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Amphibians acquire resistance to live and dead fungus overcoming fungal immunosuppression

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Abstract

Emerging fungal pathogens pose a greater threat to biodiversity than any other parasitic group[1], causing declines of many taxa, including bats, corals, bees, snakes and amphibians[1–4]. Currently, there is little evidence that wild animals can acquire resistance to these pathogens[5]. *Batrachochytrium dendrobatidis* is a pathogenic fungus implicated in the recent global decline of amphibians[6]. Here we demonstrate that three species of amphibians can acquire behavioural or immunological resistance to *B. dendrobatidis.* Frogs learned to avoid the fungus after just one *B. dendrobatidis* exposure and temperature-induced clearance. In subsequent experiments in which *B. dendrobatidis* avoidance was prevented, the number of previous exposures was a negative

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Supplementary Information is available in the online version of the paper.

predictor of *B. dendrobatidis* burden on frogs and *B. dendrobatidis*-induced mortality, and was a positive predictor of lymphocyte abundance and proliferation. These results suggest that amphibians can acquire immunity to *B. dendrobatidis* that overcomes pathogen-induced immunosuppression[7–9] and increases their survival. Importantly, exposure to dead fungus induced a similar magnitude of acquired resistance as exposure to live fungus. Exposure of frogs to *B. dendrobatidis* antigens might offer a practical way to protect pathogen-naive amphibians and facilitate the reintroduction of amphibians to locations in the wild where *B. dendrobatidis* persists. Moreover, given the conserved nature of vertebrate immune responses to fungi[5] and the fact that many animals are capable of learning to avoid natural enemies[10], these results offer hope that other wild animal taxa threatened by invasive fungi might be rescued by management approaches based on herd immunity.

In recent decades, emerging fungal pathogens have had devastating effects on agricultureand caused population declines of several plant and animal species[1]. Many plants can acquire immunological resistance to fungal pathogens, which has proven useful for managing fungal pathogens of crops[11], but acquired resistance to fungi in wild animals has not been well studied[5].If natural variation in acquired resistance to fungi exists in wild animal populations, it might partly explain why fungal pathogens cause epidemics and extirpations of some animal populations (those that have not acquired resistance) but persist in an endemic state in others (those that have acquired resistance)[12,13]. If wildlife managers could induce resistance in enough individuals of a population, they might be able to drive the basic reproductive ratio (R_0; the average number of infections one infected individual generates in a population of susceptible hosts over the course of its infectious period) of a pathogenic fungus below one, such that ‘herd immunity’ would protect even pathogen-naive members of the population, a concept that is the basis for vaccination campaigns. Consequently, acquired resistance offers a potential tool to rescue animal populations threatened by fungi[14].

Here, we investigate whether amphibians can acquire behavioural and immunological resistance to the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*). We define acquired resistance broadly as the process of reducing infection loads upon subsequent exposure to a pathogen or antigen caused by changes in behaviour, innate immunity or adaptive immunity within the lifetime of an individual. We focused on *Bd* because it is implicated in the global decline of amphibians, the most threatened vertebrate taxon[6]. *Bd* is known to hinder amphibian lymphocyte responses[7], but acquired resistance might still be detectable if its strength exceeds any immunosuppression by *Bd.* Hence, it is critical to quantify the net effect of any acquired host resistance and *Bd*-induced immunosuppression on *Bd* abundance (average number of *Bd* zoospores on *Bd*-exposed hosts) across multiple host species to evaluate the feasibility of acquired resistance as a conservation tool[14]. Although beneficial for understanding mechanisms of immunity, gene expression studies or studies that only quantify immunity to *Bd*(and not also *Bd* loads)do not capture the extended phenotype and thus cannot quantify the net effect of acquired immunity and immunosuppression on the host–parasite interaction[8,9,15,16]. Surprisingly, there are no published studies that experimentally subjected amphibians to regimes of repeated *Bd* infection followed by clearance and then tested for an association between the number of previous infections and

Bd abundance, immune parameters and behavioural avoidance (ref. 14, but see also 15–22). We hypothesized that, despite *Bd*-induced immunosuppression[7–9], effects of repeated exposures to *Bd* followed by pathogen clearance could include reduced *Bd* abundance on frog skin, increases in the abundance or efficacy of skin peptides, augmentation of the abundance of responding lymphocyte populations (mediators of *Bd* resistance[16–18]), and induction of learned avoidance of *Bd*.

To create variation in number of exposures to *Bd*, groups of frogs were exposed to *Bd* and cleared of their infection using heat zero to four times (depending on their treatment assignment and experiment) in a manner that prevented a simple decay of immune responses from accounting for our results (Extended Data Table 1; Supplementary Methods). Frogs were swabbed for *Bd* before each experiment, after each *Bd* exposure, and after each clearance period. Quantitative PCR (qPCR) of these swabs revealed that frogs were free of *Bd* before each experiment, that all unexposed control frogs were *Bd*-free after each *Bd* growth period (that is, no cross contamination), that exposed frogs generally became infected (average prevalence across exposure periods was 85%), and that heat-clearances were 100% effective at eliminating established *Bd* infections (Extended Data Table 2). Additionally, our methods ensured that we did not confound acquired resistance, a form of phenotypic plasticity, with selection (via mortality of low-resistance individuals; see Supplementary Results, Extended Data Table 3, Extended Data Fig. 1) or *Bd* inoculates (via different inoculates for each treatment), confounding factors that have hampered previous studies[14].

