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Supplementary Methods

Bd *Culture and Inoculation*

We prepared *Bd* inoculum by growing 1 ml of *Bd* stock (strain SRS 812 isolated from *Rana catesbeiana*) on 1% tryptone agar plates for 8d at 23°C. We flooded each plate with 3 ml of deionized water to suspend zoospores and then homogenized the water from each plate to generate *Bd*+ inoculum. *Bd*- inoculum was simultaneously prepared using the same method but no *Bd* was added to the agar plates. We estimated zoospore density in *Bd*+ inocula with a hemocytometer and diluted it with deionized water to the same concentration for all exposures ($3x10^6$ zoospores/ml, respectively, for the *First* and *Second Immunological Resistance Experiments* and $3x10^4$ for the *Behavioral Resistance Experiments*).

Quantitative PCR.

We followed the procedure described by Hyatt *et al.*[1] to quantify *Bd* abundance using qPCR (with a StepOne™ Real-Time PCR System; Applied Biosystems, Foster City, CA). DNA was extracted from swabs with 40 μL of PrepMan Ultra (Applied Biosystems). All samples were diluted 1:100 to reduce PCR inhibition. We added TaqMan® Exogenous Internal Positive Control Reagents (Applied Biosystems) to every reaction well to assess potential inhibition of the PCR reaction[1]. There was no inhibition in any of the reactions.

Confirmation of Bd*-negative frogs prior to experiments*

Wild caught animals were used in each experiment. We verified that the frogs (Table S2) were free of *Bd* prior to the beginning of each experiment by swabbing each frog and

testing for *Bd* with qPCR. Our lab has never detected *Bd* on frogs collected from the Tampa, FL region.

***Statistical analyses conducted for the* Behavioral Resistance Experiment**

The ideal analysis for the Behavioral Resistance Experiment would have been a mixed effects log-linear analysis but we are unaware of any such analyses. Log-linear analyses offer the option of crosstabulation of frequency tables based on a null model of expected frequencies. The underlying assumption of these models, however, is that all the observations are independent, which was not the case because we had repeated binomial observations on each animal. A mixed effects logistic regression model does not explicitly offer the option to provide a null expectation for the observed frequencies, which was critical in this case because we would not have been able to distinguish between innate and learned avoidance without demonstrating a significant change from an expected frequency. As an example, imagine a case where there is innate *Bd* avoidance but no change in the strength of *Bd* avoidance with subsequent exposures. A standard binomial mixed effects model would show no effect of treatment (i.e. number of *Bd* exposures) but would never be able to test for innate avoidance because it would not by default test whether the avoidance was greater than expected by chance within each trial.

Hence, for our behavioral analyses, we add a null expectation that 50% of the observations on each frog would be on the *Bd* side of the container and 50% would be on the opposite side. This same approach is applied when one conducts a single sample T-test based on some null expectation. In this case, we intentionally made sure that there

was always an even number of observations on each frog so the null expectation was straightforward to calculate – half the observations should be on the right and half on the left side of the chamber. To ensure that we did not inflate our sample size, we treated individual as a random effect so that the individual was the replicate and not the repeated samples made on each individual. We had two experiments with similar treatments, so we also treated experiment as a random factor allowing us to test for effects within experiments. Hence, in our spreadsheet each individual frog in each trial had an even number of observations where the frog was either on the same or opposite side of *Bd* (determined after the fact because observations were done blind to *Bd* location), a binomial response. The same number of observations was duplicated and an even number of 0s and 1s were assigned as the expected frequency. A column was added to the spreadsheet to distinguish between observed frequencies and expected frequencies. This allowed us to test whether *Bd*-naïve and/or *Bd*-experienced frogs avoided *Bd* or not and whether the response to *Bd* changed with experience. Going back to our previous example, if there was innate avoidance and no change in this response as a function of number of *Bd* exposures, then there would be a main effect of deviation from the null 50:50 expectation, no main effect of number of *Bd* exposures, and no number-of-*Bd*-exposures-by-deviation-from-null interaction. We detected a significant interaction between number of *Bd* exposures and a deviation from the null 50:50 expectation because naïve frogs showed no attraction or avoidance of *Bd* but experienced frogs significantly avoided *Bd*.

Relevance of dead* Bd *control in skin peptide efficacy analyses.

