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RESEARCH ARTICLE

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Amphibian species vary in their learned avoidance response to the deadly fungal pathogen *Batrachochytrium dendrobatidis*

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Abstract

1. Lethal and sublethal effects of pathogens should theoretically select for host avoidance of these pathogenic organisms. Some amphibians can learn to avoid the pathogenic fungus *Batrachochytrium dendrobatidis* (Bd) after one infection-clearance event.
2. Here, we investigated whether four taxonomically distinct amphibians, Cuban tree frogs *Osteopilus septentrionalis*, southern toads *Anaxyrus* (*Bufo*) *terrestris*, greenhouse frogs *Eleutherodactylus planirostris* and pine woods tree frogs *Hyla femoralis*, exhibited any innate or learned avoidance of Bd on a moist substrate and, if so, what cues they used to identify the fungus.
3. Cuban tree frogs, pine woods tree frogs and greenhouse frogs did not appear to exhibit detectable innate or learned avoidance of Bd. However, southern toads learned to avoid Bd after only one exposure. Southern toads avoided any treatment containing Bd metabolites but did not avoid treatments that lacked Bd metabolites even when dead zoospores were present.
4. Bd metabolites appeared to be the cues that amphibians use to avoid Bd. These metabolites may have a distinct smell or may cause discomfort, which would be consistent with a classical or Pavlovian conditioning response.
5. *Synthesis and applications*. Not all species of amphibians respond the same way to Bd exposure; some can learn to avoid Bd and the metabolites it produces, while others do not. These findings have important implications for both management practices and policy, and should be considered when developing disease models and conservation plans for amphibians.

KEYWORDS

amphibian decline, behavioural avoidance, behavioural ecology, behavioural resistance, chytrid fungus, chytridiomycosis, disease avoidance, disease ecology

1 | INTRODUCTION

Emerging infectious diseases are one of the largest threats to global biodiversity, but despite this, disease is historically one of the least studied issues in conservation (Lawler et al., 2006). Amphibians, for example, are one of the most threatened vertebrate taxon (Scheele et al., 2019; Stuart et al., 2004) and have experienced extreme global declines because of the pathogenic fungus *Batrachochytrium dendrobatidis* (Bd). This pathogen is associated with the extinction and extirpation of hundreds of

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Megan N. Hill, Garrett C. Lentz, Electra F. Scott and Nadia F. Tenouri contributed equally and are listed alphabetically amphibian species globally and remains an imminent and persistent threat to many extant species (Scheele et al., 2019). Additionally, Bd is unlikely to be extirpated given its nearly global distribution and the presence of non-amphibian hosts (Brannelly et al., 2015; McMahon et al., 2013; Scheele et al., 2019).

Bd has an infectious zoospore stage that produces proteolytic enzymes that degrade elastin proteins in the epidermal layer of the host (Moss et al., 2010; Symonds et al., 2008), as well as, polyamine spermidine, methylthioadenosine and tryptophan, all of which are immunomodulators (Rollins-Smith et al., 2015, 2019). When crayfish were exposed to Bd metabolites in the absence of the fungus, the metabolites induced crayfish mortality via gill damage (McMahon et al., 2013). Additionally, tadpoles exposed to Bd metabolites in the absence of infection had increased developmental speed (Cohen et al., 2019; McMahon et al., 2019; Romansic et al., 2011); tadpoles can utilize developmental plasticity to avoid an unsuitable environment (Newman, 1988; Rohr et al., 2004).

The damage to host tissue caused by the enzymes in the metabolites is likely painful. This could set the stage for learned avoidance of Bd through classical or Pavlovian conditioning. An innate response, in this case an avoidance of discomfort (e.g. pain or itching), is paired with a second stimulus, cues from Bd (e.g. metabolite odour), to induce learned avoidance of Bd. There is precedent for this, as we already know amphibians can learn to avoid pathogenic fungi. For example, bullfrog (*Rana catesbeiana*) tadpoles use chemical cues to avoid conspecifics infected with the pathogenic fungus *Candida humicola* (Kiesecker et al., 1999). Additionally, oak toads *Bufo quercicus* learn to avoid Bd-exposed substrate after experiencing just one Bd infection and clearance event (McMahon et al., 2014). For both of these examples, a behavioural avoidance experiment was used, where researchers tracked microhabitat use within a behavioural chamber; this is similar to the methods used in this current experiment. Interestingly, the oak toads were part of a larger experiment, in which researchers found that the toads only exhibited avoidance behaviour after they had experienced an actual Bd infection, not when they were Bd naïve.