Many hosts are capable of avoiding pathogens, but few studies have tested whether avoidance is learned or innate[23,24]. To determine if amphibians could learn to avoid *Bd*, we conducted two separate experiments to examine how number of exposures to *Bd* affected the amount of time that oak toads (*Bufo quercicus*) spent on a *Bd*-negative or *Bd*-inoculated side of a test chamber. *Bd*-naive frogs showed no significant avoidance or attraction to *Bd* (χ^2_1 = 0.289, P = 0.591), but frogs previously infected with *Bd* once or twice (all the exposures resulted in infections) chose the *Bd*-free substrate approximately 65% and 70% of the time, respectively (experiment 1: χ^2_1 = 6.683, P = 0.009, experiment 2: χ^2_1 = 9.693, P = 0.002; Fig. 1), resulting in a significant interaction between naivety and *Bd* avoidance (number of previous infections × deviation from 50% null interaction: χ^2_1 = 9.107, P = 0.011; Fig. 1). These results were consistent across the two experiments (that is, repeatable; Fig. 1). Importantly, this learned behavioural resistance should be unaffected by the immunosuppressive effects of *Bd*[7–9].

To test for acquired immunological resistance, we assessed whether the number of exposures to live *Bd* (0, 1, 2, 3 or 4 exposures) was a significant predictor of *Bd* abundance on Cuban treefrogs (*Osteopilus septentrionalis*) after the third and fourth *Bd* exposure and clearance regimens (Extended Data Table 1). Number of exposures to *Bd* was a significant negative predictor of *Bd* abundance on the frogs (χ^2_1 = 8.40, P = 0.003; Fig. 2a and Extended Data Fig. 2a; see Extended Data Table 3 for prevalence data), with a 75% drop in *Bd* loads from the first to third *Bd* exposure (Fig. 2a, open circles). To evaluate whether the observed resistance to *Bd* could be attributable to changes in innate or cellular immunity, we quantified the abundance and efficacy of skin peptides and splenic leukocytes (enriched for

lymphocytes) after only the fourth exposure and clearance (0–4 exposures) and then tested whether number of *Bd* exposures was a significant predictor of these responses. The observed acquired resistance to *Bd* did not seem to be strongly attributable to changes in skin peptides because the number of *Bd* exposures was not significantly correlated with skin peptide abundance ($\chi^2_1 = 0.7$, $P = 0.38$; Extended Data Fig. 3a) or efficacy at inhibiting *Bd* growth *in vitro* (Peptide treatment × number of *Bd* exposures: $\chi^2_1 = 0.329$, $P = 0.566$; Extended Data Fig. 3b, Supporting Information). However, number of *Bd* exposures was a significant positive predictor of lymphocyte abundance in the spleen ($\chi^2_1 = 5.9$, $P = 0.015$, Fig. 3a) and lymphocyte proliferation in response to *Bd* ($\chi^2_1 = 9.5$, $P = 0.002$) and phytohaemagglutinin ($\chi^2_1 = 78.4$, $P < 0.0001$; number of exposures × method of stimulation: $\chi^2_1 = 1.3$, $P = 0.24$; Fig. 3b). Phytohaemagglutinin is a mitogen that triggers T-lymphocyte cell division and thus functioned as a positive control for lymphocyte proliferation[25]. Moreover, the slope of the relationship between *Bd* abundance and lymphocyte numbers in the spleen became more positive with each *Bd* exposure, indicating that lymphocytes were stimulated to proliferate with subsequent exposures (number of exposures × lymphocyte abundance: $F_{1,54} = 4.4$, $P = 0.04$, Fig. 3c).

Two previous studies attempted to immunize frogs systemically through injections and did not find evidence of acquired protection against *Bd*[21,26], suggesting that the approach used in our study, exposure by way of the skin, might be critical for induction of acquired resistance. Consistent with this proposition are studies suggesting that the adaptive immune system of amphibians responds to *Bd.* For example, the heterozygosity of genes associated with adaptive immunity (major histocompatibility complex loci) was greater in frog populations more resistant to *Bd*[18], which would have been exposed cutaneously. Additionally, frogs irradiated to reduce their lymphocyte numbers had increased susceptibility to *Bd* infections, and frogs immunized against *Bd* via injection with heat-killed *Bd* cells had elevated levels of *Bd*-specific IgM and IgY serum antibodies[17].

The only previous study to repeatedly infect and clear amphibians of *Bd* seemed to conflict with our results[20]. In this study[20], the authors concluded that there was no evidence of acquired resistance in the critically endangered booroolong frog (*Litoria booroolongensis*) because frogs previously cleared of *Bd* with the fungicide itraconazole did not have significantly lower prevalence or mortality upon re-infection than frogs that were not previously exposed to *Bd* or the fungicide (20 of 32 versus 14 of 28 infected, respectively; $\chi^2_1 = 0.9$, $P = 0.33$). However, the fungicide alone increased *Bd* prevalence in *Bd*-naive frogs (no previous fungicide: 14 of 28 infected, previous fungicide: 10 of 11 infected, $\chi^2_1 = 5.5$, $P = 0.01$), consistent with other studies suggesting that itraconazole is immunosuppressive[27]. Hence, a better test for acquired resistance would have been to compare *Bd* prevalence of frogs previously infected and cleared of *Bd* by the fungicide to the prevalence of *Bd*-naive frogs also previously exposed to the fungicide. A re-analysis of these data show that previous infections significantly reduced prevalence from 91% to 63% ($\chi^2_1 = 3.1$, $P = 0.03$) when making this more direct comparison. In summary, despite the immunosuppressive effects of *Bd*[7–9], the net effect of previous exposures to and clearances of *Bd*, across three studies and three species (B. quercicus, O. septentrionalis, L. booroolongensis), was to reduce *Bd* prevalence or abundance on frogs. Differences in the

strength of this net effect (immunosuppression + acquired resistance) or variation in pathogenicity across *Bd* strains might explain host variation in susceptibility to *Bd*[21,26].

