological modeling, and field tests is imperative prior for scaling disease control interventions for widespread application to natural populations.

Here, motivated by experimental evidence on a promising prophylactic treatment (akin to a prototype vaccine) for chytridiomycosis, we combine a replicated whole-waterbody field experiment and mechanistic eco-epidemiological model to evaluate the efficacy of a wildlife disease intervention that could be used to slow the global decline of amphibian biodiversity. Chytridiomycosis, caused by the aquatic fungal pathogen *Batrachochytrium dendrobatidis* (Bd), is a textbook example of a disease imperiling biodiversity, having been implicated in an unprecedented level of biodiversity loss attributable to a single pathogen (18). The gravity of chytridiomycosis for amphibian conservation has prompted research into several novel disease control methods, including those based on vaccination, microbiome manipulation, and antifungal treatment (19–22). The discovery that tadpoles, metamorphic frogs, and adults could acquire resistance to Bd following topical exposure to a low concentration of Bd metabolites (non-infectious chemicals released by Bd in liquid culture) suggests the possibility of a vaccine for chytridiomycosis (15, 20, 23). We currently refer to this treatment as a prophylaxis rather than a vaccine because it is unknown whether the acquired resistance response is antibody-mediated, and a study by Siomoko et al. found that treatment with Bd metabolites is associated with

increased presence of Bd-inhibitory bacteria (24). However, given the functional equivalency of a vaccine and prophylaxis as preventative treatments, we discuss this work within the broader context of vaccination campaigns and use the term vaccination in relation to our model to indicate the generalizability of its applications.

Protection conferred by treatment with Bd metabolites is imperfect. Bd metabolites have been found to significantly reduce infection intensities (i.e. Bd genome equivalents on infected frogs) when frogs are treated prior to pathogen challenge, but are ineffective when applied post-pathogen exposure (25). Moreover, studies have shown that there is typically no difference in Bd prevalence between groups treated with Bd metabolites and those treated with a sham control (15, 25), and further investigations are necessary to determine whether the administration of Bd metabolites enhances the host's ability to tolerate infections. Despite its imperfection, this prophylaxis has important advantages: it is effective topically and it does not contain any infectious agents. Thus, it has strong potential for environmental distribution via direct application to waterbodies. Additionally, reductions in Bd loads suggest that the prophylaxis treatment reduces onward shedding and mortality given that Bd-induced mortality is dependent on infection intensity (26).

Given the partial protection conferred by Bd metabolite prophylaxis, we also built an agent-based eco-epidemiological model to generate hypotheses for how protective efficacy (magnitude of change in important epidemiological traits) and coverage would affect key epidemiological and conservation endpoints, such as population size, infection prevalence, infection intensity, and spillover capacity (defined as environmental zoospore density). We considered four mechanistic representations of imperfect immunity wherein 1) Bd metabolite treatment decreases probability of infection establishment upon pathogen exposure (anti-

infection immunity), increases pathogen clearance (anti-growth immunity), decreases rate of pathogen shedding (anti-transmission immunity), or increases the infection intensity threshold above which disease-induced mortality occurs (anti-disease immunity; i.e., tolerance). Anti-infection, anti-growth, and anti-transmission immunity are modes of acquired resistance, while anti-disease immunity is acquired tolerance (13).

Based on our simulations, we predicted that a prophylactic treatment providing anti-infection, anti-growth, or anti-transmission resistance would succeed from a disease conservation perspective by increasing population size and reducing infection intensity with increasing coverage and efficacy. However, if the prophylaxis treatment only increases tolerance, there would be no substantial change in host population size but infection intensities would increase, thereby increasing potential for disease transmission.

Finally, we tested these predictions using a Before-After-Control-Impact (BACI) experiment in which we administered the prophylaxis treatment at the whole waterbody-scale in replicated ponds in northern California. We did this following the breeding season and measured infection prevalence and load among post-metamorphic frogs 1-2 months later. Given the increased resistance observed in laboratory experiments (15, 20, 25), we predicted that ponds treated with the Bd metabolite prophylaxis would have significantly lower infection intensities and prevalence post-intervention than that of control ponds. We also conducted a live Bd challenge BACI experiment on field collected frogs from treated and untreated ponds to test for increased resistance that endured post-metamorphosis, described in *Supporting Information* (*SI*). Lastly, to strengthen our interpretation of the field experiment, we followed up with additional model simulations considering alternative mechanisms, such as the possibility of multiple partially-protective effects and scenarios in which prophylaxis could be harmful.

Materials and Methods

Field trial

Experimental design

We used a replicated whole-waterbody Before-After-Control-Impact (BACI) experimental design to test the efficacy of environmentally administered Bd metabolite prophylaxis on *Pseudacris regilla* (Pacific chorus frog) populations. Experimental units were ponds in the Blue Oaks Reserve research station in Santa Clara County, California, USA (Permit #14025, 19-940383, 21-1194611, and 1389361). We chose *P. regilla* as the focal species for this study as they have been implicated as a reservoir species for *Batrachochytrium dendrobatidis* (27), have stable populations (28), and two laboratory experiments showed that exposure to Bd metabolites can induce resistance in *P. regilla* during subsequent challenge with live Bd (23, 25). We collected pre-intervention baseline data on pond-level Bd prevalence and load ranging from 2011-2019 (2020 data unavailable due to Covid-19 pandemic restrictions); ponds varied in the number of years with pre-intervention data available but all ponds had a minimum of two years of data included (mean duration 4.6 years, range = 2 - 8 years). In the Springs of 2021 and 2022, we applied Bd metabolites (3000 zoospores removed/L pondwater per dose) to 6 ponds and a sham control treatment to 6 ponds. Ponds were distributed randomly between groups stratified by size, historical Bd prevalence and intensities, and amphibian community composition. Timing of treatment administration was chosen according to host phenology; we dosed ponds at approximated peak tadpole density after eggs hatched which was before tadpoles metamorphosed and after breeding adults had retreated. In the Summers of 2021 and 2022, we swabbed emerging metamorphs (Gosner Stage 44-46; hereon, referred to as both “metamorphs" or “frogs”) to quantify field-level infection prevalence and Bd pathogen load. Nested within this

study, we conducted a BACI-designed challenge experiment to quantify post-metamorphosis resistance given exposure to a known live pathogen dose. In 2019 (pre-intervention) and 2022 (post-intervention), we collected a subset of metamorphs from the field and dosed them with a known quantity of Bd to quantify pre- and post-intervention resistance. Additional methods for the live Bd challenge experiment can be found in the *Supporting Information* materials. Due to the scale of the project, some factors such as swabbing technique, quantitative polymerase chain reaction (qPCR) protocol, and lab varied between years within this study, but all methods were kept standard within-year and uniform across treatment groups, thus being accounted for within the BACI design structure.

Preparing and administering Bd metabolites

We prepared the Bd metabolite stock following methods previously described in Nordheim et al., (20). To summarize, we flooded Bd+ agar plates with artificial spring water (ASW; (29)) to obtain a solution containing live Bd and metabolites and then calculated the concentration of Bd zoospores in the solution using a 10 µL aliquot of the Bd+ solution hemocytometer to estimate the quantity of metabolites. Then, Bd zoospores and zoosporangia were removed from the solution by passing it through a 1.2 µm filter (GE Whatman Laboratory Products), thereby obtaining a filtrate containing only Bd metabolites suspended in ASW and no infectious material. To produce the sham control treatment, we replicated all steps for Bd metabolite stock preparation, with the exception of using Bd-, rather than Bd+, agar plates. The Bd metabolite stock and sham control were kept frozen until thawed prior to administration. Pond volume was determined to quantify the amount of Bd metabolite stock needed and was estimated using field measurements of perimeter, surface area, and depth at the pond center. We diluted the Bd

metabolite stock into pond water accordingly to attain an overall pond-level concentration of approximately 3000 zoospores-removed per L for each dosing event. We used the average Bd metabolite stock concentration for the sham control dilution factor. Tadpoles were often observed congregating at the shoreline (personal observation), thus we used watering cans to spray the diluted metabolite or sham treatment along the perimeter of each pond, administering metabolites from shoreline to approximately 1.5 m off the shore. We dosed each pond four times over two weeks in both April 2021 and April 2022.

Pond-level Bd infection prevalence and load

For field swabs collected from 2011-2021, metamorphs were swabbed using MW113 swabs (Advantage Bundling, North Carolina, USA) on the underside of their head, ventral surface, vent, cloaca, legs, and arms 10x each per location (total of 70 swab strokes). Bd infection status and load on swabs was determined by qPCR (see (30)) with plasmid standards designed to target Bd/Bsal (Pisces Molecular, Boulder, Colorado, USA). We screened for inhibition in every sample using TaqMan Exogenous Internal Control Reagents (Applied Biosystems, Foster City, California USA) and any sample with inhibition was rerun. In 2022, metamorphs were swabbed 10x on the ventral patch and 10x on each leg (a total of 30 strokes) with the same MW113 swabs and swabs were processed using the same methodology. In 2022, metamorphs were sent to the lab individually as part of the challenge experiment (see *SI: Challenge Experiment*) and, as the same swabbing and qPCR processing methods were used in the lab as in the field, the initial swabs of each individual taken upon arrival were included in the field swab dataset.

Data analysis on Bd field trial swabs

We conducted all statistical analyses in this study using R statistical software, version 4.0.3 (31). To assess if Bd metabolite addition altered infection prevalence in field-swabbed metamorphs, we used a binomial generalized linear model on binary infection status with time (before or after intervention) crossed with treatment (sham or Bd metabolites) as predictors for Bd prevalence with year and pond as random effects (package: glmmTMB, function: glmmTMB). We conducted a likelihood ratio test (package: stats, function: anova) to evaluate significance against a null model. We calculated confidence levels for Bd prevalence using the emmeans package (function: emmeans). To test for a time x treatment interaction in infection intensity, we used a zero-inflated negative binomial generalized linear model (package: glmmTMB, function: glmmTMB, ziformula = ~ treatment*before.after + (1 | pond) + (1 | year)) with time x treatment as predictors for Bd load with pond and year as random effects and fitted zero-inflation with these covariates. We also used the emmeans package (function: emmeans) to extract the mean infection intensity estimates for each treatment.

Bd-amphibian-vaccine model

We built a stochastic, stage-structured, and spatial agent-based model (ABM) of Bd-amphibian dynamics to assess pathogen and host population-level outcomes under different vaccination efficacies, coverage levels (i.e., proportion of the population immunized), and modes of imperfect immunity using NetLogo Version 6.3.0 (32). The model contained density-dependent transmission via a free-living Bd zoospore stage and infection-induced mortality elements like that of a prior non-spatial Bd-amphibian ABM (33), and included spatial structure, within-pond movement, host and pathogen development, and functional representations of imperfect vaccination (13). The model simulated within-season dynamics of a single-species

starting with tadpoles that, conditional on survival, transition into metamorphs by the end of the simulation. We used discrete daily time steps, spanning from 0-90 days to represent the aquatic Bd transmission season of ephemeral ponds in California. Each simulation began with 0.2% of tadpoles infected with an infection intensity of 100 zoosporangia.

Spatially, the model contained three types of environmental patches: 1) perimeter pond patches (light blue), 2) deep pond patches (dark blue), and 3) terrestrial patches (brown; Fig. S1). In the ponds included in our study, high densities of tadpoles are observed along the shoreline of ponds where the water is shallow, so we assumed that perimeter pond patches are hotspots of contact with pathogens whereas, while tadpoles can shed zoospores in neighboring deep pond areas, those zoospores are unlikely to contact hosts given the lower density of tadpoles per volume of water. Thus, in the model, tadpoles could move between perimeter pond patches but deposited zoospores to both perimeter and neighboring deep pond patches and metamorphs moved between land and perimeter pond patches. Given that tadpoles in the model did not move to deep pond patches and Bd is an aquatic fungal pathogen, zoospores deposited to deep pond or terrestrial patches did not contribute to onward infection.

The major processes of the model can be split into amphibian phenology and ecology, implementation of acquired immunity (vaccination), between-host transmission, and within-host infection processes (Table S1). The model tracked host survival, infection status, zoosporangium load, and the environmental zoospore density in the water body through time to obtain relevant population- and ecosystem-level outcomes such as final population size, infection prevalence, average infection intensity, and spillover risk. Parameters were selected based on values from the literature or were selected to match appropriate phenological and infection patterns (Supplemental Table 1).

Tadpoles could die with a daily chance of 6% ('tad-mort'; day^{-1}) and 25% of tadpoles moved among perimeter pond patches each day. They developed into metamorphs starting on day 55 with a daily probability of 11% and all tadpoles remaining on day 74 transitioned to metamorphs. Metamorphs could die with a baseline probability of 2% ('meta-mort'; day^{-1}) at each time step and moved between patches daily with 10% of the population on land patches and 90% on perimeter pond patches. Zoospores could be removed from the environment through contact with a host via an exposure parameter (i.e., amount of environmental units each host is exposed to) of 0.25 or by background death rate of 2 day^{-1} (34). Upon contact with a host, each zoospore infected the host with a baseline establishment probability of 0.25 ('est'; unitless). Successful zoospores developped into reproductive zoosporangia over a fixed 4-day period (35). Depending on the life stage of the host (i.e., tadpole or metamorph), vaccination status, the mode of vaccine protection and degree of vaccine efficacy, and history of exposure to zoospores, they could vary in zoosporangia load ('Spn'). Infected amphibians, those with a zoosporangia load > 0, shed zoospores into the environment with a baseline rate of 17.8 zoospores per zoosporangium per day and could clear zoosporangia with a baseline probability of 0.2 per day (33). Metamorphs retained immune traits, infection status, and zoosporangia load from their tadpole state (36) and could die due to Bd infection if their zoosporangia load equaled or surpassed the maximum threshold ('smax'). Depending on the mode of vaccine protection, the zoospore shedding rate, zoospore establishment probability, zoosporangium clearance probability, or Bd-induced mortality threshold of a vaccinated frog may differ from baseline values proportional to the degree of vaccine efficacy. Using this model, we ran the below scenarios and display the model results as contour plots created in R statistical software (31) using generalized additive models GAM models with a gaussian distribution (package: mgcv, function: fvisgam).