While oak toads can learn to avoid Bd after exposure (McMahon et al., 2014), we do not know how common this learned avoidance response is across amphibian species, nor do we know what specific Bd cues elicit the avoidance response. Here, we tested whether four species of amphibians—Cuban tree frogs *Osteopilus septentrionalis* (family: Hylidae), southern toads (*Anaxyrus* [*Bufo*] *terrestris*; family: Bufonidae), greenhouse frogs *Eleutherodactylus planirostris* (family: Eleutherodactylidae) and pine woods tree frogs *Hyla femoralis* (family: Hylidae)—exhibited innate or learned avoidance of Bd. In brief, we conducted innate versus learned behavioural trials, where we exposed Bd-naïve and Bd-experienced individuals to Bd or different components of Bd to determine whether individuals could use these cues to avoid the pathogen. We hypothesized that, like oak toads, all four species would learn to avoid Bd after exposure to and clearance of the pathogen.

Generally, we crossed the presence and absence of Bd metabolites with the presence and absence of live and dead zoospores. Given that metabolites may have a distinct odour and are likely painful because they damage amphibian tissue, we predicted that the metabolites would be an important component of Bd that triggered any learned avoidance response. If amphibians can learn to avoid Bd metabolites alone, then they could learn to identify Bd without direct contact with infectious stages. This learned behavioural avoidance could have an impact on both pathogen and host population dynamics.

2 | MATERIALS AND METHODS

2.1 | Animal husbandry

Cuban tree frogs (*O. septentrionalis*; adults), southern toads (*A. terrestris*; metamorphs), greenhouse frogs (*E. planirostris*; adults) and pine woods tree frogs (*H. femoralis*; metamorphs) were collected within 10 km of the University of Tampa (Tampa, FL). The large arboreal species (Cuban tree frogs) were housed individually in 1-L plastic cups and all other species (pine woods tree frogs, greenhouse frogs and southern toads) were housed in smaller plastic containers (~8 × 11 cm). All animals were maintained with paper towels moistened with artificial spring water (ASW; Cohen et al., 1980) and fed gut-loaded crickets dusted with Rep-Cal vitamins ad libitum. Their bedding and containers were changed weekly (new containers were used for each behavioural trial). Animals were maintained at 23℃ until the start of the experiment and were then maintained at 18℃ unless otherwise noted.

2.2 | Treatment inoculate preparation

We cultured two strains of Bd (JEL 423 isolated in Panamá from *Hylomantis lemur* and SRS 810 isolated in the United States from *Rana catesbeiana*) on 1% tryptone agar plates. Each amphibian species tested was exposed to the strain that was isolated closest to their native range (Table 1). Immediately before use in the experiments, the Bd positive (Bd+) plates were flooded with ASW to suspend the infectious zoospores. The ASW from all Bd+ plates of a particular strain was homogenized, zoospore concentration was quantified using a haemocytometer and the stock was diluted with ASW to 1.5×10^5 zoospores/mL (Bd+ stock). Bd-free 1% tryptone agar plates were flooded using the same technique to create a control (Bd negative (Bd-) stock).

We used the same methodology to prepare the different treatment inoculates for all the amphibian species. To create *Dead zoospores with metabolites*, the Bd+ stock was flash frozen with liquid nitrogen. To create *Metabolites alone*, the *Dead zoospores with metabolites* stock was filtered through a 1.2-μm filter (GE Whatman Laboratory Products) to separate the zoospores from the liquid containing the metabolites. That zoospore-free liquid was used as the *Metabolites alone* treatment. We then washed the zoospores off the filter with an equivalent amount of ASW to create the *Dead*

TABLE 1 The Bd strain, treatment inoculation, and sample sizes used for Bd-naïve and Bd-experienced amphibians

	Greenhouse frogs		Pinewoods tree frogs		Cuban tree frogs		Southern toads	
Treatments	Treatments used	*n*	Treatments used	*n*	Treatments used	*n*	Treatments used	*n*
Live Bd	+	13	+	13	+	15	+	20
Control	+	9	+	9	+	15	+	20
Dead zoospores with metabolites	–		–		+	15	+	20
Dead zoospores alone	–		–		+	15	+	20
Metabolites alone	–		–		+	15	+	20
Bd strain used:	JEL 423		SRS 810		JEL 423		SRS 810	