Next, we sought to test whether amphibians could acquire resistance to dead *Bd* because induction of acquired resistance through exposure to dead *Bd* might offer a practical management tool to protect amphibian populations[14]. We repeated our first immunological resistance experiment described above but added a dead *Bd* exposure treatment and tracked frog survival for 6 weeks after the last *Bd* inoculation to enhance our survival estimates. Number of exposures to live *Bd* in this second immunological resistance experiment was again a significant negative predictor of *Bd* abundance on *O. septentrionalis* ($\chi^2_1 = 4.9$, $P = 0.02$; Fig. 2b, Extended Data Fig. 2b), replicating our previous findings. Additionally, when blocking by experiment ($P < 0.001$), number of exposures to live *Bd* was a significant positive predictor of the probability of surviving the experiment (logistic regression: $\chi^2_1 = 4.4$, $P = 0.03$; Fig. 2c) and a positive predictor of time of death (Cox survival analysis: $\chi^2_1 = 4.6$, $P = 0.03$, parameter ± s.e. = 0.224 ± 0.105, hazard ratio = 0.799; Supplementary Fig. 3). Indeed, frogs with previous exposure to *Bd* were 5.57 (odds ratio) times more likely to survive until the end of the experiment than frogs that were naive to *Bd.*

Similar to our findings for exposure to live *Bd*, number of previous exposures to dead *Bd* was a significant negative predictor of *Bd* abundance on frogs ($\chi^2_1 = 11.3$, $P < 0.001$) and the magnitude of acquired resistance to dead and live *Bd* did not significantly differ ($\chi^2_1 = 0.99$, $P = 0.32$; Fig. 2b, Extended Data Fig. 2b). By the end of six weeks of *Bd* growth, frogs previously exposed to dead *Bd* three or four times lived longer than those exposed to dead *Bd* two times ($df = 2$, Wald = 7.22, $P = 0.027$), but overall, number of exposures to dead *Bd* was not a significant predictor of time of death ($\chi^2_1 = 0.02$, $P = 0.900$; Extended Data Fig. 4). Our power for detecting an effect of dead *Bd* on survival, however, was lower than it was for live *Bd* because we only conducted one rather than two experiments with dead *Bd.*

As a result of efforts by the IUCN Amphibian Ark network and other conservation initiatives, hundreds of threatened amphibian species have been removed from their *Bd*-positive habitats and are being bred in captivity[14,28]. However, these amphibians often failto re-establish when released at their sites of collection, presumably because of the persistence of *Bd* at these sites on tolerant hosts[14,28,29]. Inducing acquired resistance in these captive-bred amphibians might allow for their successful re-establishment[14,28]. Although additional research is necessary to quantify the efficacy of releasing dead *Bd* into water bodies to protect amphibians and the non-target effects of these releases, mathematical models suggest that, despite biotic and abiotic reservoirs for *Bd*, this strategy offers a promising management tool to reduce R_0 of *Bd* below one, which could be used to proactively prevent *Bd* epidemics and to rescue host populations already threatened by this pathogen[12,13]. However, the efficacy of this management option will depend on several factors, such as whether *Bd*-naive larval amphibians can also acquire immunity, the role of biotic and abiotic reservoirs in maintaining *Bd*, and the extent and magnitude of variation in acquired resistance among amphibian species. Perhaps most importantly, given the conserved nature of the immune responses of vertebrates to fungi[5] and that many animals are capable of learning to avoid natural enemies[10], the results presented here offer hope that other wild animal taxa threatened by invasive fungi, such as bats, bees, and snakes[1–4], might be capable

of acquiring resistance and might also be rescued by management approaches based on herd immunity.

METHODS

Bd culture and inoculation

For all experiments we prepared *Bd* inoculum (strain SRS 812) according to the methods used in McMahon *et al.*[28] (see Supplementary Methods for additional details).

First and second immunological resistance experiments

We collected *O. septentrionalis* eggs from *Bd*-free wading pools at the University of South Florida Botanical Gardens (Tampa, FL) and reared them through metamorphosis in a *Bd*-free laboratory. All frogs were approximately five months post-metamorphosis before the first and second immunological resistance experiments were initiated and all frogs were assigned randomly to treatments so that time since metamorphosis should not be confounded with number of *Bd* exposures. For the first immunological resistance experiment, we exposed frogs to and cleared frogs of *Bd* 0–4 times (n = 10–20/treatment; see Extended Data Table 3 for sample sizes). After each exposure and clearance period, each frog was weighed and swabbed 10 times from hip to toe (left leg after *Bd* growth period, right leg after clearance period) to determine the abundance of *Bd* via qPCR. Frogs were fed crickets *ad libitum*, mortality was monitored daily, and moist paper towels were changed weekly. These methods were repeated in the second immunological resistance experiment, except that frogs were exposed to live or flash frozen (culture in flask placedin liquid nitrogenfor 15 min) *Bd* 0–4 times (n = 20 per treatment; Extended Data Tables 1, 2). For each exposure period, we plated 1 ml of the previously frozen *Bd* inoculum on three 1% tryptone agar plates and incubated each at 23 °C for at least 8 days (see Supplementary Methods). No *Bd* grew confirming that the *Bd* was successfully killed. Frogs were exposed to dead *Bd* every 2 days for 11 days to match the live *Bd* exposure in this same experiment (see Supplementary Methods and Supplementary Results for a preliminary experiment demonstrating that dead *Bd* DNA was not detectable after 2 days). In the first immunological resistance experiment, frogs were cleared after the last 11-day *Bd* exposure period to ensure that we had adequate frog survival and sample sizes for immune assays. In the second immunological resistance experiment, we quantified frog survival for six weeks after the last live *Bd* inoculation so we could better assess the effects of acquired resistance on survival. In the fourth exposure period after exposure to dead *Bd,* frogs were challenged with live *Bd* to test whether previous exposure to dead *Bd* induced acquired resistance. In the first immunological resistance experiment, we quantified *Bd* abundance on frogs after each exposure period but only present the data for exposure periods three and four. In the second immunological resistance experiment, we did not conduct the qPCR to quantify *Bd* loads in the third exposure period because the dead *Bd* treatment had not yet received the live *Bd* challenge. Thus, we only conducted the qPCR for the fourth exposure period in this experiment.