Modes of protection

Vaccine-induced immunity could provide four types of functional protective phenotypes (i.e., "modes of protection") by 1) reducing successful infection establishment (anti-infection resistance), 2) reducing pathogen shedding (anti-transmission resistance), 3) increasing infection clearance (anti-growth resistance), or 4) increasing a host's ability to survive infection (tolerance). First, we ran our model where vaccination modulated one mode of protection over varying vaccine coverages (i.e., proportion of the population vaccinated). In these scenarios, vaccination reduced infection establishment, decreased pathogen shedding, increased infection clearance, or boosted the threshold for infection-induced mortality by a 10-100% change to the baseline immune parameter in increments of 10. The degree of change to the baseline parameter is defined as efficacy. Vaccine coverage also varied 10-100% in increments of 10 across these scenarios. Each combination of mode of immunity, level of efficacy, and vaccine coverage was replicated 25 times for a total of 10,000 runs across the experiment. Additionally, we ran a baseline control scenario without vaccination 250 times. We compared outcomes from the varying vaccination scenarios to outputs of this control scenario to calculate relative differences to evaluate hypothetical intervention success (defined as an increase to population size and reduction in infection intensity, infection prevalence, and zoospore density).

Two-way interactions with tolerance

To investigate the possibility that vaccination simultaneously affected tolerance and a mode of resistance, we ran two-way interaction scenarios where vaccination increased tolerance by 0-100% in increments of 10, in conjunction with also either decreasing infection establishment,

reducing shedding, or increasing zoosporangia clearance from 0-100% in increments of 10. Vaccination coverage remained constant at 75% across these simulations. Each tolerance by mode of resistance combination was run 25 times for a total of 9,075 runs across the experiment. We then compared these scenario outputs to the control scenarios as described (see *Modes of protection*).

Vaccination caused harm

Given the adverse outcome of the field experiment, we explored the possibility that vaccination could be harmful rather than protective. In these scenarios, vaccination reduced immunity instead of boosting it, thus, vaccination either increased infection establishment, increased sporangia shedding, decreased tolerance or reduced infection clearance by 10-100% in increments of 10. We ran these scenarios across a gradient of coverages ranging from 10-100% in increments of 10. Again, each mode of harm by degree of harm by coverage combination was replicated 25 times for a total of 10,000 runs across the experiment and outputs were compared to results from unvaccinated control scenarios.

Results

Field trial and challenge experiment

There was a significant time (before vs. after intervention) by treatment interaction ($p = 0.001$) wherein Bd infection intensity increased after ponds were treated with Bd metabolites (Fig. 1). We found no significant time by treatment interaction in Bd prevalence for both field-swabbed (Fig. S2) and lab-challenged frogs (Fig S3a*)* and no significant time by treatment interaction in infection intensity for frogs challenged with live Bd (Fig S3b*)*.

Bd-amphibian-vaccine model

Modes of protection

Infection intensities decreased in model scenarios where vaccination boosts resistance (e.g., by reducing infection establishment, increasing infection clearance, and reducing pathogen shedding; Figs. 2a and S4), while infection intensities increased when vaccination boosts tolerance (Fig. 2c). Prevalence only decreased at very high levels of coverage and anti-infection or anti-transmission immunity, otherwise, prevalence remained unchanged (Fig. S5). Frog population size increased with increasing levels of coverage and resistance (Figs. 2b and S6), but the effect was less strong for tolerance as high levels of coverage and efficacy were needed to increase the population size by 20% as compared to an untreated population (Fig. 2d). Zoospore density (i.e., high zoospore densities indicate greater risk of pathogen spillover) decreased with boosts to resistance (Fig. S7 a-c). However, zoospore densities remained unchanged or increased when vaccination boosted tolerance (Fig. S7d).

Two-way interactions with tolerance

When vaccination enhances both tolerance and resistance, the effect of tolerance on increasing Bd infection intensities was counteracted with increasing efficacy of the boosted resistance phenotype (Figs. 3a and S8). When combined with boosted tolerance, prevalence only decreased with a high degree of anti-infection resistance, otherwise, prevalence remained unchanged (Fig. S9). Population sizes increased with increasing resistance (Figs. 3b and S10). While resistance phenotypes appeared to drive this boost in population size irrespective of the degree of enhanced tolerance (Figs. 3b and S10), there appeared to be a slight observable

interaction wherein when vaccination only provided weak anti-growth resistance, boosting tolerance increased population sizes above that of increasing clearance alone (Fig. 3b). Lastly, zoospore densities decreased with increasing resistance efficacy and zoospore densities were slightly lower when tolerance was also low (Fig. S11).

Vaccination caused harm

Infection intensities decreased with increasing reductions in resistance and increasing population coverage (Figs. 4a and S12). The pattern of decreased infection intensities with increasing coverage and reduced resistance is also observed when vaccination reduced tolerance, though greater levels of coverage and harm are needed to see this effect strongly (Fig. S12). Similarly, infection prevalence decreased with increasing coverage and harm when vaccination reduced resistance or tolerance (Fig. S13), though again in the case of decreasing tolerance, high coverage and harm was needed to see this effect. This pattern was also seen for population sizes, wherein lowered resistance and tolerance led to lower resulting population sizes as compared to untreated populations (Figs. 4b and S14). Lastly, zoospore densities increased in scenarios where vaccination reduced resistance (Fig. S15, a-c). Conversely, zoospore densities decreased in scenarios where vaccination decreased tolerance (Fig. S15d).

Discussion

Here, we conducted a field evaluation of a Bd prophylaxis that has previously been shown to induce resistance, indicated by protection against high Bd infection intensities, in several laboratory trials (15, 20, 25). Counter to previous findings from laboratory trials, Bd infection intensity significantly increased ($p = 0.001$) after ponds were treated with Bd

metabolites (Fig. 1). We found no change in infection prevalence in field swabbed frogs (Fig. S1*)*; additionally, we found no significant change in infection intensity or Bd prevalence in frogs experimentally challenged with live Bd (Fig. S2). We then used mechanistic modeling of amphibian-Bd-vaccine dynamics to aide interpretation of these field results. All modeled scenarios where vaccination boosted resistance (e.g., reduced infection establishment, increased infection clearance, and reduced pathogen shedding) led to a decrease in infection loads (Figs. 2a and S4), while vaccination which strongly enhanced tolerance led to an increase in infection intensities (Fig. 2c). Thus, of the four modes of protection modelled, scenarios in which vaccination boosts tolerance (i.e. increases a host's ability to survive high infection burdens) were the only scenarios consistent with our field results. Also consistent with our field results, model scenarios showed enhanced tolerance did not change infection prevalence (Fig. S5). Additionally, our model results show that while all three modes of resistance increased frog population sizes compared to an untreated control population, boosting tolerance was not effective at notably increasing frog population size (Fig. 2b). Thus, we speculate that vaccination is ineffective at meeting the key conservation goal of increasing frog population size if the only mode of protection is enhanced tolerance. Unfortunately, we were not able to measure population sizes in the field given that *P. regilla* disperse as they metamorphose, thus we are unable to compare these model results with field observations. However, we did not detect notable die-offs at any ponds throughout the duration of our study. While tolerance is beneficial at the individual level, it can be deleterious at the population-level when, rather than dying, highly infected individuals are able to continue shedding for prolonged durations of time (10, 27). Increased zoospore (i.e., the infectious stage of the pathogen) density as a consequence of enabling higher infection intensities may increase risk of spillover to susceptible sympatric hosts

(Fig. S6). Thus, not only is enhanced tolerance alone unlikely to successfully aid amphibian conservation given our model results, it can be deleterious.

Vaccination can provide more than one mechanism of partial protection (8, 37, 38). Given that our model results indicate tolerance is a driving mechanism in determining vaccination backfiring, we tested scenarios where vaccination both enhanced tolerance and a mode of resistance. When vaccination at least moderately increases resistance (with or without increasing tolerance), infection intensities decrease and frog population sizes increase (Figs. 3, S7 and S9). However, in scenarios where vaccination provides only a weak boost to resistance but a strong boost to tolerance, infection intensities are higher than unvaccinated scenarios (Figs. 3a and S7). Therefore, results from our field trial are consistent with the hypothesis that the Bd metabolite prophylaxis has a greater impact on enhancing tolerance compared to its effect on increasing resistance. This proposed explanation helps to reconcile previous findings that Bd metabolites provide some mechanism of acquired resistance, evidenced by significantly lower infection intensities in frogs treated prophylactically in laboratory experiments (15, 20, 25), with the opposite result of higher infection intensities in field-treated frogs (Fig. 1).

While evolutionary trade-offs between resistance and tolerance mechanisms of immunity are well-documented, both mechanisms of protection need not necessarily be mutually exclusive in the case of acquired immunity. Specifically, it is common that vaccines targeting toxins (e.g., toxoid vaccines like those for pertussis (37, 39) and diphtheria (38)) do not prevent infection but reduce disease and partially limit transmission. Given this, we postulate a potential mechanism for a combined tolerance-resistance acquired immune response. As Bd metabolites are known to facilitate immune system dysregulation caused by Bd infection (40, 41), it is possible that prophylactic use of Bd metabolites in low concentration elicits an anti-toxin immune response,

reducing immunopathology associated with Bd infection and thereby boosting infection tolerance (42). Consequently, it may be possible that if prophylactic treatment reduces immunopathology, immune systems of treated frogs could be more competent in providing anti-Bd resistance.

Alternatively, heightened infection intensities in field-treated frogs could be the result of an environmental interaction wherein prophylactic treatment is harmful to hosts when applied in field conditions. This has been observed with antibody-dependent enhancement in response to dengue vaccination (14). To investigate this hypothetical possibility, we explored model scenarios in which vaccination increased disease susceptibility by decreasing tolerance, reducing infection clearance, exacerbating infection establishment or increasing pathogen shedding. However, under our model parameterization, these scenarios led to die-offs (Figs. 4b and S14) and subsequently lower infection intensities (Figs. 4a and S12) and infection prevalence (Fig. S13) in surviving frogs, and thus are inconsistent with our field observations. Notably, in simulated scenarios where vaccination caused harm by decreasing tolerance, zoospore densities decreased as a result of highly infected individuals being culled from the population and in all other scenarios vaccination-induced harm increased zoospore densities (Fig. S15).

Herd immunity, wherein vaccinated individuals indirectly protect susceptible individuals by disrupting transmission, is considered a central benefit to vaccination but is only achievable under certain conditions of coverage, efficacy, and modes of protection. While significant attention has been paid to determining coverage thresholds required to achieve herd immunity, we must also emphasize that herd immunity is a function of the extent to which vaccination reduces the transmission potential of an immunized host and that not all vaccines or prophylactic treatments can produce herd immunity. Studies often determine vaccine success by reductions in

infection risk, disease severity, pathogen load, or neutralizing antibody titers (9, 43–45). However, our model results draw attention to how the relative strength of interacting functional modes of vaccine-induced protection (anti-infection, anti-growth, anti-transmission and anti-disease immunity) can lead to divergent outcomes, specifically in relation to how changes in host tolerance translate to greater transmission per infected host. Assessing differences in survival based on treatment is often thought to be a measure of tolerance, but survival is confounded by resistance as lowered infection burdens due to boosted resistance can also lead to enhanced survival (42). It can be challenging to empirically untangle the relative contributions of resistance versus tolerance that result in increased survival, but specifically attributing outcomes to resistance versus tolerance mechanisms is not necessary for quantifying the impact of these interacting effects on herd immunity. To achieve this, we strongly suggest more focus be placed on directly quantifying differences in pathogen transmission by measuring duration of transmission period and productivity of pathogen shedding in immunized individuals compared to untreated individuals to better project the success of vaccination or prophylaxis in reducing infection prevalence and burdens at the population-level.

Duration of protection is also a key factor in determining vaccine efficacy. While our challenge experiment did not find evidence that induced resistance during the larval period endures past metamorphosis, our experiment (see *SI Challenge Experiment*) was limited by sample size (number of frogs collected per pond and number of years replicated) and our inability to confirm that the frogs collected from Bd metabolite treated ponds were sufficiently exposed to those metabolites. Thus, future controlled laboratory studies should investigate if and to what degree protection provided by Bd metabolite exposure wanes through time and development. Additionally, this model can be adapted in the future to explore scenarios varying

durations of acquired protection. Furthermore, studies should evaluate potential non-target impacts of Bd metabolite addition on other species in these aquatic communities, including invertebrates, some of which have exhibited pathology in response to extremely high doses of Bd metabolites (46, 47).