Before analyses to test for an effect of number of *Bd* exposures on the efficacy of skin peptides, we first tested whether there was any reason to include the dead *Bd* control. We conducted a general mixed effects model with experimental day (0 or 7) nested within frog (i.e., frog identity was treated as a random effect to ensure we had the proper degrees of freedom). Our response variable was the absorbance measurement in each well and we tested for the main and interactive effects of number of *Bd* exposures (treated as a categorical variable) and day of the measurement (0 or 7). There were no main effects or interactions (χ^2_1<0.997, P>0.318), so we did not include the dead *Bd* treatment in our subsequent analyses where we used a mixed effects model (package: nlme, function: lme) 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 on days 7 and 0, respectively] in culture treating frog identity as a random effect.

Bootstrapped-adjusted means for Bd abundance.

Gregory and Woolhouse[2] used a simulation study to demonstrate that sample means of parasite burden systematically underestimate the true population mean parasite burden when sample sizes are low, especially when parasite distribution is highly aggregated, as is the case for the negative binomial distribution observed in our *Bd* abundance data. After Exposure Period 4 in the *First Immunological Resistance Experiment*, our sample sizes in each treatment were low and highly uneven (n= 9, 15, 17, 19 for the 1, 2, 3, 4 *Bd* exposure groups, respectively). To estimate the degree to which our low sample sizes

may have been underestimating the 'true' treatment mean, we employed a bootstrapping procedure identical to the simulation used by Gregory and Woolhouse[2].

Gregory and Woolhouse[2] simulated the effects of undersampling by randomly selecting 1,000 samples of *n* observations of parasite burden from a negative binomial distribution defined by the mean (μ) and the aggregation parameter (κ). We calculated the mean *Bd* abundance from 1,000 bootstrapped samples of size n_x taken from *Bd* abundance pool *x*, where *x* refers to the number of exposures, and n_x is the sample size for Group *x* after Exposure Period 4 (e.g. $n = 9$ for Group 1). We used the median of the 1,000 bootstrapped sample means ($\tilde{a}_{n_x}$, where *a* represents *Bd* abundance) as an estimate of the degree to which a sample of size n_x underestimates the observed mean of the *Bd* abundance pool ($\bar{a}_{pool\,x}$) from which it was drawn. We then generated a proportion underestimation factor, calculated as:

$$\hat{c}_{n_x} = \frac{(\bar{a}_{pool\,x} - \tilde{a}_{n_x})}{\bar{a}_{pool\,x}} \qquad \text{eqn. 1}$$

and then corrected the mean of each treatment for this bias by multiplying the uncorrected mean by $1 + \hat{c}_{n_x}$. We repeated the above bootstrapping procedure 100 times to generate a mean and upper and lower 95% confidence interval limit for $\hat{c}_{n_x}$. These bootstrapped means and confidence intervals are what is shown in Figure 2a.

The same bootstrapping procedure was performed on the immune parameter data from each treatment to assess if any adjustments were necessary. However, in no case was any correction necessary for immune parameters because the immune parameters were not nearly as right skewed as *Bd* load and most immune parameters had multiple replicates per frog and thus the central limit theorem applied.

Collection and quantification of skin peptides

One week after Clearance Period 4 of the *First Immunological Resistance Experiment*, we shipped the frogs overnight to the Rollins-Smith lab at Vanderbilt University (Nashville, TN, USA). Seven days later, we conducted skin peptide and lymphocyte proliferation assays. Frogs were weighed within 0.1 g and were placed in 50 ml of collection buffer (50 mM sodium chloride, 25 mM sodium acetate, pH 7.0; [3] for 15 min after injection with 40 nmol/gbw (gram body weight) of norepinephrine (HCl salt, Sigma-Aldrich, St. Louis, MO) to stimulate the release of skin secretions containing skin peptides. The buffer containing skin peptides was acidified (final volume of 1% HPLC grade trifluoroacetic acid [TFA]; Thermo Scientific, Rockford, IL) to inactivate endogenous peptidases[4]. The acidified collection buffer with peptides was passed over C-18 Sep-Pak cartridges (Waters Corporation, Milford, MA, USA; [4]. Peptides bound to Sep-Paks were eluted with 70% HPLC grade acetonitrile, 29.9% HPLC grade water, 0.1% HPLC grade TFA (v/v/v), and were then dried completely by vacuum centrifugation. The total concentration of skin peptides recovered after Sep-Pak separation was determined by Micro BCA™ (bicinchoninic acid) Assay (Thermo Scientific, Rockford, IL, USA) according to manufacturer's directions except that bradykinin (RPPGFSPFR; Sigma-Aldrich, St. Louis, MO) was used to establish a standard curve[4,5]. Consequently, concentrations of peptide mixtures are expressed as mg equivalents/ml with reference to the bradykinin standard.