Antimicrobial skin peptide and splenic lymphocyte collection and assays

In the first immunological resistance experiment, we used the methods of Rollins-Smith *et al.*[25,30] to quantify skin peptide abundance and efficacy at inhibiting *Bd* growth and to

quantify lymphocyte abundance in the spleen and lymphocyte proliferation in response to freshly killed *Bd* zoospores (60 °C 10 min) and phytohaemagglutinin (see Supplementary Methods for additional details).

Behavioural resistance experiment

We field collected adult oak toads (*B. quercicus*; $n = 30$, Extended Data Table 3) from Hillsborough County, FL and reared and monitored them individually in the laboratory as described in the first immunological resistance experiment. Toads were placed in a test chamber (9.53 × 7.0 cm) containing two 3.03 × 9.5 cm paper towels on each side of the chamber separated by a 1.0 cm gap. We randomly dosed one side with 2.0 ml of a *Bd*+ inoculum and the other side with 2.0 ml of Bd^- inoculum. We then placed a toad in the centre of the chamber, allowed it to acclimate for 30 min, and then conducted double blind scan samples every 5 min for 40 min, recording the side of the chamber containing each toad. After the behavioural trials, the toads were transferred to new containers and experienced the *Bd* exposure-clearance regime described in the first immunological resistance experiment. Behavioural observations were repeated after each clearance period so that by the end of behavioural resistance experiment 1 and 2, we quantified toad avoidance of *Bd* after 0 or 1 and 0, 1, or 2 infections, respectively (a dysfunctional environmental chamber caused a mass mortality event preventing the second exposure for experiment 1).

Quantitative PCR

We followed the procedure described by Hyatt *et al.*[30] to quantify *Bd* abundance using qPCR (with a StepOne Real-Time PCR System; Applied Biosystems, Foster City, CA). See Supplementary Methods for additional details.

Statistical analyses

All animals were randomly assigned to treatments, statistics were analysed with R statistical software, significance was attributed when $P < 0.05$, frog mass was used a covariate when significant (unless the response was mass standardized), and all analyses were conducted within rather than across exposure periods so that treatment comparisons were made among frogs exposed to the same *Bd* inoculate. For the first and second immunological resistance experiments, the effect of number of live *Bd* exposures on survival was analysed using Cox proportional hazards regression (package: survival, function: coxph), blocking by experiment. A generalized linear model (package: stats, function: glm), was used to determine whether number of *Bd* exposures affected frog growth, lymphocyte densities, and skin peptide abundance. A mixed effects model (package: nlme, function: lme) was used to test for the main and interactive effects of skin peptides (presence/absence) and number of *Bd* exposures on *Bd* growth (defined as$(\ln(OD_7) - \ln(OD_0))/7$ where OD_7 and OD_0 refer to optical density measurements at 490 nm on days 7 and 0, respectively) in culture, treating frog identity as a random effect (see Supplementary Information). A mixed effects negative binomial model (package: glmmADMB, function: glmmadmb) was used to determine if there was a difference in lymphocyte proliferation in response to *Bd* and PHA for each frog (individuals frogs treated as a random effect) compared to their lymphocyte proliferation in

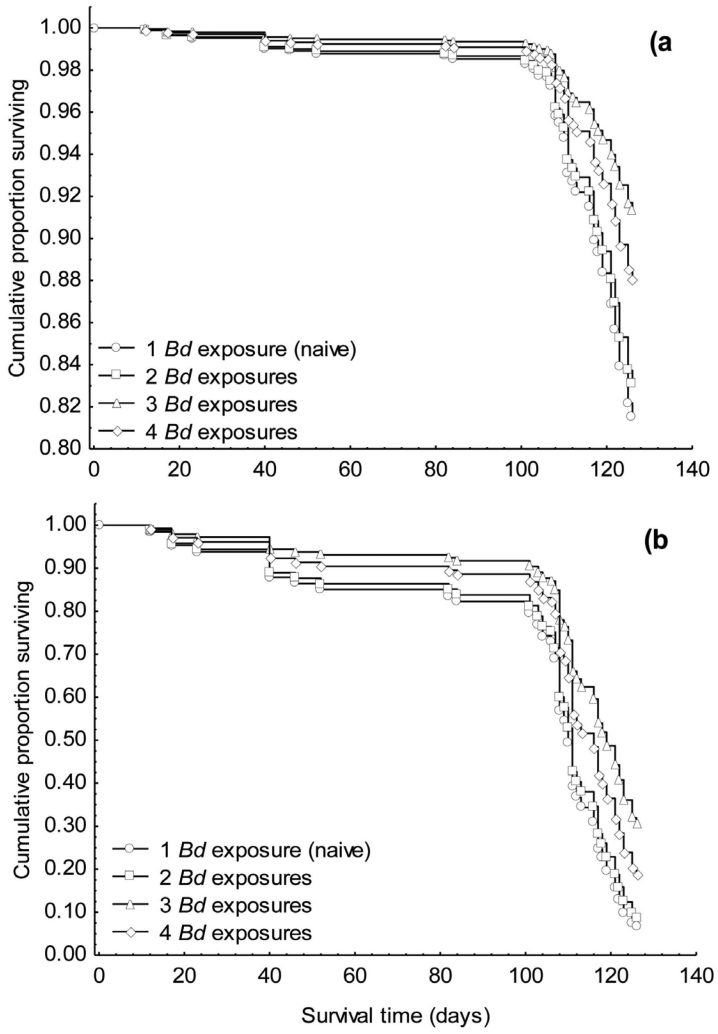
the absence of either stimulus. A general linear model was used to test whether number of *Bd* exposures was a predictor of the slope of the relationship between *Bd* abundance and lymphocyte densities. We used a zero-inflated negative binomial error model (package: pscl, function: zeroinf) to test for the effect of number of exposures on *Bd* abundance within the third exposure period for the first immunological resistance experiment and within the fourth exposure period for the second immunological resistance experiment. Bootstrapping analyses were used to test for the effect of number of *Bd* exposures on *Bd* abundance within the fourth exposure period for the first immunological resistance experiment (see Supplementary Methods for a justification and for additional details).