Overall, our findings emphasize that determining the effectiveness of a partially protective vaccine or prophylaxis requires a cross-scale (from individual to population-level) approach and specific attention to the degree to which vaccination affects transmission. Importantly, aims of vaccination campaigns can differ from management of disease morbidity and mortality (i.e., public health campaigns), reduction in disease prevalence (i.e., for disease spillover prevention), or reversal of disease-induced population declines (i.e., for wildlife conservation) and these differing ideals of success correspond with different priorities for vaccine-induced protection. Given its global distribution and complex ecology, eradication of Bd is unrealistic. Tools that facilitate endemic Bd presence, where it does not cause outbreaks resulting in large-scale die-offs (33), are the priority. As Bd metabolite treatment increased infection intensities in the field, it is unlikely that this treatment will be useful or safe for amphibian conservation when used as a sole intervention. However, our model results suggest that the deleterious impacts of enhanced tolerance on increasing infection loads be overcome with increased resistance (Figs. 3a and S8). Thus, if direct evidence is found that the prophylaxis enhances tolerance (e.g. reduces Bd-associated immunopathology), it may be considered for use in combination with other interventions which strongly boost resistance.

Additionally, it is possible that an environmental interaction caused the increase in Bd metabolites and future research should elucidate if environmental factors such as temperature, sunlight, water chemistry, alternative circulating Bd strains, transmission seasonality, pond size

and tadpole density impact treatment efficacy. If an environmental interaction caused this prophylaxis to backfire in California ponds, it is possible Bd metabolite addition may be suitable in other amphibian communities given that Bd is found across diverse ecosystems.

Acknowledgements

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Figures

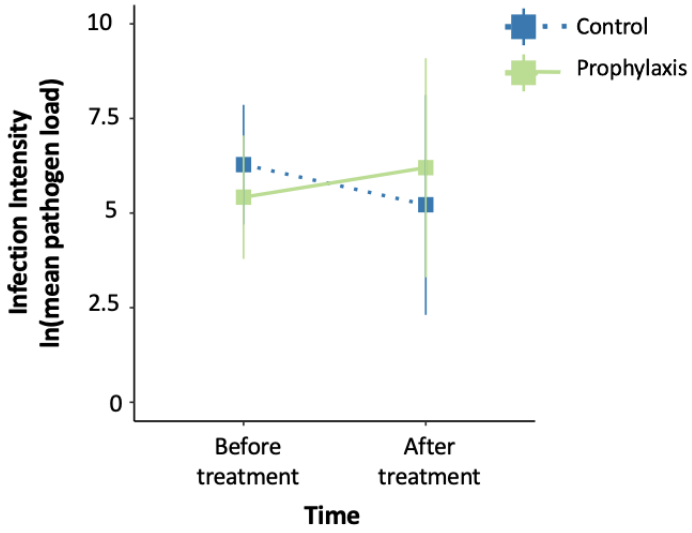

Figure 1. Estimated mean infection intensity (i.e., Bd load of infected individuals transformed to natural log scale) before and after Bd metabolite addition in *Pseudacris regilla* metamorphic frogs. There was a significant time by treatment interaction ($p = 0.001$) wherein frogs from ponds treated with Bd metabolites had significantly higher Bd loads after treatment than frogs in ponds treated with the sham treatment.

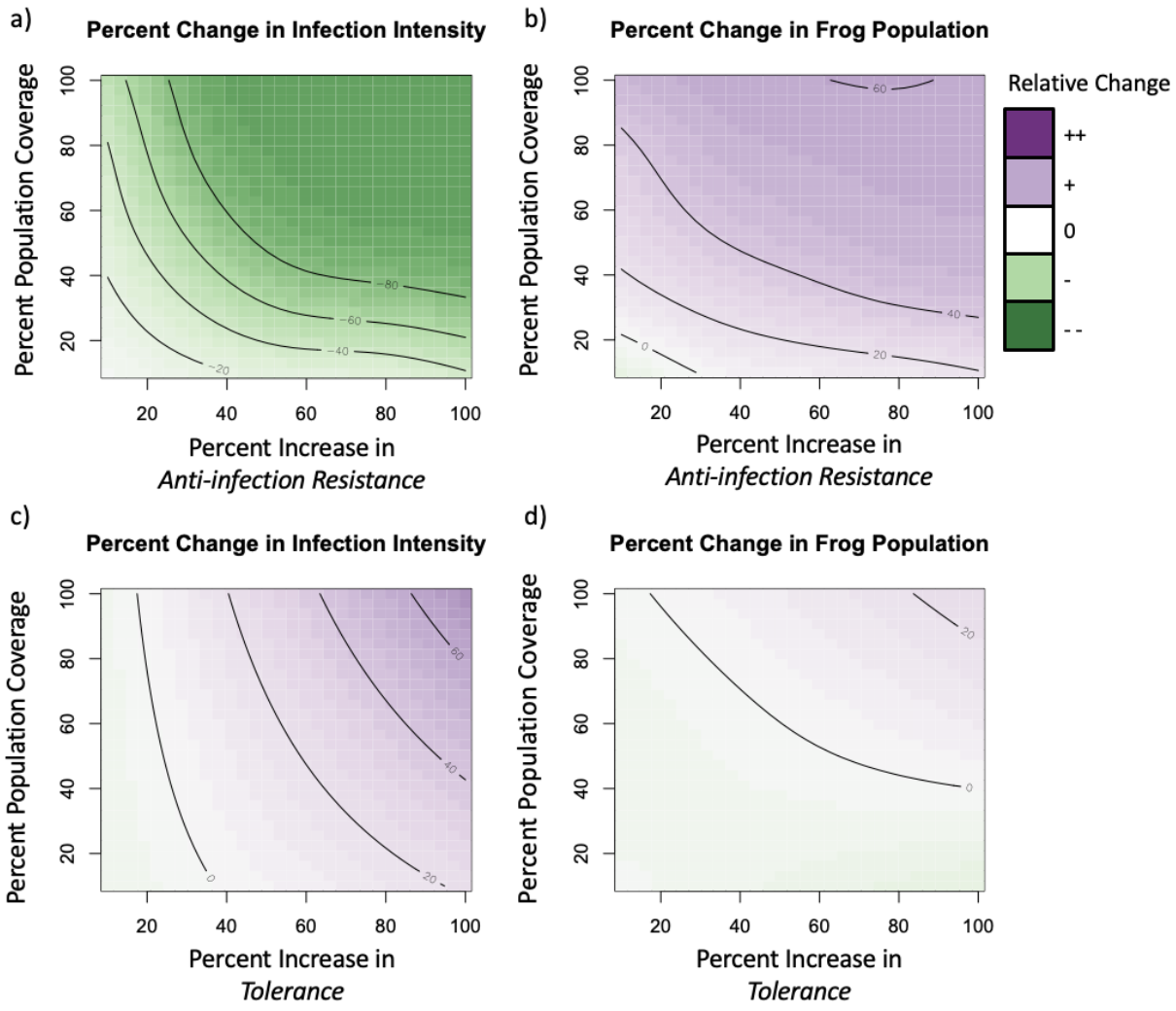

Figure 2. Changes to infection intensity and frog population size when vaccination increases resistance or tolerance. Generalized Additive Model (GAM) summary of modeled changes in (a) infection intensity and (b) final frog population (green-purple color scale) as a function of increasing vaccination-induced anti-infection resistance (decrease in infection establishment; x-axis) and population coverage (y-axis), relative to simulations of an untreated control population. GAM summary of modeled changes in c) infection intensity and d) final frog population as vaccination increases host tolerance (increase in infection induced mortality threshold; x-axis) and population coverage on the y-axis relative to a modeled untreated control population. Deeper green shades represent reductions and deeper purples represent increases

compared to unvaccinated populations. Contour lines define increments of 20% change relative to vaccine-free simulations. (a) Infection intensities decrease and, correspondingly, (b) frog population sizes increase as population coverage and efficacy of vaccine-induced resistance increases. Alternatively, (c) infection intensities increase as population coverage and efficacy of vaccine-induced tolerance increase and (d) population size only substantially increases with high levels of population coverage and boosted tolerance.

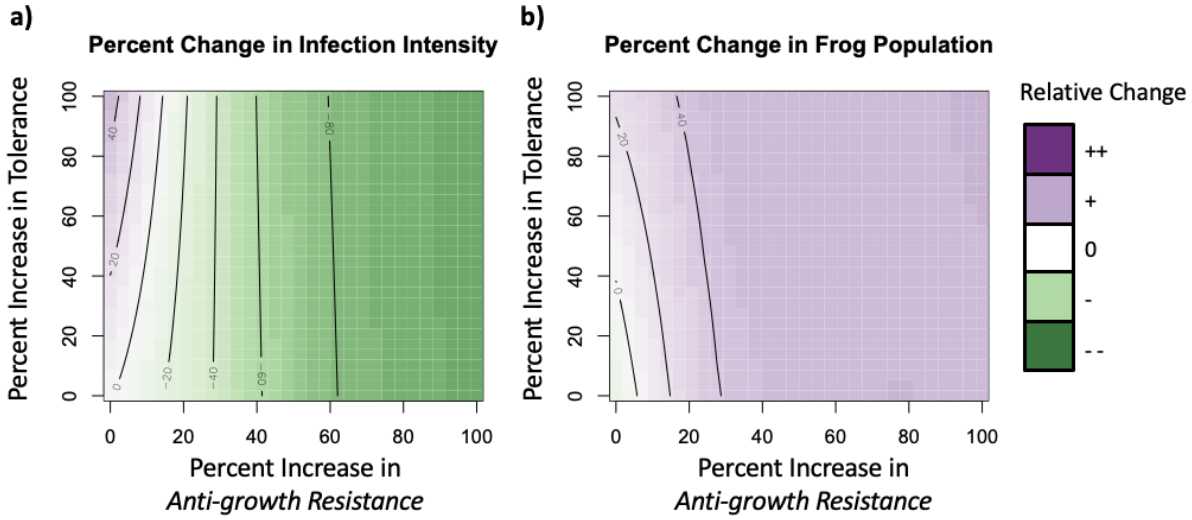

Figure 3. Changes to infection intensity and frog population size when vaccination provides both tolerance and resistance. Generalized Additive Model summary of modeled changes in a) infection intensity and b) final frog population (green-purple color scale) as vaccination boosts both anti-growth resistance (increase in pathogen clearance; x-axis) and tolerance (increase in infection induced mortality threshold; y-axis) in a population where 75% of hosts are treated, relative to simulations of an untreated control population. Deeper green shades represent reductions and deeper purples represent increases compared to unvaccinated populations. Contour lines define increments of 20% change relative to vaccine-free simulations. (a) When

vaccination strongly boosts tolerance and only provides a minor increase to anti-infection resistance, infection intensities increase but when vaccination provides at least a small boost in anti-growth resistance, regardless of the degree of enhanced tolerance, infection intensities decrease. (b) Frog population sizes increase with increasing anti-growth resistance, with a minor boost from increasing tolerance at low levels of anti-growth resistance.

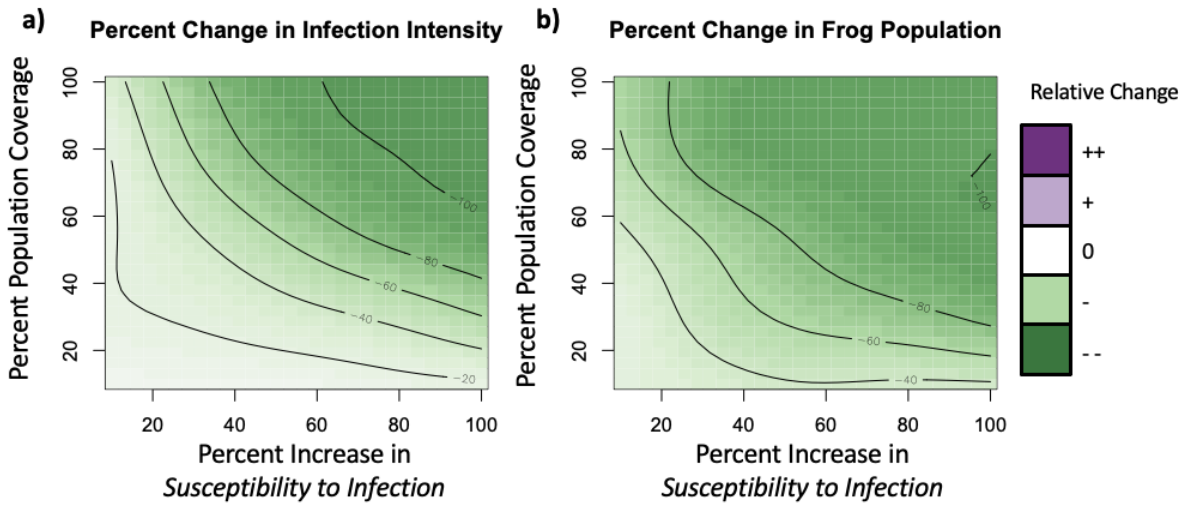

Figure 4. Changes to infection intensity and frog population size when vaccination is harmful. Generalized Additive Model summary of modeled changes in a) infection intensity and b) final frog population (green-purple color scale) as vaccination increases susceptibility to infection (increases infection establishment; x-axis) and population coverage (y-axis), relative to simulations of an untreated control population. Deeper green shades represent reductions and deeper purples represent increases compared to unvaccinated populations. Contour lines define increments of 20% change relative to vaccine-free simulations. (a) Infection intensities and b) frog populations decrease as susceptibility and population coverage increase.