Antimicrobial peptide-mediated inhibition of Bd

We adapted the *Bd* growth inhibition assay of Mor and Nicolas[5,6] to determine the efficacy of the skin peptides of each *O. septentrionalis*. *Bd* zoospores were isolated by filtration and diluted to $1x10^6$ zoospores/ml broth (*Bd*+ inoculum). *Bd*+ inoculum (50 μl) was plated in replicates of five in a sterile, 96-well, flat bottom, tissue culture plate (Becton Dickinson, Franklin Lakes, NJ). Antimicrobial peptides diluted in sterile HPLC water were added in 50 μl/well at a final concentration of 25 μg/ml. Positive control wells received 50 μl of HPLC water and 50 μl of *Bd*+ inoculum, and negative control wells received 50 μl of HPLC water and 50 μl of heat-killed (60°C for 10 min) *Bd*+ inoculum. The plate was incubated at 21°C for 7 d, optical density (OD) was measured at 0 and 7d at 490 nm with a Dynex Technologies (Chantilly, VA) plate reader, and growth was measured as increased OD.

Leukocyte isolation and quantification

To quantify leukocyte numbers in the spleen, we euthanized each *O. septentrionalis* in 5 g/ml ethyl 3-aminobenzoate methanesulfonate, and removed and dissociated their spleens. Spleen leukocytes were suspended in 300 μl Leibovitz (L-15) culture medium (Sigma, St. Louis, MO) supplemented with 100 international units (I.U.) penicillin, 100 μg/ml streptomycin, 12.5 mM sodium bicarbonate, 50 μM 2-mercaptoethanol, 2 mM L-glutamine, and 1% heat-inactivated fetal calf serum (Atlanta Biologicals, Lawrenceville, GA). To determine the number of leukocytes in each frog spleen, 10 μL of the cells from each frog were counted on a hemocytometer after staining with 10 μL of Trypan blue dye

to determine viability. Trypan blue stains dead cells and so we counted only non-stained live cells with lymphocyte morphology.

Leukocyte proliferation assay

In addition to quantifying the abundance of leukocytes, we also determined their ability to proliferate in response to *Bd* (n = 19, 17, 17, 10, and 7, respectively for 0-4 *Bd* exposures for the control and *Bd* treatments and n = 15, 13, 11, 2, and 4, respectively for 0-4 *Bd* exposures for the PHA treatment)[7]. Leukocytes from the spleen of each frog (n = 2-8 subsamples/frog/treatment) were cultured in 96-well round-bottom plates at $4x10^4$ leukocytes/well (or $2x10^4$ if there were not enough cells for $4x10^4$ leukocytes/well) in the presence or absence of phytohemagglutinin (PHA, positive control mitogen) at a final concentration of 2 μg/ml or heat-killed zoospores (60°C bath for 10 min.)[8] at a ratio of two zoospores for each leukocyte ($8x10^4$ per well). Leukocyte cultures were incubated at 26°C in 5% CO_2 for 3d. Leukocytes were pulsed with 0.5 μCi ^{3}H-thymidine (PerkinElmer, Waltham, MA) 24 hrs before harvesting (incubation 2d). Proliferation was quantified as counts per min (CPM) of harvested cells on filters using a scintillation counter (Wallac 1205 Betaplate Beta Liquid Scintillation Counter, Perkin-Elmer, Waltham, MA).

***Verification that liquid nitrogen kills* Bd**

To verify that exposing a culture of *Bd* to liquid nitrogen successfully killed all *Bd* cells, we first prepared a *Bd* inoculum as described above. Half of this *Bd*+ inoculum was sealed in a beaker and submerged in liquid nitrogen for 15 min, providing a live and

"dead" *Bd* inoculum. We then plated 1 mL of each inoculum on 1% tryptone agar plates (n = 3 for live and dead inocula), incubated these six plates at 23°C for 20d, and regularly checked the plates for live zoospores. To verify that *Bd* was successfully killed for each exposure in the *Second Immunological Resistance Experiment*, the process above was repeated for each exposure period. For this part of the experiment the plates were only monitored for *Bd* growth for 8d.