In our behavioural resistance experiment, we tested for avoidance of *Bd* using a linear mixed-effects model (package: lme4, function: lmer) with a binomial error distribution. We nested number of *Bd* exposures within frog and frog within experiment (that is, treated frog and experiment as random effects) and tested whether the proportion of observations on the *Bd*+ side of the container differed from a 50:50 expectation, allowing us to discriminate between innate and learned avoidance. We tested for main effects of number of *Bd* exposures (continuous predictor), experiment, deviation from a null 50:50 expectation, and a number-of-*Bd*-exposures-by-deviation-from-null interaction (see Supplementary Information for additional details). We also conducted post-hoc analyses to determine when frogs significantly avoided *Bd*, with 0, 1, or 2 *Bd* infections (with a Bonferroni alpha adjustment; $\alpha = 0.016$).

Compliance statement

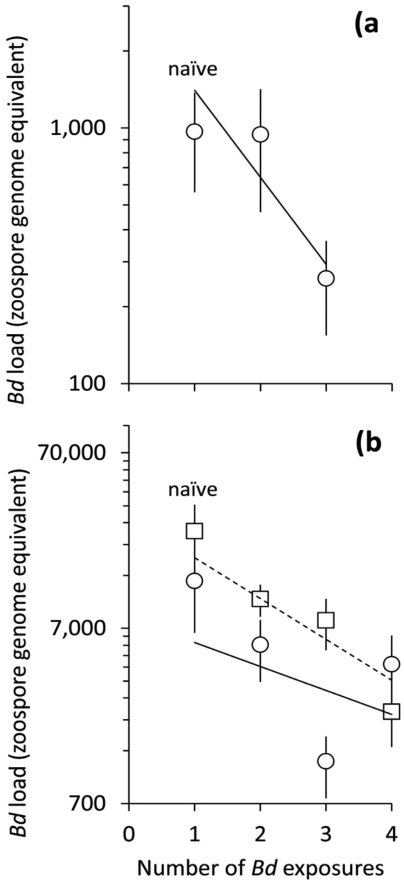
All experiments were approved by the Institutional Animal Care and Use Committee of the University of South Florida.

Extended Data



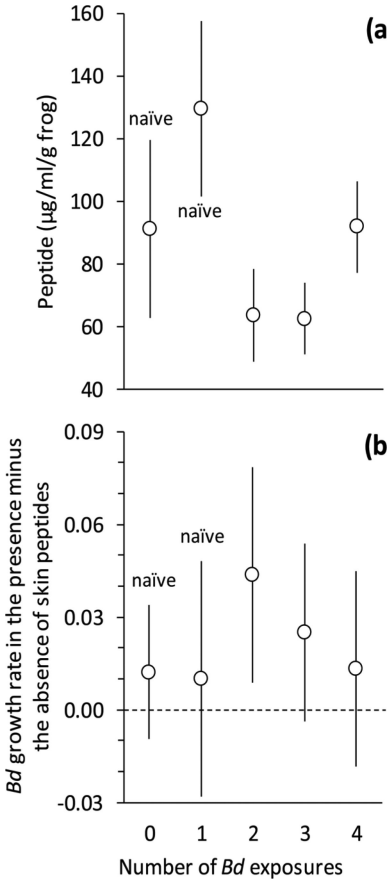
Extended Data Figure 1. Cumulative survival of *Osteopilus septentrionalis*

a, b, Cuban treefrog survival in the first (**a**) and second immunological resistance experiments (**b**) with 1, 2, 3 or 4 exposures (previous infections were cleared with heat) to live *Batrachochytrium dendrobatidis* (*Bd*). Mortality was greater in the second immunological resistance experiment because we provided six weeks for *Bd* to grow on the frogs after the final *Bd* exposure, whereas the frogs were cleared of *Bd* after only 11 days of growth in the first immunological resistance experiment so that we had ample frog survival for subsequent immunological analyses. Naivety was based on the state of the frog before *Bd* exposure during the fourth exposure period; thus frogs exposed to *Bd* for the first time during the fourth exposure period were classified as naive because they had not previously been exposed to *Bd.*



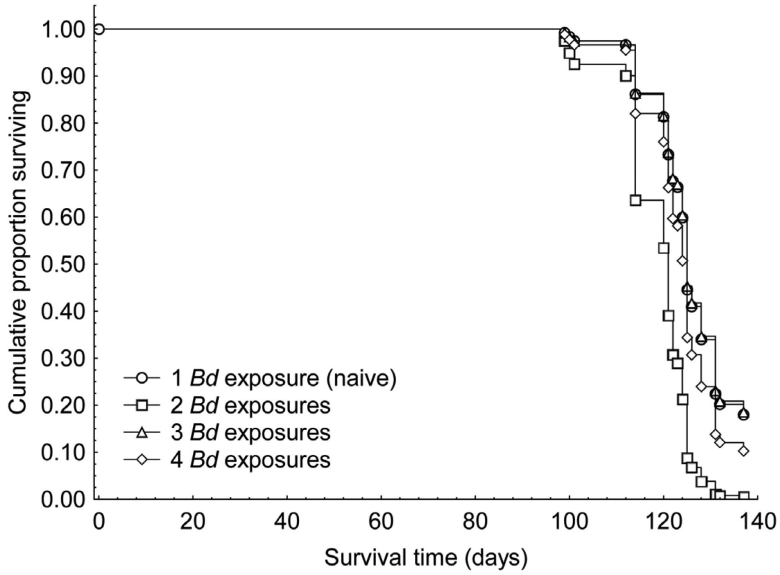
Extended Data Figure 2. Effects of 1–4 exposures to *Batrachochytrium dendrobatidis* (*Bd*) on *Bd* abundance on frogs (*Osteopilus septentrionalis*; not standardized by weight)