Supporting Information

Challenge Experiment

Materials and Methods

As a subset of the Before-After-Control-Impact (BACI) field trial, we conducted a live Bd challenge experiment to assess if there were differences in post-metamorphosis resistance between frogs from ponds treated with Bd metabolites or sham.

Pre-intervention: Pseudacris regilla metamorphs (defined as Gosner Stage 44-46; n= 30-50 per pond, average = 40) from each pond were sent overnight on ice in groups of 15-26 frogs per 1000-3000mL Tupperware containers to the McMahon Lab at University of Tampa, Tampa, Florida, USA (IACUC #2018-2). Each container contained a moistened paper towel and air holes in the lid. On day of arrival, metamorphs were swabbed 10x on the left leg, weighed, and placed in 12 oz clear deli cups with air holes and paper towel dampened with artificial spring water (ASW; (1)) on the bottom. They were kept on a 12 hr light/dark cycle at 21 degrees Celsius. Metamorphs were fed live calcium-dusted crickets 3x per week and container changes were done weekly. All metamorphs were dosed with 6×10^4 zoospores of live Bd JEL-270 and were swabbed, weighed and euthanized on the 10th day after live Bd exposure using Orajel (20% benzocaine gel was placed on the head and dorsal side of the frog (2)). To quantify Bd infection status and load, all swabs were processed using qPCR with plasmid standards designed to target Bd/Bsal (Pisces Molecular, Boulder, Colorado, USA) (3). All samples were screened for

inhibition using TaqMan Exogenous Internal Control Reagents (Applied Biosystems, Foster City, California USA) and reran if found to be inhibited.

Post-intervention: Pseudacris regilla metamorphs (n= 9-45 per pond, average = 28) were sent overnight on ice in individual falcon tubes, with a moistened cotton ball and air hole, to the Rohr Lab at University of Notre Dame, South Bend, Indiana, USA (IACUC #19-04-5328). Upon arrival, each metamorph was swabbed according to the protocol used for the 2022 field swabs and weighed. Metamorphs were maintained in the same housing conditions as those in 2019. Metamorphs were split into two batches per arrival date – half of the frogs were challenged with 2.5×10^5 zoospores of live JEL-270 Bd on day of arrival and the other half were challenged with the same dose of live Bd a week after to standardize effects of Bd batch. As in 2019, frogs were swabbed, weighed and euthanized on the 10th day after Bd exposure using Orajel (20% benzocaine gel was placed on the head and dorsal side of the frog) (2). Bd infection status and load was diagnosed using the same methodology as that used for the pre-intervention swabs.

Data analysis: To test if Bd metabolite addition altered infection outcomes in field-collected metamorphs challenged with a known dose of live Bd, we used a binomial generalized linear model on binary infection status of the post-challenge swab with time crossed with treatment as predictors for probability of Bd infection and pond as a random effect (package: glmmTMB, function: glmmTMB) in R statistical software, version 4.0.3 (4). We again used a likelihood ratio test (package: stats, function: anova) to evaluate significance against a null model and calculated confidence levels using the emmeans package (function: emmeans). Then, we used a zero-inflated negative binomial generalized linear model (package: glmmTMB, function: glmmTMB) with time x treatment as predictors for infection intensity with pond as a random effect and fitted

zero-inflation with these covariates. We estimated mean infection intensities for each treatment using the emmeans package (function: emmeans).

Results and Discussion

We found no significant time by treatment interaction in infection intensity or probability of infection for field-collected frogs challenged with live Bd (Fig. S2). There are several reasons that could explain why we did not see any effect of Bd metabolite addition in the challenge experiment: 1) resistance induced by Bd metabolites may not carry through metamorphosis, 2) prophylaxis coverage may not have been high and frogs collected for the challenge experiment may not have been directly exposed to the metabolites even if from metabolite-treated ponds, and 3) sample size was considerably lower for the challenge experiment (e.g., data from only a single pre- and post-intervention year, compared with field experiment which had data from multiple pre-intervention years, two post-intervention years, and a larger number of animals swabbed).

Figures

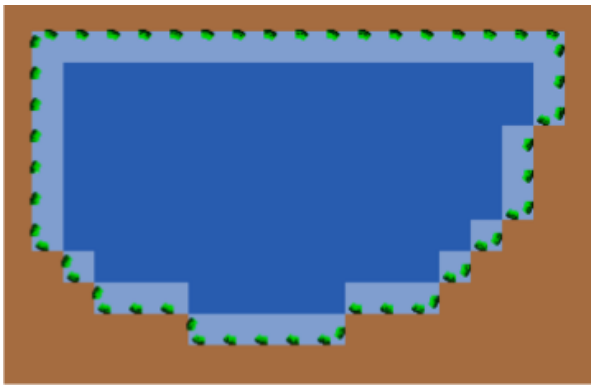

Figure S1. Netlogo user interface graphical display of the Bd-amphibian-vaccine model's spatial structure. There are types of environmental patches in this model: 1) perimeter pond patches (light blue), 2) deep pond patches (dark blue), and 3) terrestrial patches (brown). Amphibians are represented as green and black objects.

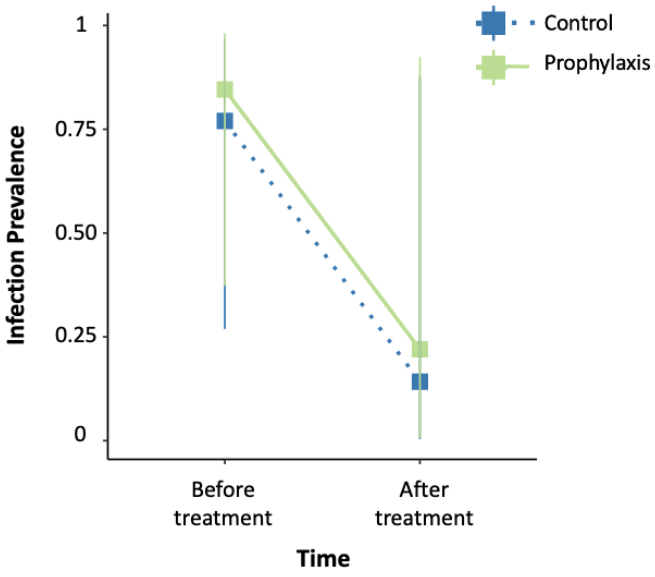

Figure S2. No significant interaction ($p > 0.05$) between time (before/after treatment addition) and treatment type for infection prevalence in field swabbed frogs.

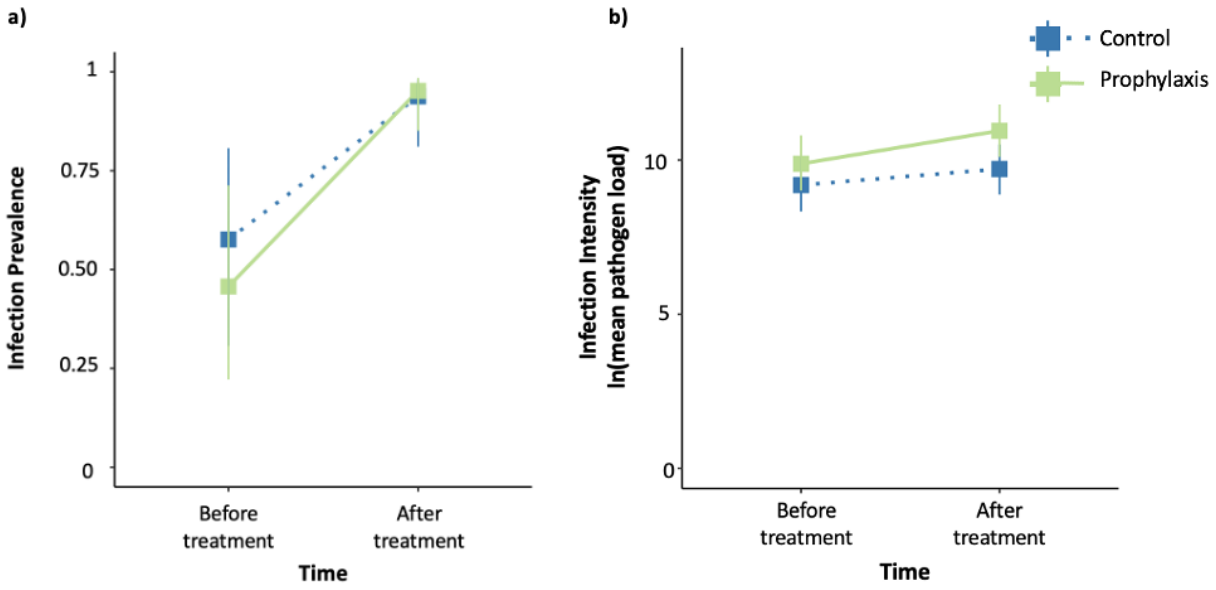

Figure S3. In the Bd challenge experiment, there was no significant interaction ($p>0.05$ for Fig S2 a and b) between time (before/after treatment addition) and treatment in a) estimated mean infection intensity (Bd load (GE) of infected individuals transformed to natural log scale) or b) infection prevalence.

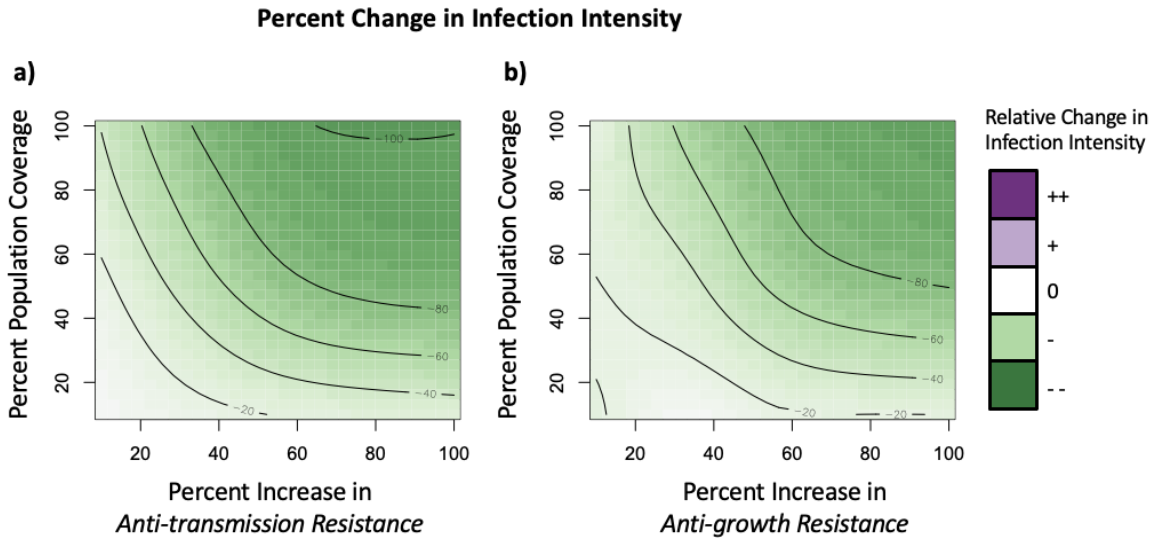

Figure S4. Changes in infection intensity when vaccination provides either anti-transmission or anti-growth resistance across increasing population coverage. Generalized Additive Model summary of modeled changes in infection intensity (green-purple color scale) as a function of vaccination-induced increase in (a) anti-transmission resistance (i.e., decrease in pathogen shedding) or (b) anti-growth resistance (increase in pathogen clearance; x-axis) and population coverage (y-axis), relative to simulations of an untreated control population. Deeper green shades represent reductions and deeper purples represent increases compared to unvaccinated populations. Contour lines define increments of 20% change relative to vaccine-free simulations. Infection intensities decrease as (a) anti-transmission resistance or (b) anti-growth resistance, as well as, population coverage increase.

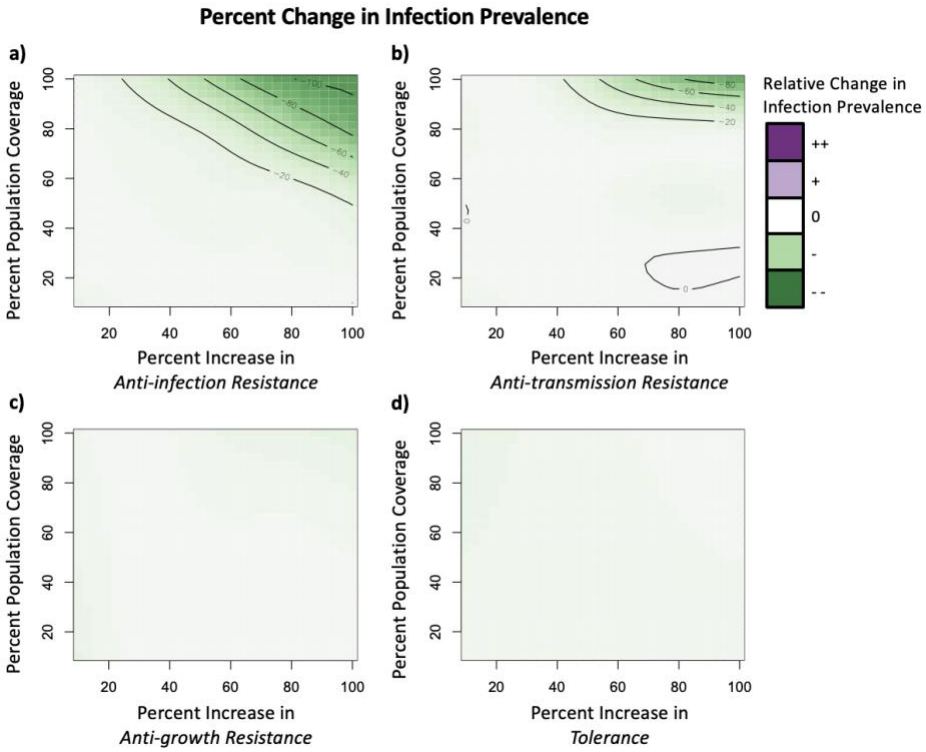

Figure S5. Changes in infection prevalence when vaccination provides anti-infection resistance, anti-transmission resistance, anti-growth resistance or tolerance across increasing levels of population coverage. Generalized Additive Model summary of modeled changes in infection prevalence (green-purple color scale) as a function of vaccination-induced increase in (a) anti-infection resistance (decrease in infection establishment), (b) anti-transmission resistance (decrease in pathogen shedding), (c) anti-growth resistance (increase in pathogen clearance), or (d) tolerance (increase in infection induced mortality threshold; x-axis) and population coverage (y-axis), relative to simulations of an untreated control population. Deeper green shades represent reductions and deeper purples represent increases in infection prevalence compared to unvaccinated populations. Contour lines define increments of 20% change relative to vaccine-free simulations. Infection prevalence decreases at high levels of (a) anti-infection resistance or (b) anti-transmission resistance and coverage but does not change under any scenarios of (c) anti-growth resistance or (d) tolerance boosting vaccines.