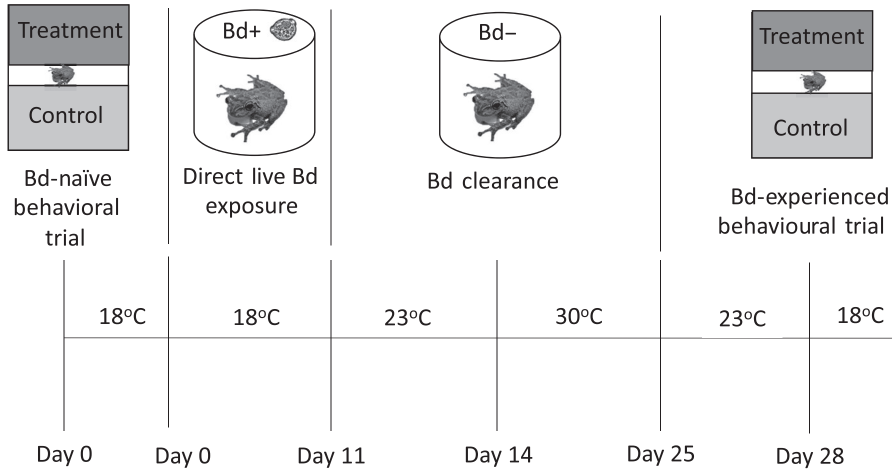

FIGURE 1 Timeline for experimental design

zoospores alone treatment. See the *Treatments Used for Each Species* and Table 1 for information on which treatments were used with each species.

For all treatments, we used a haemocytometer to verify that we had what we expected, either live zoospores, dead zoospores or no zoospores (for methods see McMahon & Rohr, 2014). Additionally, for the dead zoospores and metabolite treatments, we verified there was no live fungus by plating 1 ml of each treatment inoculate on 1% tryptone agar plates ($n = 3$) and monitored growth for 2 weeks (there was no Bd growth).

2.3 | Bd infection and clearance

Each frog was exposed to one of the following treatments: *Live Bd*, *Dead zoospores with metabolites*, *Dead zoospores alone*, *Metabolites alone* or *Control* (see Table 1 for which species received which treatments), as a Bd-naïve individual and then again as a Bd-experienced individual (except for the control treatment groups) to determine changes in individual behaviour. To create Bd-experienced hosts, individuals were infected with and cleared of a sublethal dose of live Bd (Figure 1). Live Bd from the Bd+ stock (1 ml) was pipetted on the dorsal surface of each amphibian within their housing chamber (not the observation chamber) so the Bd could not be avoided. After exposure, all amphibians (including all uninfected controls) were then held at 18°C for 11 days, allowing enough time for growth of the Bd infection but not enough time for Bd to cause morbidity (McMahon et al., 2014). Amphibians were then cleared of infections using the heat clearance method described by McMahon et al. (2014). Briefly, all amphibians were held at 23°C for 3 days to reduce temperature shock, they were then moved to a 30°C incubator for 11 days to clear the infection. They were moved to 23°C for 3 days again to reduce temperature shock and then they were returned to 18°C for the remainder of the experiment (see Figure 1 for experimental timeline).

2.4 | Treatments used with each species

Greenhouse frogs and pine woods tree frogs were indirectly exposed to *Live Bd* or *Control* treatments (see Table 1 for treatments used, sample sizes and strains) as Bd-naïve and Bd-experienced individuals. Southern toads and Cuban tree frogs were indirectly exposed to one of the five treatments: *Live Bd*, *Control*, *Dead zoospores with metabolites*, *Dead zoospores alone* and *Metabolites alone*. For all species, each individual was randomly assigned to their respective treatment (see Table 1 for treatments, sample sizes and strains used).

2.5 | Behavioural observations for all amphibian species

Observation chambers were created by placing ASW-moistened paper towels on each side of the container leaving a 1 cm gap in the centre. For all observation chambers, the paper towels and gap covered the bottom of the container and extended vertically up the walls so that the frogs could not avoid the two towels at the same time. Within each container, the ASW-moistened paper towel on one side of the chamber was inoculated with 1 ml of the treatment inoculate (see Sections 2.2 and 2.4; Table 1) and the ASW-moistened paper towel on the other side received 1 ml of ASW. The side inoculated with the treatment was randomly selected, and we implemented a double-blind inoculation process so that the person running the behavioural trials did not know the treatment location.