Dead* Bd *persistence

Frogs infected with live *Bd* are exposed to *Bd* continuously. To make the live and dead *Bd* exposures comparable, we desired to have the exposure to dead *Bd* also be continuous for the 11d *Bd* growth periods. Hence, we had to determine how long dead *Bd* persisted in the containers in which frogs were housed to determine how often we had to reapply the dead *Bd* to ensure a continuous 11d exposure. To meet this objective, we inoculated six individually-housed *O. septentrionalis* with 1 mL of dead *Bd* each. We then swabbed each frog (10 swabs along the ventral and dorsal length of the frog) and its substrate daily (10 swabs across the paper towel substrate) to determine when the DNA of the *Bd* was no longer detectable using qPCR of the swabs.

Supplementary Results

Verification that liquid nitrogen kills Bd

Each of the three plates inoculated with live *Bd* had viable zoospores until the cultures crashed around 15d (this is typical of sealed plate cultures with strong *Bd* growth). However, no live zoospores were ever observed on the plates inoculated with the flash frozen *Bd*, verifying that the freezing successfully killed the *Bd* before each inoculation on the frogs.

Dead Bd *persistence*

Bd DNA was detectable on the frogs' skin for up to 1d after exposure to dead *Bd*, whereas *Bd* DNA was detectable on the substrate for up to 2d after exposure to dead *Bd*. Hence, we re-exposed the frogs to dead *Bd* every 2d to ensure a continuous exposure to dead *Bd*, matching the continuous exposure to live *Bd*.

Mortality and clearance verification

There was no mortality in the *Behavioral Resistance Experiments*. In the *First* and *Second Immunological Resistance Experiments,* mortality was kept low (11 frogs died in each experiment). Moreover, in both of these experiments, there was no significant association between the number of previous *Bd* infections and frog mortality ($\chi^2_1 = 2.9$, $P = 0.08$, Fig. S3) or body mass change ($\chi^2_1 = 0.12$, $P = 0.71$). Additionally, in both the *First* and *Second Immunological Resistance Experiment*s, all heat-clearances were 100% effective (Table S2).

Effects of number of previous Bd *exposure on peptide efficacy*

The number of previous exposures to *Bd* did not alter the effect of skin peptides on *Bd* growth ($N = 47$, $P = 0.77$). Two treatments exhibited Δr values that significantly differed from 0: Two exposures ($N = 10$, $P < 0.0001$) and three previous exposures ($N = 11$, $P < 0.001$). In both cases the Δr values were positive, indicating a stimulatory effect of skin peptides on *Bd* growth.

Bd *abundance versus* Bd *intensity*

In the main manuscript, we reported that the number of *Bd* exposures was a significant negative predictor of *Bd* abundance on frogs after Exposure Period 3 and 4 for the *First Immunological Resistance Experiment* and after Exposure Period 4 for the *Second Immunological Resistance Experiment*. We also tested whether the number of *Bd* exposures was a significant negative predictor of *Bd* intensity on frogs (i.e., excluding any frogs that had a *Bd* abundance of zero for any exposure period). The results qualitatively matched analyses on *Bd* abundance. The number of previous *Bd* infections was a significant negative predictor of *Bd* intensity on the frogs in both the *First* and *Second Immunological Resistance Experiments* (*First Experiment*: $x^2_1 = 4.0$, $P = 0.04$; *Second Experiment*: live exposures: $x^2_1 = 9.1$, $P = 0.002$; dead exposures: $x^2_1 = 4.9$, $P = 0.02$). There was 100% prevalence for the *Behavioral Resistance Experiment* and so no statistics on intensity were conducted. Because we found the same effect of number of exposures on *Bd* abundance and *Bd* intensity, we only report the results for abundance in the main manuscript.

Supplementary Discussion

Demonstrating a mechanism of acquired immunological resistance

We isolated lymphocytes from the spleen, the only central lymphoid organ that frogs possess. Thus, increased numbers of lymphocytes in the spleen would indicate amplification of the lymphocyte response, which increased with multiple exposures. This is consistent with the Ramsey et al.[9] in which it was shown that irradiation reduced lymphocyte numbers in frog spleens correlated with increased burden of *Bd* on the skin. Although there is always more that could be done to prove a mechanism of protection, we believe we provide strong evidence for an adaptive immune response following repeat exposure and clearance.

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