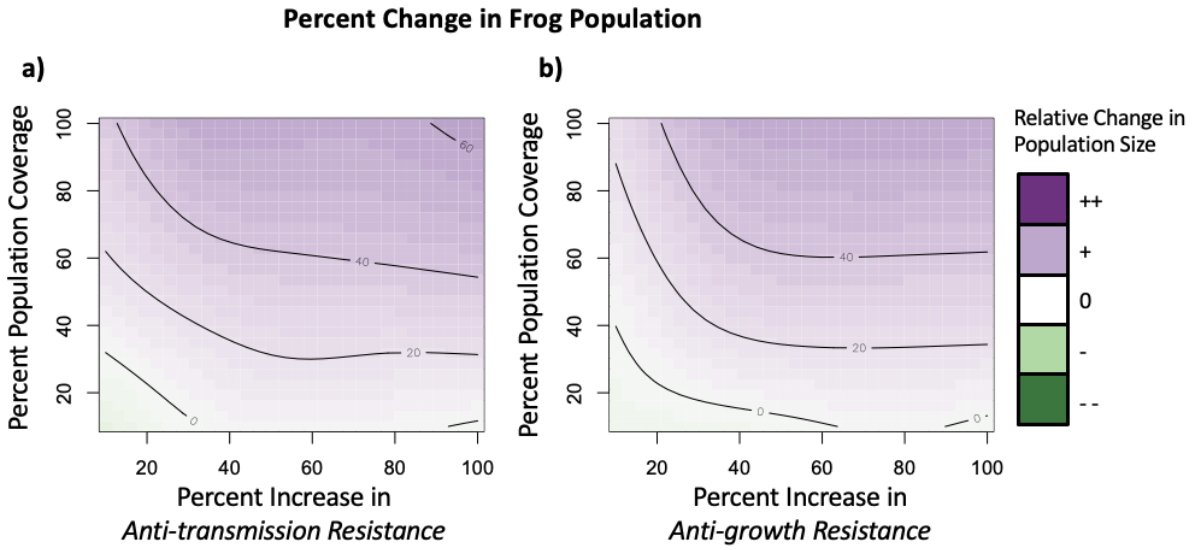

Figure S6. Changes to frog population size when vaccination provides either anti-transmission or anti-growth resistance across increasing population coverage. Generalized Additive Model summary of modeled changes in surviving population size (green-purple color scale) as a function of vaccination-induced increase in (a) anti-transmission resistance (i.e., decrease in pathogen shedding) or (b) anti-growth resistance (increase in pathogen clearance; x-axis) and population coverage (y-axis), relative to simulations of an untreated control population. Deeper green shades represent reductions and deeper purples represent increases in frog population compared to unvaccinated populations. Contour lines define increments of 20% change relative to vaccine-free simulations. Frog population sizes increase as (a) anti-transmission resistance or (b) anti-growth resistance and population coverage increase.

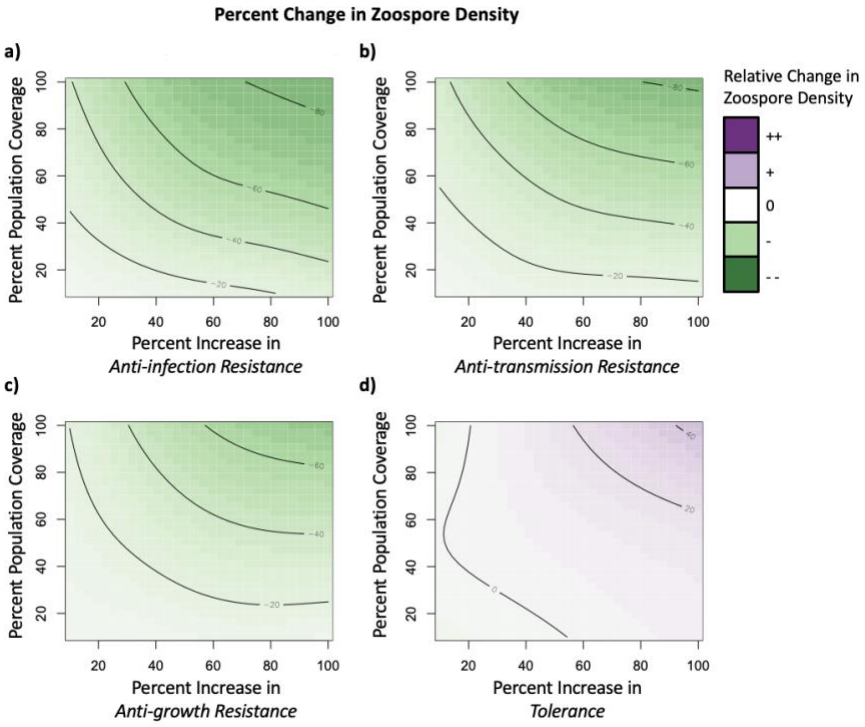

Figure S7. Changes in zoospore density when vaccination provides anti-infection resistance, anti-transmission resistance, anti-growth resistance or tolerance across increasing levels of population coverage. Generalized Additive Model summary of modeled changes in zoospore density (green-purple color scale) as a function of vaccination-induced increase in (a) anti-infection resistance (decrease in infection establishment), (b) anti-transmission resistance (decrease in pathogen shedding), (c) anti-growth resistance (increase in pathogen clearance), or (d) tolerance (increase in infection induced mortality threshold; x-axis) and population coverage (y-axis), relative to simulations of an untreated control population. Deeper green shades represent reductions and deeper purples represent increases in zoospore density compared to unvaccinated populations. Contour lines define increments of 20% change relative to vaccine-free simulations. Zoospore densities decrease as (a) anti-infection resistance, (b) anti-transmission resistance, or (c) anti-growth resistance and coverage increase but zoospore density increases with high levels of enhanced tolerance and coverage.

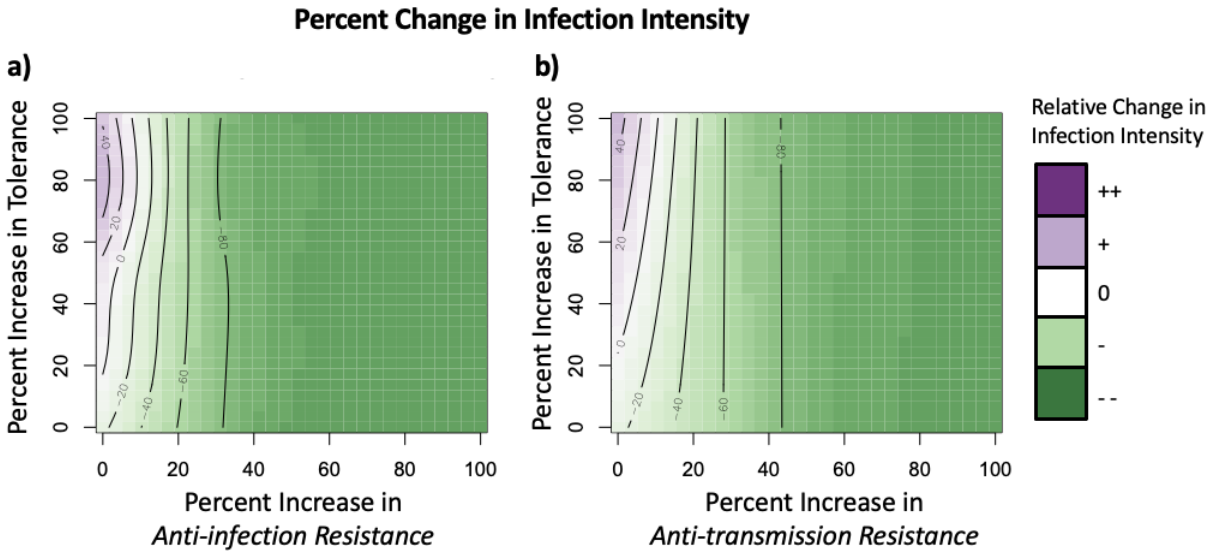

Figure S8. Changes to infection intensity when vaccination provides both tolerance and either anti-infection or anti-transmission resistance. Generalized Additive Model summary of modeled changes in infection intensity (green-purple color scale) as a function of vaccination-induced increases in (a) anti-infection resistance (decrease in infection establishment) or (b) anti-transmission resistance (decrease in pathogen shedding; x-axis) and enhanced tolerance (y-axis), relative to simulations of an untreated control population. Deeper green shades represent reductions and deeper purples represent increases in infection intensity compared to unvaccinated populations. Contour lines define increments of 20% change relative to vaccine-free simulations. Infection intensities decrease as (a) anti-infection resistance or (b) anti-transmission resistance increase, but infection intensities increase at high levels of enhanced tolerance and low levels of resistance. When (a) anti-infection resistance is low, it appears there is a slightly greater decrease in infection intensities when vaccination provides only a minor boost to tolerance.

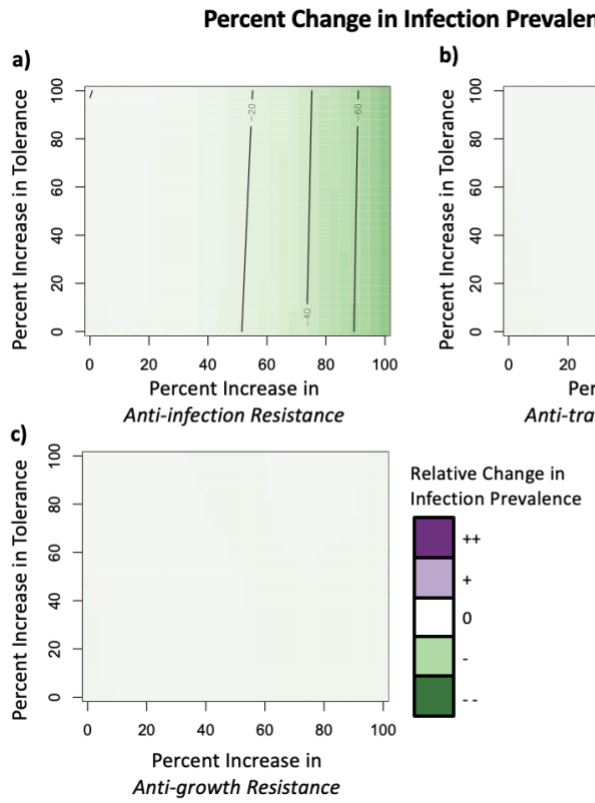

Figure S9. Changes to infection prevalence when vaccination provides both tolerance and resistance. Generalized Additive Model summary of modeled changes in infection prevalence (green-purple color scale) as a function of vaccination-induced increases in (a) anti-infection resistance (decrease in infection establishment), (b) anti-transmission resistance (decrease in pathogen shedding), (c) anti-growth resistance (increase in pathogen clearance; x-axis) and increase in tolerance (infection induced mortality threshold; y-axis) where 75% of hosts are vaccinated, relative to simulations of an untreated control population. Deeper green shades represent reductions and deeper purples represent increases in infection prevalence compared to unvaccinated populations. Contour lines define increments of 20% change relative to vaccine-free simulations. Infection prevalence decreases at high levels of (a) anti-infection resistance, regardless of level of boosted tolerance, but does not change under any combinations of boosted (b) anti-transmission resistance or (c) anti-growth resistance and enhanced tolerance.