For each behavioural trial, each paper towel was inoculated with either the treatment or ASW and then the amphibian was placed in the centre of the container. After a 20-min acclimation period, we recorded the location of the amphibians in the container (left, centre or right) every 15 min for 3–4 hr depending on the species (southern toads: 4 hr; all other species: 3 hr). The observer remained sitting still during the entire observation period so that they did not disturb the animals during the experiment. The animal was considered to be avoiding if it was in the centre or on the side opposite of the treatment dose location.

2.6 | Statistical analysis

Statistics were analysed with R statistical software (R Development Core Team, 2020) and significance was attributed at $p < 0.05$ using the likelihood ratio tests based on Type II sum of squares (package: CAR, function: ANOVA). For the southern toad experiment, we used the glmer function in the LME4 package to column bind the number of observations on the treatment side and non-treatment side for each toad (i.e. a binomially distributed response variable) and tested for a three-way interaction among toad experience with Bd infections, zoospore presence in a treatment and metabolite presence in a treatment. Additionally, we treated individual frogs as a random effect to account for the non-independence associated with repeated measurements (i.e. when Bd-naïve and Bd-experienced) on individuals. We used the emmeans function in the EMMEANS package to conduct post hoc comparison tests evaluating whether there were differences in avoidance between Bd-naïve and Bd-experienced animals for each of the five treatments. Unfortunately, for the Cuban tree frog experiments, binomial mixed effects models would not converge. Thus, we arcsine square root transformed the proportions and conducted mixed effects GLMs (i.e. with a Gaussian error distribution; package: NLME, function: lme) analogous to those that were conducted for the southern toads (including mass as a covariate).

There were too few greenhouse frogs and pine woods tree frogs to have frogs assigned to treatments with control solutions assigned to both sides of the container as we did for the southern toads and Cuban tree frogs. Thus, for the greenhouse frogs and pine woods tree frogs, we used the same models as described above for the southern toads (binomial linear model with frog as a random effect) but we tested for deviations between the observed and expected null (which had no error) spatial distributions rather than from the observed spatial distribution in the control treatment (which had error). Thus, we were testing for an interaction between experience with Bd infections and whether an avoidance response was observed or expected. We analysed the greenhouse frogs and pine woods tree frogs separately, even though they were tested simultaneously, because the pine woods tree frogs had considerable mortality during the Bd infection and clearing process (see Section 3).

3 | RESULTS

3.1 | Cuban tree frog

Cuban tree frogs did not avoid any treatments (*Live Bd*, *Dead zoospores with metabolites*, *Dead zoospores alone*, *Metabolites alone* or *Control)*, regardless of whether they were Bd-naïve or Bd-experienced (Treatment: $\chi^2_4 = 1.57$, $p = 0.814$; Bd experience: $\chi^2_1 = 0.04$, $p = 0.8430$; Treatment × Bd experience: $\chi^2_4 = 0.6036$, $p = 0.9627$; Figure 2a), and there were no significant main effects or interactions among Bd experience, metabolite presence or zoospore presence (Metabolites: $\chi^2_1 = 0.0001$, $p = 0.992$; Zoospores: $\chi^2_1 = 0.0002$, $p = 0.987$; Bd experience: $\chi^2_1 = 0.046$, $p = 0.8305$, Interactions: $\chi^2_1 < 0.283$, $p > 0.595$; Figure 2a). Hence, Cuban tree frogs exhibited no evidence of innate or learned avoidance to any treatments.

3.2 | Southern toad

Bd-naïve toads did not avoid any of the treatments (Treatment: $df = 4$, $\chi^2 = 1.326$, $p = 0.857$; Figure 2b), and there was no evidence of a change in their avoidance response over the 4-hr trial (Table S1). However, there was a significant main effect of Bd experience and a significant interaction between Bd experience and metabolite presence in a treatment (Bd experience: $\chi^2_1 = 12.93$, $p < 0.0004$; Bd experience × Metabolites: $\chi^2_1 = 16.08$, $p < 0.0001$; Figure 2b). There were no other significant main effects or interactions ($\chi^2_1 < 2.51$, $p > 0.113$). The avoidance of all three treatments containing Bd metabolites was significantly greater when toads were Bd-experienced than Bd-naïve (Figure 2b). In contrast, neither Bd-naïve nor Bd-experienced toads exhibited any avoidance of zoospores without metabolites (Figure 2b).