a, b, Effects of exposures on mean *Bd* abundance (zoospore genome equivalents (GE) ± s.e.m.) after exposure period 3 in the first immunological resistance experiment (**a**) and exposure period 4 in the second immunological resistance experiment (live *Bd* exposures: circles and solid line; dead *Bd* exposures: squares and dotted line) (**b**). The best-fit lines are based on predicted values from the implemented zero-inflated negative binomial. Naivety was based on the state of the frog before *Bd* exposure during the focal exposure period (third or fourth exposure period depending on what is being displayed). Thus, frogs exposed to *Bd* for the first time during the focal exposure period were classified as naive because they had not previously been exposed to *Bd*.



Extended Data Figure 3. Effects of 0–4 exposures to live *Batrachochytrium dendrobatidis* (*Bd*) on the abundance and efficacy of skin peptides extracted from frogs (*Osteopilus septentrionalis*)

a, Mean (± s.e.) skin peptide abundance (n = 8, 9, 17, 17, and 18 for 0–4 *Bd* exposures, respectively). **b**, Mean (± 95% confidence interval) efficacy of skin peptides at inhibiting *Bd*, measured as the difference between *Bd* growth rate in the presence and absence of a standardized concentration of skin peptides (n = 8, 8, 10, 11, and 10 for 0–4 *Bd* exposures, respectively). See Methods for details on how *Bd* was quantified and growth rates were calculated. Naivety was based on the state of the frog before *Bd* exposure during the fourth exposure period; thus frogs exposed to *Bd* for the first time during the fourth exposure period were classified as naive because they had not previously been exposed to *Bd*.



Extended Data Figure 4. Cumulative survival of *Osteopilus septentrionalis* in the second immunological resistance experiment with 1, 2, 3 or 4 exposures to dead *Batrachochytrium dendrobatidis* (*Bd*) followed by an exposure to live *Bd*

Naivety was based on the state of the frog before *Bd* exposure during the fourth exposure period; thus frogs exposed to *Bd* for the first time during the fourth exposure period were classified as naive because they had not previously been exposed to *Bd.*

Extended Data Table 1

Description of when exposure to live or dead *Batrachochytrium dendrobatidis* (*Bd*) occurred (designated by x) during the first and second immunological resistance experiments

	Bd* exposure-clearance period			
Total no. of exposures to live or dead *Bd*	**1**	**2**	**3†**	**4†‡**
0§				
1§				X
2//			X	X
3	X		X	X
4	X	X	X	X

*All frogs, regardless of treatment, were exposed to the same temperature regimes.

†Statistical analyses only occurred within the third or fourth exposure-clearance periods rather than across exposure-clearance periods.

‡For the first immunological resistance experiment, immunological assays occurred after the fourth exposure-clearance period.

§Frogs in this treatment were *Bd*-naive throughout the experiment.

//Frogs in this treatment were *Bd*-naive during the third but not the fourth exposure-clearance period.

Extended Data Table 2

Number of frogs swabbed for, and infected with, *Batrachochytrium dendrobatidis* (*Bd*) before each experiment began, and clearance temperatures, number of frogs cleared of *Bd*, and number of *Bd* clearances (includes multiple clearances per frog) in each experiment

Experiment	No. of frogs swabbed before experiment	*Bd* prevalence before experiment (%)	Clearance temperature (°C)	No. of frogs cleared of *Bd*[*]	Incidents of *Bd* clearance (counts multiple clearances per frog)[*]
First Behavioral Resistance	20	0	30	30	30
Second Behavioral Resistance	20	0	30	30	60
First Immunological Resistance	20	0	32	71	172[†]
Second Immunological Resistance	20	0	30	80	117[†]

[*] Heat-induced clearance of *Bd* infections was 100% effective.

[†] The difference in *Bd* clearance incidents between the first and second immunological resistance experiments was because all surviving frogs were cleared of their infections at the end of the first immunological resistance experiment before being shipped to Vanderbilt University for immunological assays and because of minor differences in survival between experiments.

Extended Data Table 3

Sample size, *Batrachochytrium dendrobatidis* (*Bd*) prevalence, and mortality from each experiment

Experiment	Species tested	Treatment[*]	*n*	Prevalence (%)[†]	No. of frogs that died[‡]	Mortality (%)
First Behavioral Resistance	*Bufo quercicus*	0 exposures to live *Bd* (naïve)[§]	10	-	0	0
First Behavioral Resistance	*Bufo quercicus*	1 exposure to live *Bd* (naïve)[§//]	10	100	0	0
Second Behavioral Resistance	*Bufo quercicus*	0 exposures to live *Bd* (naïve)[§]	10	-	0	0
Second Behavioral Resistance	*Bufo quercicus*	1 exposure to live *Bd* (naïve)[§//]	10	100	0	0
Second Behavioral Resistance	*Bufo quercicus*	2 exposures to live *Bd*[§//]	10	100	0	0
First Immunological Resistance	*Osteopilus septentrionalis*	0 exposures to live *Bd* (naïve)[§]	10	-	1	10
First Immunological Resistance	*Osteopilus septentrionalis*	1 exposure to live *Bd* (naïve)[§]	10	100	3	30
First Immunological Resistance	*Osteopilus septentrionalis*	2 exposures to live *Bd*[§//]	20	100	3	15
First Immunological Resistance	*Osteopilus septentrionalis*	3 exposures to live *Bd*[§//]	20	100	2	10