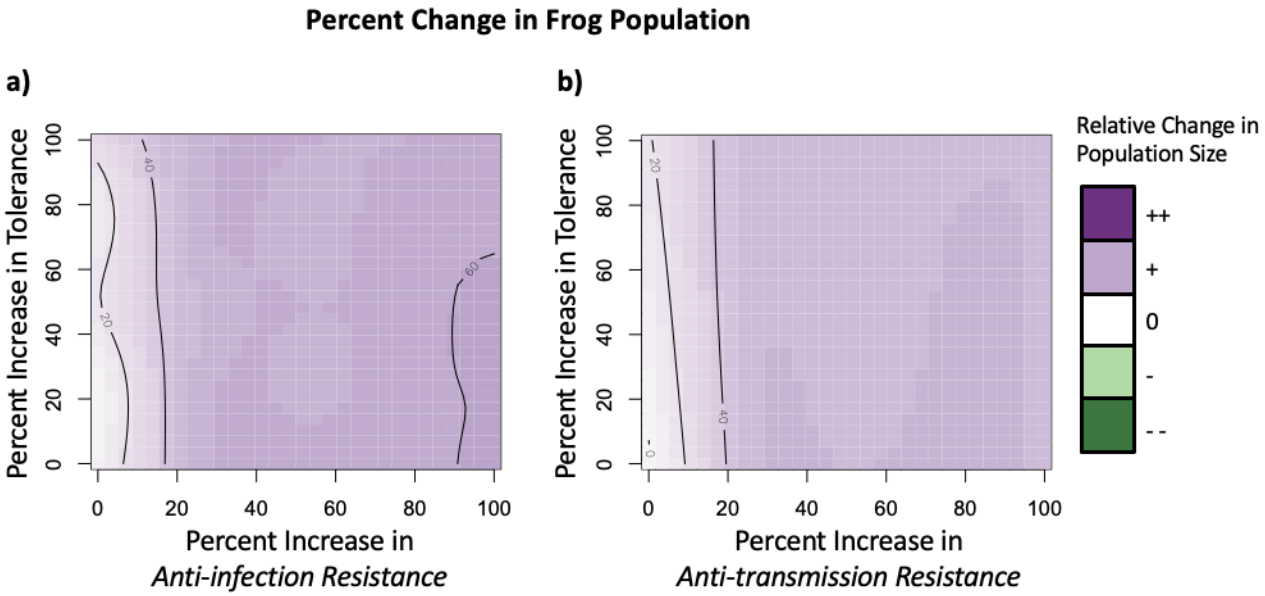

Figure S10. Changes to frog population size when vaccination provides both tolerance and either anti-infection or anti-transmission resistance. Generalized Additive Model summary of modeled changes in surviving frog population size (green-purple color scale) as a function of vaccination-induced increases in (a) anti-infection resistance (decrease in infection establishment) or (b) anti-transmission resistance; (decrease in pathogen shedding; x-axis) and enhanced tolerance (y-axis) in a population where 75% of hosts are treated, relative to simulations of an untreated control population. Deeper green shades represent reductions and deeper purples represent increases in frog population size compared to unvaccinated populations. Contour lines define increments of 20% change relative to vaccine-free simulations. Population sizes increase as (a) anti-infection resistance or (b) anti-transmission resistance, with negligible effects of increasing tolerance.

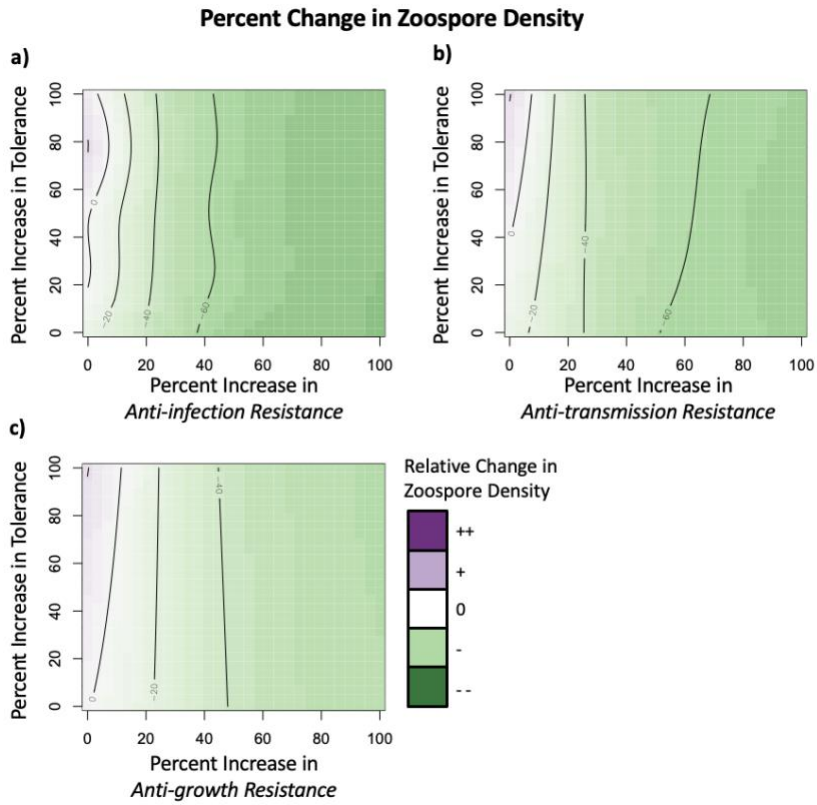

Figure S11. Changes to zoospore density when vaccination provides both tolerance and resistance. Generalized Additive Model summary of modeled changes in zoospore density (green-purple color scale) as a function of vaccination-induced increases in a) anti-infection resistance (decrease in infection establishment), b) anti-transmission resistance (decrease in pathogen shedding), or c) anti-growth resistance (increase in pathogen clearance; x-axis) and tolerance (increase in infection induced mortality threshold; y-axis), relative to simulations of an untreated control population. Population coverage was kept at 75% across all simulations. Deeper green shades represent reductions and deeper purples represent increases in zoospore density compared to unvaccinated populations. Contour lines define increments of 20% change relative to vaccine-free simulations. Zoospore density decreases at moderate to high levels of resistance, especially when increases in anti-transmission resistance are greater than increases in tolerance (b), but increases when resistance is low and tolerance is high.

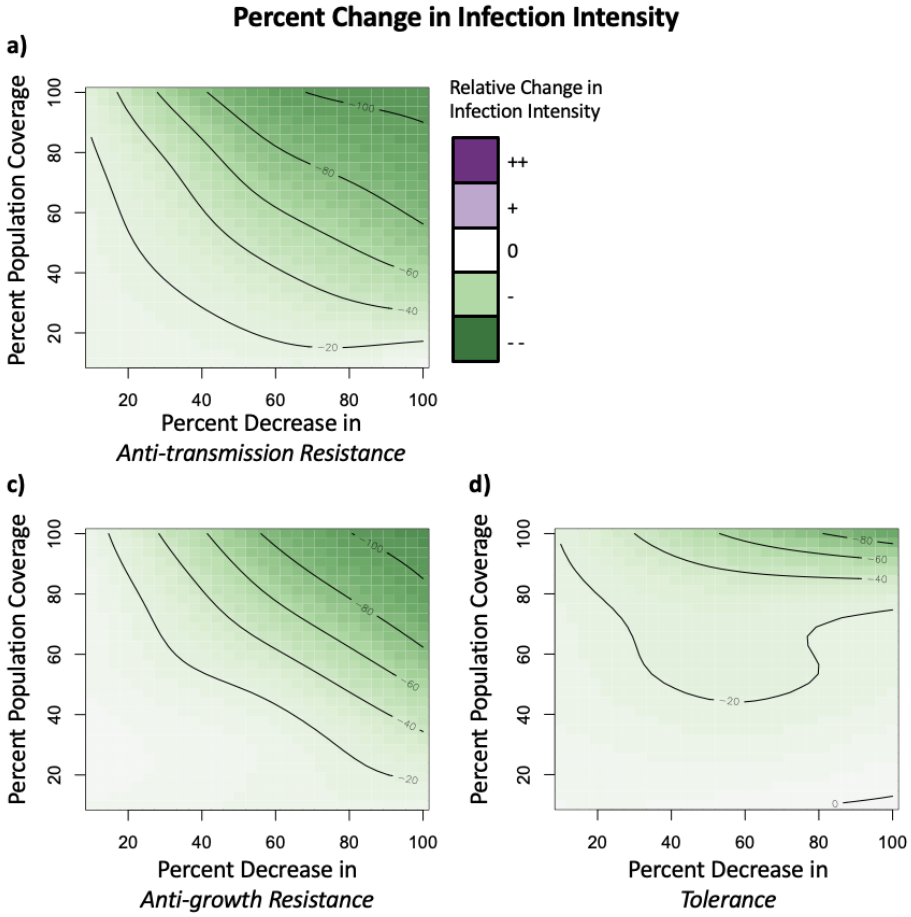

Figure S12. Changes to infection intensity when vaccination is harmful, across increasing population coverage. Generalized Additive Model summary of modeled changes in infection prevalence (green-purple color scale) as a function of vaccination-induced decreases in a) anti-transmission resistance, b) anti-growth resistance (decrease in pathogen clearance) or c) tolerance (decrease in infection induced mortality threshold; x-axis) and increasing population coverage (y-axis), relative to simulations of an untreated control population. Deeper green shades represent reductions and deeper purples represent increases in infection intensity compared to unvaccinated populations. Contour lines define increments of 20% change relative to vaccine-free simulations. Infection intensities decrease with decreasing levels of resistance or tolerance.

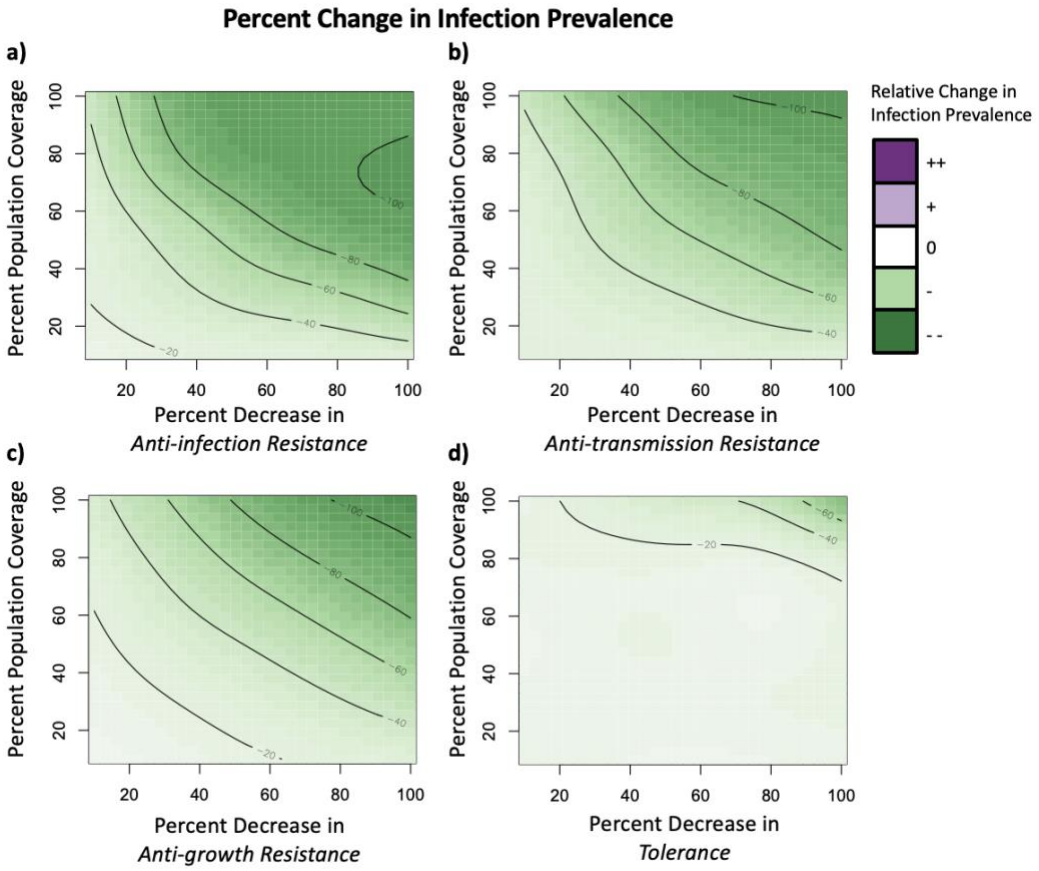

Figure S13. Changes to infection prevalence when vaccination is harmful, across increasing population coverage. Generalized Additive Model summary of modeled changes in infection prevalence (green-purple color scale) as a function of vaccination-induced decreases in a) anti-infection resistance (increase in infection establishment), b) anti-transmission resistance (increase in pathogen shedding), c) anti-growth resistance (decrease in pathogen clearance) or d) tolerance (decrease in infection induced mortality threshold; x-axis) and increasing population coverage (y-axis), relative to simulations of an untreated control population. Deeper green shades represent reductions and deeper purples represent increases in infection prevalence compared to unvaccinated populations. Contour lines define increments of 20% change relative to vaccine-free simulations. Infection prevalence decreases with decreasing levels of resistance or tolerance.