3.3 | Pine woods tree frog

Nine out of 10 pine woods tree frog died during the live Bd exposure and so we could not determine whether experience with a Bd infection affected their behavioural response. Thus, we only tested for an innate avoidance response in this species. Bd-naïve pine woods

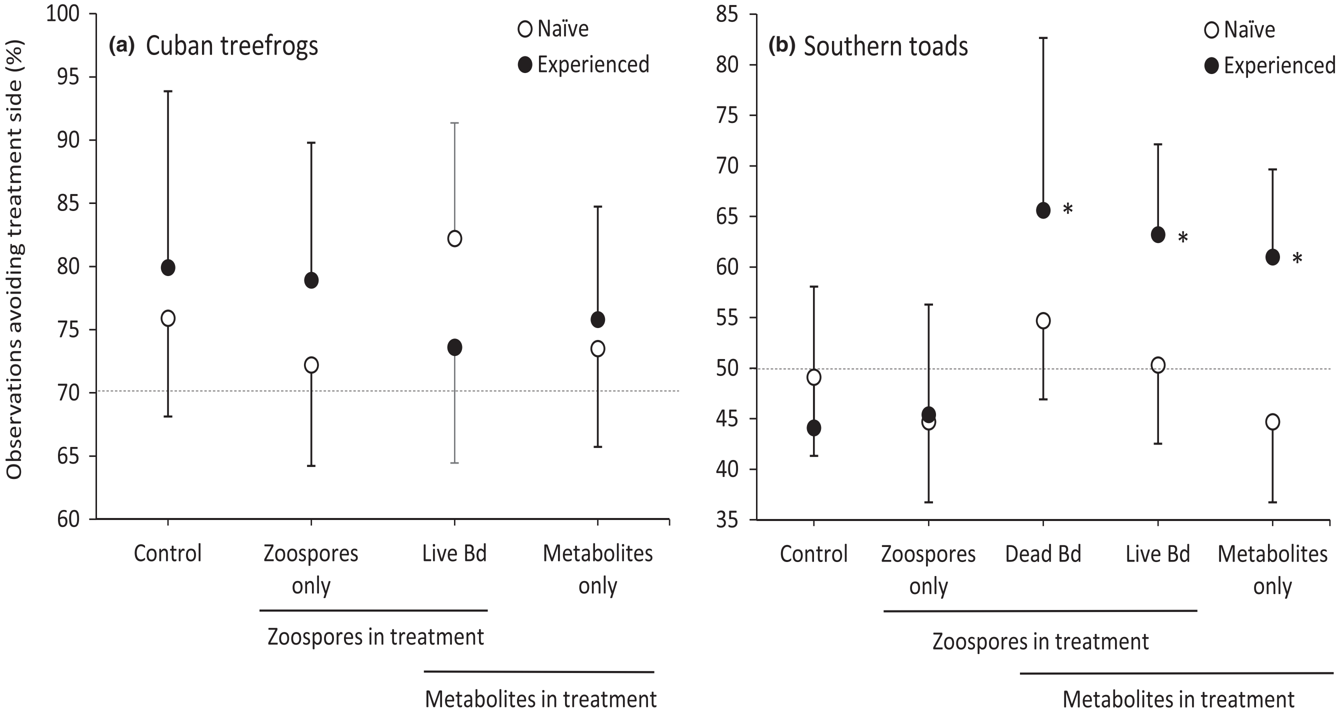

FIGURE 2 (a) Cuban tree frogs *Osteopilus septentrionalis* did not exhibit innate or learned avoidance to *Batrachochytrium dendrobatidis* (Bd) zoospores or metabolites ($p > 0.05$); whereas (b) southern toads (*Anaxyrus terrestris*) did learn to avoid Bd metabolites after being cleared of a Bd infection (Bd experienced*metabolites: $\chi^2 = 16.08$, $p < 0.0001$). Shown are marginal means (±*SE*) and null expected locations (dashed lines) of 70% and 50% in the Cuban tree frog and southern toad experiments, respectively. Asterisks signify greater avoidance of a treatment when Bd-experienced relative to Bd-naïve based on post hoc tests conducted using the emmeans function in *R*

tree frogs did not significantly avoid the live Bd treatment ($\chi^2 = 1.86$, $p = 0.173$; Figure 3a).