Experiment	Species tested	Treatment*	*n*	Prevalence (%)†	No. of frogs that died‡	Mortality (%)
First Immunological Resistance	*Osteopilus septentrionalis*	4 exposures to live *Bd*§//	20	95	1	5
Second Immunological Resistance	*Osteopilus septentrionalis*	0 exposures to live *Bd* (naïve)§	20	-	0	0
Second Immunological Resistance	*Osteopilus septentrionalis*	1 exposure to live *Bd* (naïve)§	20	95	1	5
Second Immunological Resistance	*Osteopilus septentrionalis*	2 exposures to live *Bd*§//	20	77	2	10
Second Immunological Resistance	*Osteopilus septentrionalis*	3 exposures to live *Bd*§//	20	85	0	0
Second Immunological Resistance	*Osteopilus septentrionalis*	4 exposures to live *Bd*§//	20	70	0	0
Second Immunological Resistance	*Osteopilus septentrionalis*	0 exposures to dead *Bd* (naïve)§¶	20	-	0	0
Second Immunological Resistance	*Osteopilus septentrionalis*	1 exposure to dead *Bd* (naïve)§¶	20	100	3	15
Second Immunological Resistance	*Osteopilus septentrionalis*	2 exposures to dead *Bd*§¶	20	94	2	10
Second Immunological Resistance	*Osteopilus septentrionalis*	3 exposures to dead *Bd*§¶	20	71	1	5
Second Immunological Resistance	*Osteopilus septentrionalis*	4 exposures to dead *Bd*§¶	20	71	3	15
Cashins *et al.* 2013#	*Litoria booroolongensis*	1 exposure to live *Bd*, no exposure to itraconazole (naïve)	28	50	4	14.3
Cashins *et al.* 2013#	*Litoria booroolongensis*	1 exposure to live *Bd*, after previous exposure to itraconazole (naïve)	11	91	2	18.2
Cashins *et al*. 2013#	*Litoria booroolongensis*	2 exposures to live *Bd*, first exposure cleared with itraconazole	32	63	5	15.6

*Immunological defences of frogs were naïve to *Bd* upon the first exposure because they had not previously been exposed to *Bd*. Do not confuse the treatments in this column for the behavioural resistance experiments with the *x*-axis label on Fig. 1, which emphasizes number of previous not present exposures to *Bd*.

†Values are for prevalence at the end of the behavioural resistance and Cashin *et al.* experiments, at the end of exposure period 3 for the first immunological resistance experiment, and at the end of exposure period 4 for the second immunological resistance experiment.

[‡] Cumulative number of frogs that died up to the final swabbing period, with the exception of the second immunological resistance experiment, where data represent cumulative number of frogs that died up to 11 days after the final *Bd* exposure to match the growth periods in the behavioural and first immunological resistance experiments.

[§] Frogs in these treatments were exposed to exactly the same temperature conditions regardless of whether they were exposed to live *Bd* or not.

[//] Frogs exposed to live *Bd* more than once had their previous infections cleared using heat exposures.

[¶] Frogs in these treatments were challenged with live *Bd* at the end of the experiment to evaluate how previous exposures to dead *Bd* affected actual *Bd* growth on the frogs.

[#] Ref. 20.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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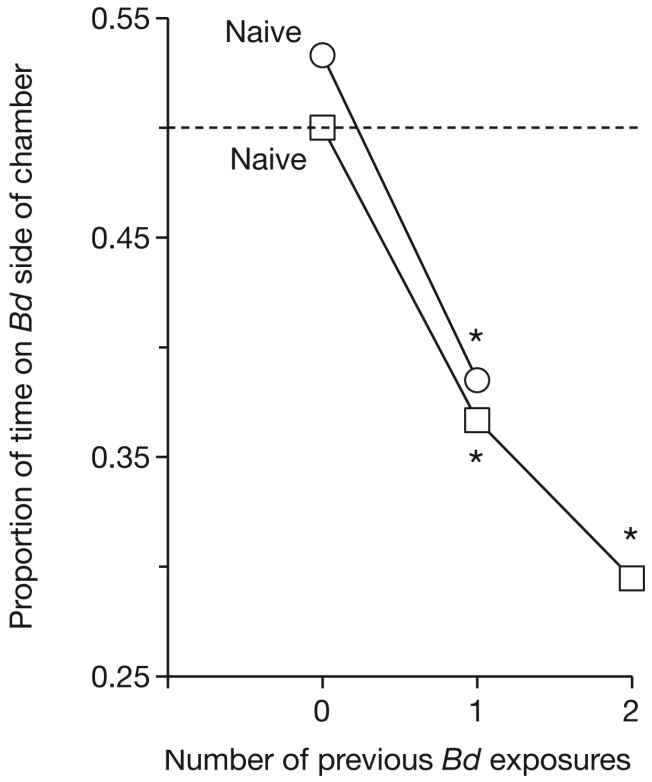

Figure 1. Effects of 0, 1 or 2 previous exposures to live *Batrachochytrium dendrobatidis* (*Bd*) on the proportion of time that toads (*Bufo quercicus*) spent on the *Bd*-positive side of a test chamber in two experiments (Experiment 1: circles, Experiment 2: squares)

In both experiments (n = 30 toads), *Bd*-naive frogs showed no significant avoidance or attraction to *Bd* ($\chi^2_1 = 0.29$, $P = 0.59$), but frogs previously infected with *Bd* once or twice chose the *Bd*-free substrate more frequently than expected by chance ($\chi^2_1 = 6.7$, $P = 0.009$ and $\chi^2_1 = 9.7$, $P = 0.002$, respectively). Asterisks represent significant avoidance of *Bd*.