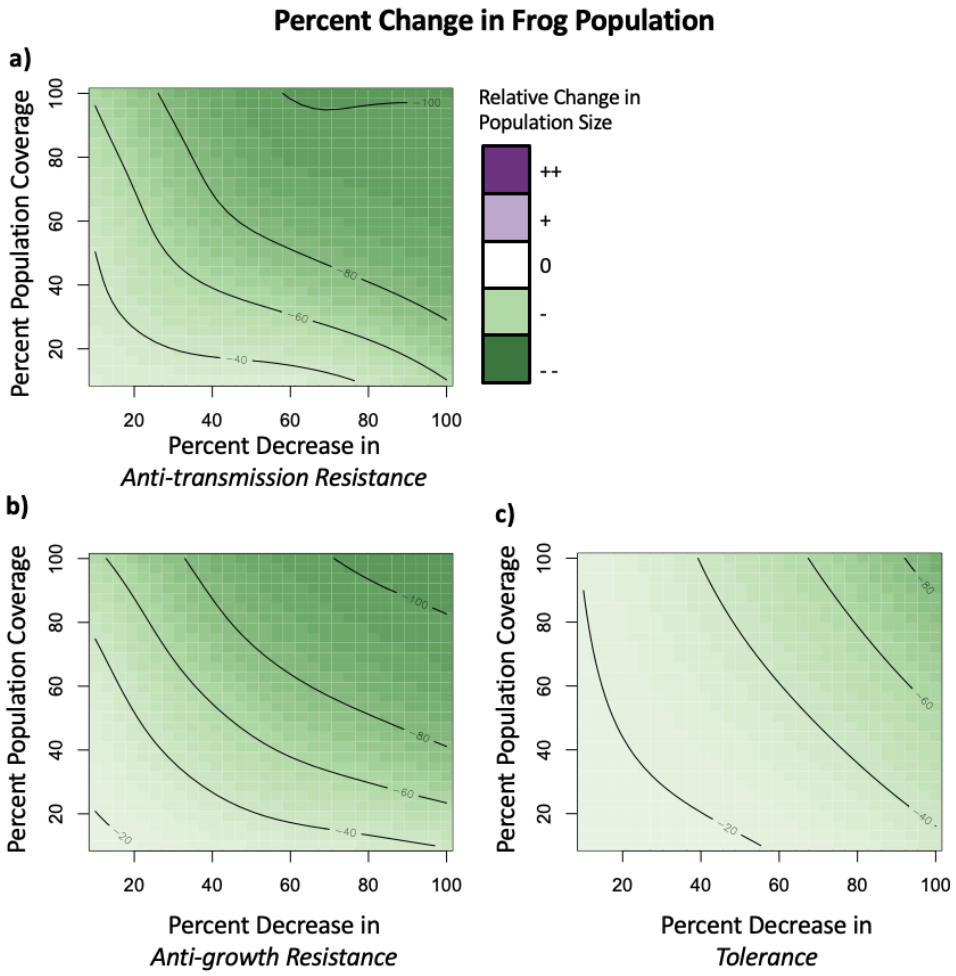

Figure S14. Changes to frog population size when vaccination is harmful, across increasing population coverage. Generalized Additive Model summary of modeled changes in infection prevalence (green-purple color scale) as a function of vaccination-induced decreases in a) anti-transmission resistance, b) anti-growth resistance (decrease in pathogen clearance) or c) tolerance (decrease in infection induced mortality threshold; x-axis) and increasing population coverage (y-axis), relative to simulations of an untreated control population. Deeper green shades represent reductions and deeper purples represent increases in frog population size compared to unvaccinated populations. Contour lines define increments of 20% change relative to vaccine-free simulations. Frog populations decrease with decreasing levels of resistance or tolerance.

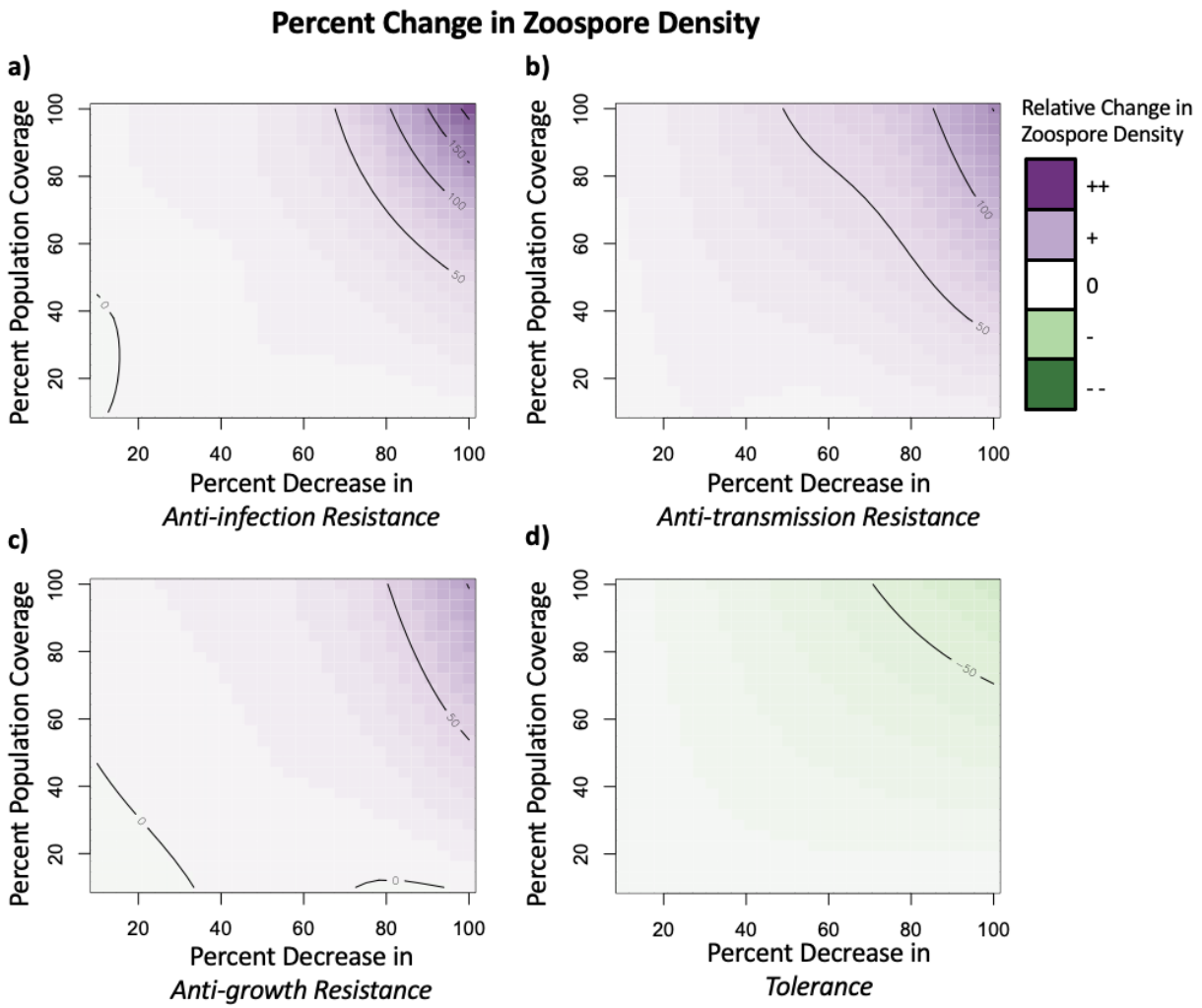

Figure S15. Changes to zoospore density when vaccination is harmful, across increasing population coverage. Generalized Additive Model summary of modeled changes in infection prevalence (green-purple color scale) as a function of vaccination-induced decreases in a) anti-infection resistance (increase in infection establishment), b) anti-transmission resistance (increase in pathogen shedding), c) anti-growth resistance (decrease in pathogen clearance) or d) tolerance (decrease in infection induced mortality threshold; x-axis) and increasing population coverage (y-axis), relative to simulations of an untreated control population. Deeper green shades represent reductions and deeper purples represent increases in zoospore density compared to unvaccinated populations. Contour lines define increments of 50% change relative to vaccine-

free simulations. Zoospore densities increase as resistance decreases (a-c), but zoospore densities decrease with high reductions in tolerance.

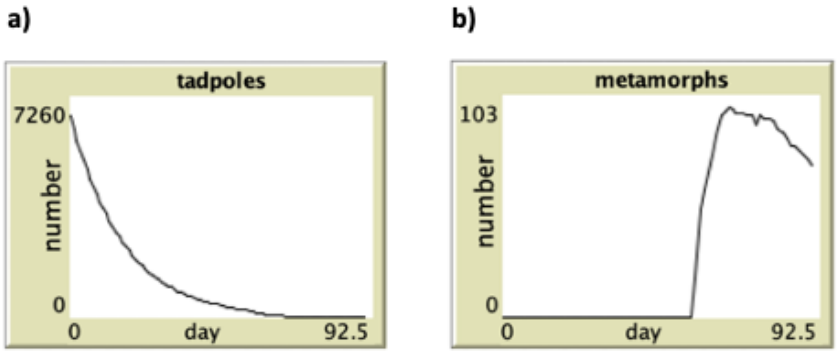

Figure S16. Netlogo visualization amphibian abundance data from a run of the model. *Pseudacris regilla* tadpoles hatch during spring and metamorphose by mid-summer ((5); personal observation). Here, we show that our modeled stage-structured population mimics this phenology so that a) the number of tadpoles decline over time through baseline mortality or metamorphosis and no tadpoles remain by the end of the simulation. Likewise, b) metamorphs emerge within approximately two months of hatching and populations decline through baseline or disease-induced mortality. The x-axis demonstrates the day within the simulation and the y-axis represents the number of (a) tadpoles or (b) metamorphs.

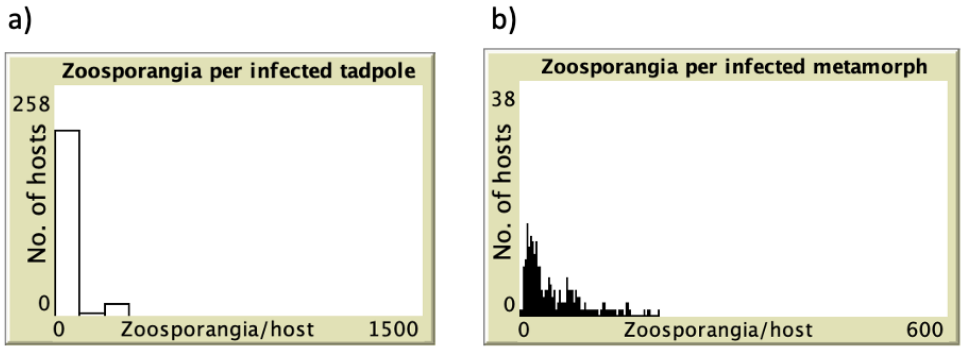

Figure S17. Netlogo visualization of a snapshot in time of Bd abundance data from a run of the model. This histogram displays the number of hosts with a given zoosporangia load across a) tadpoles on day 18 or b) metamorphs on day 75. In natural host populations, Bd infections are aggregated (i.e., few hosts have high infection burdens and most hosts have low infection burdens) (6). Here, we show that our model population also exhibits this overdispersed distribution in both life stages.

Table S1. Major model processes, defined as the following: amphibian phenology and ecology, implementation of acquired immunity (vaccination), between-host transmission, and within-host infection processes.

Major Model Processes			
Process	**Baseline parameter**	**Variation in parameter**	**Procedural info**
Amphibian phenology and ecology			
Within-season dynamics	Last-day = 90 ticks (to match 3 month transmission season from tadpole hatching to metamorphosis).	No	Simulation ends at day 90.
Natural tadpole mortality	tad-mort = 0.06 day^{-1} (7)	No	Probability of mortality each time step.
Natural metamorph mortality	meta-mort = 0.02 day^{-1} Determined through pattern-matching (Fig. S16).	No	Probability of mortality each time step.

Tadpole movement	Proportion of tadpole population that moves each time step ('t-movement') = 0.25.	No	25% of tadpoles move to a new perimeter pond patch each time step.
Metamorph movement	Proportion of metamorphs on land at each time step ('m-land') = 0.1.	No	Metamorphs move to a new patch each time step. 10% of metamorphs are on terrestrial patches and 90% are on perimeter pond patches.
Metamorphosis	Tadpoles transition to metamorphs between day 55-74. Determined through pattern-matching (Fig. S16).	No	Beginning on day 55, each tadpole has a 11% chance of transitioning to a metamorph. On day 74, all remaining tadpoles become metamorphs. Metamorphs retain infections (8) and all immune traits from tadpole state.
Acquired immunity			
Vaccination	Host vaccination status is 'immunized' = 1 if host is	To allow for a range of individual variation	We use constants to modulate baseline

	vaccinated or 0 if host is unvaccinated. Vaccine constants (c_est, c_clear, c_shedding, and c_smax) given vaccination status are specified per scenario. v-efficacy = 1 relative_variation = 10%.	in response vaccination, we allow for +/- 10% differences in response to the baseline vaccine efficacies specified in each scenario.	parameters regarding infection establishment (‘c_est’), infection clearance (‘c_clear’), infection shedding (‘c_shedding’), and infection-induced mortality (‘c_smax’). Vaccine parameter c_est, c_clear, c_shedding, and c_smax serve as exponential modulating factors (exponential to prevent values from becoming negative) that adjust the baseline values for probability of successful infection establishment given zoospore exposure, probability of zoosporangia clearance, zoospores shed per zoosporangia, or

			zoosporangia threshold above which mortality occurs, respectively, given a host's vaccination status and 'imm' parameter. Positive values of constants increase baseline parameters, while negative values decrease baseline parameters, and constants = 0 when hosts are unvaccinated or vaccination does not impact a given process.
Vaccination coverage	Specified per scenario.	No	On day of vaccination (default = day 0), the specified proportion (70% coverage = 0.7) of the tadpole population is randomly selected. Immune parameters of selected uninfected tadpoles will be adjusted according to the scenario's vaccine efficacy (see

			"vaccination" process). Immune parameters of selected but infected tadpoles will remain unchanged given that Bd-metabolites have been found to be ineffective at inducing acquired resistance in frogs previously challenged with Bd (9).
Between-host infection processes			
Host exposure to zoospores	amount of environmental units each host is exposed to per day = 0.25 (unitless). Determined through pattern-matching (Fig. S17).	No	Transmission is determined by draws from a multinomial probability distribution where each host's likelihood of infection with a zoospore is determined by its establishment and exposure parameter. (10)
Infection Establishment	probability of successful infection establishment upon exposure ('est') = 25%.	Varies depending on host vaccination status and vaccine efficacy scenario.	