3.4 | Greenhouse frogs

Two out of 14 of the greenhouse frogs died during the live Bd exposure. Greenhouse frogs showed no significant avoidance of live Bd when Bd-naïve or Bd-experienced (Interaction: $\chi^2 = 0.09$, $p = 0.767$; Figure 3b). In fact, they exhibited 4.5% more attraction to the Bd after experience, indicating that there was no trend for learned avoidance.

4 | DISCUSSION

We found that amphibian species had different abilities to learn to avoid Bd. Neither Bd-naïve nor Bd-experienced Cuban tree frogs, greenhouse frogs and pine woods tree frogs avoided Bd. Bd-naïve southern toads exhibited no avoidance of Bd zoospores or metabolites, but after one Bd infection and clearance, they avoided treatments containing Bd metabolites. Oak toads were also capable of learning to avoid Bd (McMahon et al., 2014) and thus this is the second species in Bufonidae capable of acquired behavioural avoidance of Bd. More research is needed to explore whether the ability to learn avoidance behaviours is influenced by phylogeny.

Bd metabolites triggered the associational behavioural avoidance in southern toads, but we do not know why this avoidance behaviour was triggered specifically. Many vertebrate species are capable learning to avoid a cue that is accompanied by a painful stimulus (Dunlop et al., 2006) and the metabolites contain proteolytic enzymes and other chemicals that degrade host tissue (McMahon et al., 2013; Moss et al., 2010; Rollins-Smith et al., 2015, 2019), and thus exposure to metabolites might indeed be painful. Vertebrates are also capable of avoiding certain odours (Hansen et al., 2015). Bd produces the metabolite putrescine (Rollins-Smith et al., 2019), which is responsible for the smell of putrefying tissue, and therefore the frogs may be avoiding the metabolites if this odour is repulsive (Nielsen et al., 2015). More research is needed to understand the mechanisms underlying the behavioural response demonstrated by the toads.

Amphibians may be able to detect these chemical metabolites through the chemo- or pain receptors in their skin (Hillyard & Willumsen, 2011), which could facilitate the detection and avoidance of external stimuli through epidermal contact (for example see Kiesecker et al., 1999). Pavlovian conditioning predicts that chemicals that induce negative stimuli, for example pain or itching, or are associated with allelochemicals, for example odours, would produce a strong behavioural avoidance response. This is not only consistent with what we found here but is also consistent with amphibian behavioural avoidance of other directly transmitted pathogens (Kiesecker et al., 1999; Zylberberg et al., 2013). In all of these cases,