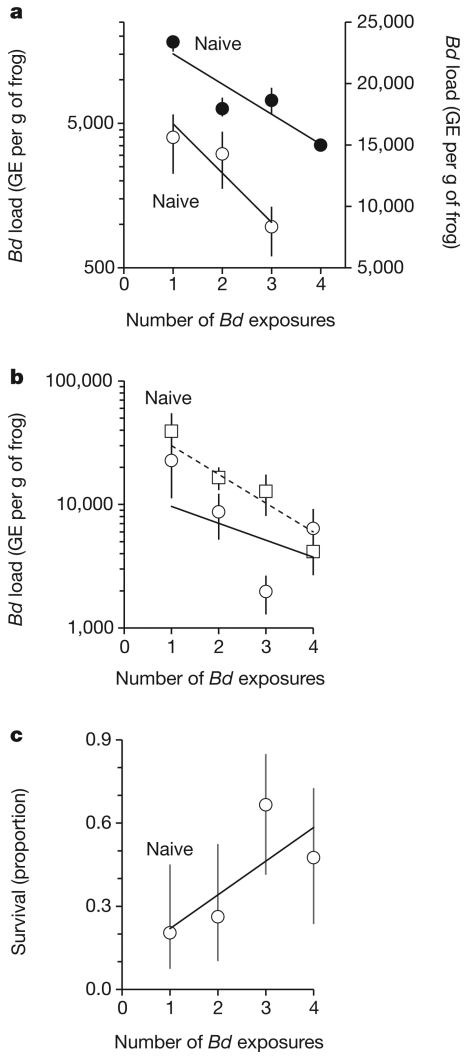

Figure 2. Effects of 1–4 exposures to *Batrachochytrium dendrobatidis* (*Bd*) on *Bd* abundance per gram of frog and frog (*Osteopilus septentrionalis*) survival

a, b, Effects of exposures on mean *Bd* abundance (zoospore genome equivalents (GE)/g of frog) after exposure period 3 (± s.e.m., open circles and left axis; $\chi^2_1 = 8.40$, $P = 0.003$) and exposure period 4 (closed circles, right axis; bootstrapped means ± bootstrapped 95% confidence interval, see Supplementary Methods for details) in the first immunological resistance experiment (**a**) and exposure period 4 in the second immunological resistance experiment (± s.e.m.; live *Bd* exposures (circles and solid line): $\chi^2_1 = 4.9$, $P = 0.02$; dead *Bd* exposures (squares and dotted line): $\chi^2_1 = 11.3$, $P < 0.001$) (**b**). **c**, Effects of live *Bd* exposures on mean frog survival (± 95% confidence interval; odds ratio: 1.66, $\chi^2_1 = 4.45$, $P = 0.035$; experiment treated as a temporal block). The best-fit lines are based on predicted values from the implemented zero-inflated negative binomial (*Bd* abundance) and binomial statistical models (frog survival). Naivety was based on the state of the frog before *Bd* exposure during the focal exposure period (third or fourth exposure period depending on what is being displayed). Thus, frogs exposed to *Bd* for the first time during the focal

exposure period were classified as naive because they had not previously been exposed to *Bd*.

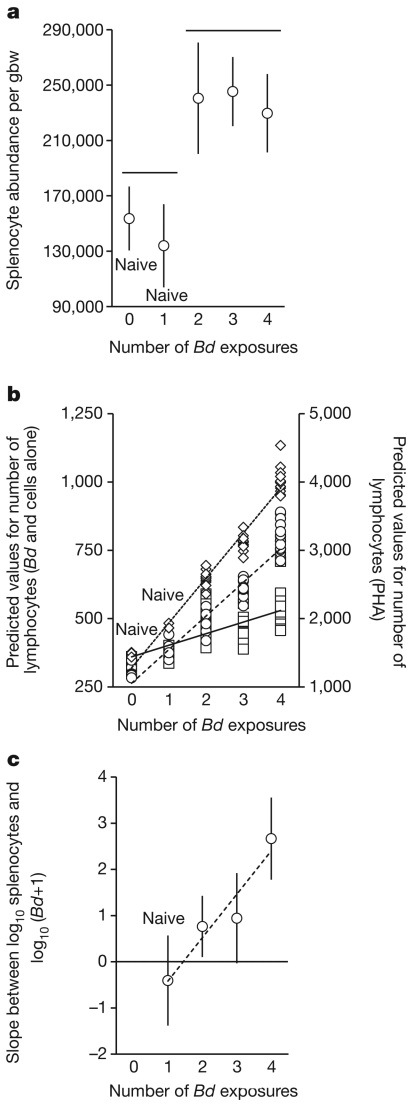

Figure 3. Effects of 0–4 exposures to live *Batrachochytrium dendrobatidis* (*Bd*) on immune parameters of frogs (*Osteopilus septentrionalis*)

a, Mean (± s.e.m.) lymphocyte abundance in the spleen (that is, splenocytes) per gram body weight (gbw). Splenocyte densities did not differ within the naive ($\chi^2_1 = 0.35$, $P = 0.56$) or experienced groups ($\chi^2_1 = 0.14$, $P = 0.93$) but differed significantly between these two groups ($\chi^2_1 = 11.35$, $P < 0.001$; designated by the horizontal lines). Frogs exposed to *Bd* for the first time during the fourth exposure period were classified as naive because they had not previously been exposed to *Bd*. **b**, Lymphocyte proliferation when cultured as splenocytes alone (squares and solid line), or with heat-killed *Bd* zoospores (circles and dashed line) or phytohaemagglutinin (PHA; diamonds and dotted line). Values provided are predicted values of proliferated lymphocytes (measured as counts per minute by a scintillation counter) based on a negative binomial model (n = 19, 17, 17, 10 and 7, respectively for 0–4 previous live *Bd* exposures for both the splenocytes alone (that is, control) and splenocytes plus live *Bd* and n = 15, 13, 11, 2 and 4, respectively, for splenocytes plus PHA). See text for statistics. **c**, Slope (± s.e.) of the relationship between *Bd* abundance and splenocyte

abundance ($F_{1,54}$ = 4.4, P = 0.04), showing greater lymphocyte proliferation in response to *Bd* load with each previous *Bd* exposure.