Zoospore removal from pool	zoospore mortality factor ('z-mort') = 2 day^{-1} (11).	No	Zoospores are removed from patches as a function of exposure to hosts and exponential decay determined by the mortality factor.
Within-host infection			
Zoosporangia maturation	NA	No	Upon a zoospore successfully establishing an infection, it moves between one of 4 prezoosporangia stages (pz0-pz4) per day to approximate the 4 days it takes for a zoosporangia to mature before shedding (12).
Infection clearance	baseline_spn_clearance = 0.20 day^{-1} (13).	Varies depending on host vaccination status and vaccine efficacy scenario.	Binomial draw from probability of zoosporangia clearance to determine how many sporangia survive on each host.

Zoosporangia shedding	17.8 zoospores produced per zoosporangium per day (13).	Varies depending on host vaccination status, ‘imm’ value, and c_shedding.	Zoosporangia shed 40% of zoospores to the patch the host is currently on, 50% of zoospores to a neighboring perimeter or inner patch, and 10% of zoospores directly re-expose the host that produced them.
Self-reinfection	Proportion of zoospores that a host releases and is re-exposed to = 0.1 (13).	Varies depending on host vaccination status, ‘imm’ value, and c_est.	Zoospores that reinfect host is calculated by multiplying the number of zoospores that host releases with the probability of infection given establishment (‘est’)
Infection-induced mortality	Threshold of sporangia above which mortality occurs (‘smax’) = 562. Derived from (13) which cites maximum 10,000 zoospores released per day. We divided 10,000 zoospores by 17.8	Varies depending on host vaccination status, ‘imm’ value, and c_smax.	Metamorph dies if the zoosporangia load reaches threshold value (‘smax’).

	zoospores shed per zoospore per day to approximate 562 as the maximum number of zoosporangia per host.		
Tadpole sporangia carrying capacity	Maximum zoosporangia burden for a tadpole (‘s_k’) = 10,000. Determined through pattern-matching (Fig. S17).	No	If a tadpole has 10,000 zoosporangia, it cannot be further infected. However, it can continue to be exposed to zoospores.

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Chapter 5: Conclusion

As I conclude my dissertation, I believe it is useful to situate this work within the context it was undertaken. The COVID-19 pandemic began to unfold during the second year of my PhD, and stay-at-home quarantine measures began to take effect in the U.S. a month before my oral qualifying exam. Similar to SARS-CoV-2, *Batrachochytrium dendrobatidis* (Bd) is a globally-distributed, newly emergent pathogen. In researching a prophylactic treatment (akin to a prototype vaccine) for chytridiomycosis, the questions of my dissertation have in many ways mirrored those that followed the rollout of COVID-19 vaccines: What functional modes of protection does this novel vaccine provide and what are its limitations? What conditions are necessary for this vaccine to be efficacious for disrupting transmission and achieving the overall goal of outbreak mitigation? Is protection provided by this vaccine strain- or variant-specific? Which host characteristics, such as age or previous pathogen exposure history, influence the efficacy of this vaccine? As is necessary when assessing recently developed treatments for use in a rapidly transpiring pandemic or panzootic, these questions ranged from broad to specific, invoked attention to ecological scale, and required the use of multiple methods.

Despite overarching similarities, the goals of SARS-CoV-2 and chytridiomycosis disease control are quite different. Control measures for COVID-19 seek to end human suffering caused by the pathogen with the ideal of locally eliminating the virus, whereas Bd control measures aim to mitigate the threat of disease-induced extinction, which does not necessarily require pathogen elimination [1]. In my introduction (Chapter 1), I discuss how wildlife vaccination campaigns can be motivated by conservation or spillover reduction and I highlight how these differing aims influence distinctions in how much and what kind of vaccine-induced protection is needed for intervention success [2]. Additionally, I discuss how wildlife vaccination campaigns are often

complicated by a multitude of uncertainties, such as a limited understanding of vaccine efficacy, host immunity and ecology. In my dissertation, I have investigated uncertainties surrounding the efficacy and feasibility of a Bd metabolite prophylaxis for chytridiomycosis with the desire that this new knowledge will inform ongoing amphibian conservation efforts. In the below sections, I summarize my findings.

Vaccine or prophylaxis efficacy can be influenced by pathogen strain variation. In Chapter 2, we investigated if efficacy provided by Bd metabolite prophylaxis is sensitive to Bd strain [3]. Contrary to our hypothesis that protection from Bd metabolite prophylaxis would be highest in same-strain treatments (i.e., exposure to metabolites of the same strain as that used for the live pathogen challenge), we found a result of asymmetric cross-protection wherein frogs treated with metabolites from a California-isolated strain JEL-270 and challenged with a live Panamá-isolated strain JEL-419 had significantly lower Bd loads than frogs treated with a sham control treatment, but no other treatments were found to confer protective effects. Given that this cross-strain result was asymmetric (i.e., occurring in one cross-strain treatment, but not the other), it is possible that metabolites from some Bd strains provide broader and more effective protection than others. We theorize that differences in virulence between strains may influence metabolite immunogenicity, suggesting that potentially less virulent strains may provide better immunity. Future studies should identify metabolite characteristics that drive the observed acquired resistance response.

In Bd-endemic ecosystems, metabolite administration may occur after amphibians have been exposed to or infected with Bd. Thus, in Chapter 3, we assessed whether treatment with Bd metabolites could provide resistance in tadpoles that have already been exposed to live Bd [4]. I was also motivated to conduct this study to ensure that Bd metabolites would not exacerbate

infections, given that some Bd metabolites have been found to be immunosuppressive and aid in Bd infection establishment [5]. While the results of this experiment confirmed that Bd metabolites provide protection in Bd-naïve individuals, Bd metabolite treatment administered to tadpoles post-live Bd challenge did not provide acquired resistance nor exacerbate infections. Thus, it is important to time Bd metabolite administration early in the transmission season for best results. Additionally, this study provides evidence that low-dose Bd metabolite treatment should not amplify infections in tadpoles previously exposed to Bd.

Chapter 4 is the capstone of my dissertation, wherein I conducted model simulation experiments to investigate potential outcomes of varying Bd metabolite prophylaxis scenarios, and then conducted a large-scale field manipulation experiment to empirically test Bd metabolite prophylaxis effectiveness in a natural setting. We developed a stochastic, stage-structured agent-based model and used the model to explore the general behavior of the Bd-frog-vaccine system to identify factors and scenarios important for vaccination success or failure. Specifically, we modeled varying forms and degrees of vaccine-induced protection (anti-infection resistance, anti-growth resistance, anti-transmission resistance, or enhanced tolerance) across a range of coverage levels to generate insights regarding logistically feasible wildlife vaccination programs. We followed this with a Before-After-Control-Impact (BACI) designed field trial to assess the effectiveness of Bd metabolite administration at reducing Bd prevalence and infection intensities in a field setting and we used model projections to aid in the interpretation of its results. Unexpectedly, we found that infection intensities significantly increased ($p = 0.001$) in frogs from ponds treated with Bd metabolites relative to frogs from ponds treated with a sham control; additionally, probability of infection did not differ between treated and untreated ponds. Model scenarios in which vaccination greatly enhanced tolerance and provided only a negligible to

weak boost in resistance were most consistent with the field experimental results. We also modeled scenarios in which vaccination caused harm, but found that simulated frog populations steadily declined with increasing vaccine-induced harm and thus surviving frogs were those that had low infection burdens. Thus, model scenarios in which vaccination backfired and caused harm were inconsistent with our field findings.

The results of Chapter 4 directly address the throughline of my dissertation: is partial protection provided by Bd metabolite prophylaxis sufficient to be useful for amphibian conservation? Unfortunately, the observed increase in infection intensities following Bd metabolite addition calls into question the safety of Bd metabolite addition at the population-level. Even if this result is due to enhanced tolerance in treated frogs, increased infection intensities indicate greater onward transmission and thus exacerbated risk of infection to untreated co-habiting amphibians. Therefore, for the purpose of amphibian conservation, I conclude that partial protection provided by Bd metabolite addition is inadequate when used as a singular intervention in Bd-endemic environments. Given this research, I strongly recommend that, when methods are available, future studies prioritize quantifying net transmission output (i.e., the total number of zoospores produced, which is the product of the duration and rate of pathogen shedding) of treated versus untreated individuals, to properly parameterize vaccine transmission models. For vaccines or prophylactic treatments that do not significantly prevent infection, this assessment will identify potential increases in transmission that may occur from boosted tolerance and will inform more realistic projections for conditions needed to increase host populations sizes or attain herd immunity – if herd immunity is possible at all.

Further, it is also important to investigate the possibility that Bd metabolite addition backfired due to an environmental interaction when applied in these ponds. For example, we

calculated a target dose based on the pond volume but applied the treatment to the pond perimeter, yielding less precise control over metabolite concentrations at very small scales. Additionally, we did not account for baseline levels of pre-existing metabolites, if there are significant concentrations of metabolites already present in these sites, then our perimeter dosing strategy and their prior occurrence could have created realized doses far greater than we expected. Future studies should use environmental DNA (eDNA) techniques to quantify baseline levels of Bd, from which Bd metabolite concentrations can be back-calculated, and lab experiments should evaluate if over-dosing causes harm to hosts. Bd eDNA approaches could also assess another alternative interpretation – the possibility that spuriously, Bd exposure risk happened to be higher in treatment ponds than control ponds for both post-intervention years.

Additionally, we used metabolites isolated from the JEL 270 strain for the field trial as that is the Bd strain assumed to be circulating in the East Bay, California ponds, and results from my third chapter showed that *Pseudacris regilla* tadpoles can acquire same-strain JEL 270 resistance. However, as my second chapter showed that Bd metabolite treatment efficacy is sensitive to strain, the assumption that the circulating strain is JEL 270 should be verified. Ideally, future studies would sequence Bd from each pond to confirm JEL 270 is the circulating strain or identify alternative circulating strains. If alternative strains are identified, follow up laboratory studies should assess the efficacy of JEL 270 metabolites against those strains.

Moreover, molecular studies should characterize the specific metabolite compounds driving the acquired immune response. Then, if possible, these compounds alone should be tested as a prophylaxis to determine if it is possible to use them isolation, thereby removing risk attributable to adding unnecessary non-antigenic and potentially toxic compounds to waterbodies. Additionally, further studies should investigate if tadpole density, pond size,

metabolite addition timing, temperature, sunlight, and water chemistry affect the effectiveness of Bd metabolite prophylaxis. As Bd is found across many diverse ecosystems, understanding the impact of these factors on prophylaxis efficacy could aid in the consideration of alternative amphibian communities where Bd metabolite treatment could be useful.

I hypothesize that the anti-toxin nature of our prophylaxis is relevant to the results of each of my research chapters. As I mentioned earlier, some Bd metabolites are known virulence factors which elicit immunopathology to facilitate successful Bd infection establishment [5]. By using Bd metabolites, rather than whole-cells of killed Bd, we are effectively inducing acquired resistance to the pathogen's toxins rather than the pathogen itself. In Chapter 2, I postulate that more virulent strains of Bd may contain a greater concentration or composition of immunomodulatory toxins, and thus potentially metabolites from less virulent strains may allow for the host's immune system to mount a more effective response. In Chapter 3, I hypothesize that Bd metabolites are ineffective post-live Bd exposure because once amphibians have been exposed to a high dose of immunosuppressive compounds, the ability of their immune system to subsequently mount a response to those same compounds may be inhibited. While I was originally concerned that additional exposure to Bd metabolites may exacerbate infections in frogs previously challenged with live Bd, I expect we did not see this harmful response given that the dose of metabolites used for our post-exposure prophylactic treatment was 1000x lesser than that which accompanied the live Bd challenge. Lastly, the results of my fourth chapter suggest that Bd metabolite prophylaxis may boost tolerance and weakly impact resistance, which is characteristic of other vaccines targeting virulence factors, referred to as toxoid vaccines [6,7].

Disease control tools for the purpose of amphibian conservation are urgently needed. Though Bd metabolites appeared promising for use as a chytridiomycosis prophylactic treatment

as they can be distributed environmentally and were found to be effective in multiple amphibian life stages and species, further work is needed to determine what caused Bd metabolites to increase infection loads when applied in the field for the treatment to be reconsidered for potential use as a conservation tool. If Bd metabolite prophylaxis is found to reduce immunopathology and hence enhance tolerance, it may be considered for use in combination with a resistance-boosting intervention.

Overall, this dissertation provides a critical evaluation of the feasibility and effectiveness of a conservation-motivated prophylactic treatment under realistic ecological conditions. While this research was specific to the Bd-amphibian system, it also raises broader insights regarding the conditions required for partially protective vaccines to be useful and safe for conservation purposes. The unexpected results of this work reinforce that, even if individual-level outcomes are consistently promising in laboratory conditions, it is highly important to first rigorously evaluate intervention outcomes at the population-level in natural conditions prior to implementing it at scale.

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