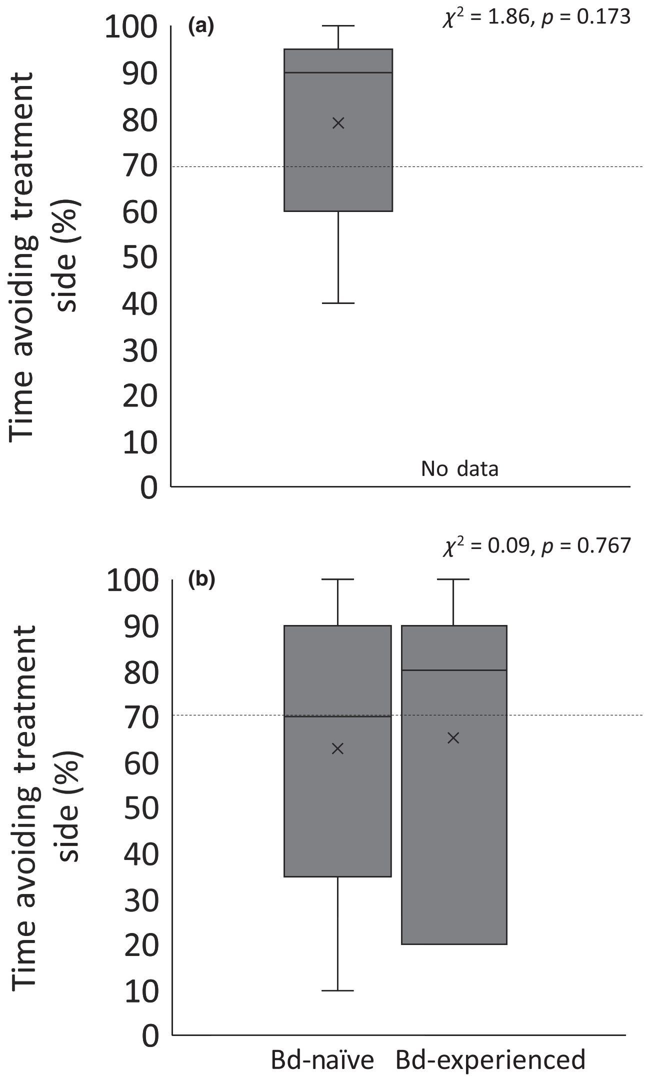

FIGURE 3 (a) Bd-naïve pine woods tree frogs *Hyla femoralis* did not avoid live Bd. Due to mortality, there were no Bd-experienced pine woods tree frogs. (b) Neither Bd-naïve nor Bd-experienced greenhouse frogs *Eleutherodactylus planirostris* avoided live Bd. The top and bottom of the box represents the 75th and 25th percentiles, respectively, line represents median, *x* represents mean and whiskers represent the minimum and maximum values not included in the outliers. The dashed line at 70% represents expected avoidance to treatments

the behavioural response is likely to lower negative stimuli, which may also lower the risk of infection.

Interestingly, the other species of amphibians tested did not exhibit the avoidance of Bd when Bd-naïve or Bd-experienced. The high Bd-induced mortality in the pine woods tree frogs was not unusual for species in the family Hylidae (Scheele et al., 2019). Given this high susceptibility to Bd, we would have expected strong selective pressure for innate behavioural avoidance, but none was detected in this species. This is possibly because these populations may not have an evolutionary history with Bd, which would be necessary for selection of avoidance responses.

We did not see high Bd-induced mortality in the greenhouse frogs and Cuban tree frogs. Because these species appear to be somewhat tolerant to Bd, they may not have experienced strong selective pressures to exhibit innate or learned avoidance of the fungus. Additionally, Cuban tree frogs can acquire resistance to Bd after a Bd infection (McMahon et al., 2014), and so this species may utilize its adaptive immune response as opposed to innate or learned avoidance.

Given that human-assisted migration is believed to have introduced Bd to new locations around the planet in the last 150 years (for example see Ouellet et al., 2005; Padgett-Flohr & Hopkins, 2009; Talley et al., 2015), we suspect that many hosts might not have had the evolutionary history with Bd needed for avoidance behaviours to have become fixed (i.e. innate) traits. Selection pressures are a product of variation in both exposure and susceptibility to the fungus (Sears et al., 2015). In addition to different selection pressures for avoidance, there might also be differences among species in their ability to learn. Hence, the combination of variation in selection pressures and learning abilities might explain the observed variation among amphibian species in their learned avoidance responses to Bd. We encourage additional research that screens for the ability to discriminate between innate and learned avoidance of pathogens, explores what the animals are avoiding (e.g. pain response or allelochemicals) and identifies the cues used to induce any avoidance response. This would give researchers a better understanding of the strength and importance of behavioural avoidance in the field and the degree of species-level variation in this avoidance.

This research has important management and policy implications for the applied conservation practices for amphibian populations threatened by Bd. The IUCN Amphibian Ark network and other conservation initiatives rescued hundreds of amphibian species threatened by Bd and are maintaining these species in captivity in the hopes that they might be released back into the wild (Venesky et al., 2012, 2014). Reintroduction attempts have often failed presumably because of the persistence of Bd in the environment on tolerant hosts (McMahon et al., 2013; Venesky et al., 2012, 2014). Previous work has suggested that immunization with killed Bd might induce acquired immunological resistance that could facilitate successful reintroductions of captively bred amphibians (McMahon et al., 2014). Given that some amphibians can also learn to avoid Bd metabolites, our work opens the door to using non-infectious cues to induce an acquired behavioural resistance response in captively bred and perhaps even wild amphibians. Mitigation plans that incorporate the use of acquired behavioural resistance protocols, which could be developed from this work, could facilitate amphibian reintroductions. Acquired behavioural resistance to Bd could increase the likelihood that certain species can be reintroduced, establish and persist in nature with Bd. For this to be successful, a better understanding of which species of amphibians are capable of acquiring immunological or behavioural resistance to Bd is needed. We hope that the induction of acquired immunological and behavioural resistance using Bd metabolites can reduce Bd-induced amphibian declines and can facilitate the successful re-establishment of captively bred amphibians into the wild.

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AUTHORS' CONTRIBUTIONS

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

DATA AVAILABILITY STATEMENT

Data available via the Dryad Digital Repository https://doi.org/10.5061/dryad.7h44j0zth (McMahon et al., 2021).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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