Supporting Information

Effects of chytrid and carbaryl exposure on survival, growth and skin peptide defenses in foothill yellow-legged frogs

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Seven pages, including two tables and one figure

Additional details of methods

Animal care

Animals were randomly placed in 31.5 cm long by 17 cm wide by 9.6 cm high plastic box placed on a slope so that approximately 300 ml of artificial pond water (25% Holtfreter's solution) formed a pool. Post-metamorphic *R. boylii* were maintained at 20–22°C and fed early-instar crickets daily.

Determination of lethal levels of Carbaryl.

We conducted up-down tests to determine the 24-hour minimum lethal level of carbaryl exposure. In each test, a single animal was placed in a one liter glass jar with 70 ml of artificial pond water and a specific concentration of carbaryl. Individuals were observed every hour for twelve hours, and then were observed again twelve hours later. After 24 hours, each animal was rinsed in a bath of water, returned to its cage, and observed for 24 hours. If an individual died during this process, we diluted the concentration 10-fold and repeated the test with a new individual until a level was found that did not kill the animal. Once a non-lethal pesticide concentration was found, we conducted a new assay with a concentration that was five times the non-lethal concentration (that is, midway between the lowest lethal concentration and its non-lethal, ten-fold dilution). We conducted a final test with a level midway between the lowest lethal level and the highest non-lethal level. The estimated minimum lethal concentration was then calculated as the midpoint between the lowest concentration that killed an animal, and the highest concentration in which an animal survived (a two-fold difference). As an additional check, eight new frogs were then exposed to 10% of the estimated minimum lethal concentration

using the same testing protocol to ensure that all survived. Our initial carbaryl level was 7.7 mg/l which killed the test animal. We reduced the level ten-fold to 0.77 mg/l and the next animal survived. We then increased the level five-fold to 3.8 mg/l which again resulted in survival of the test animal. We then tested the midpoint level of 5.8 mg/l which killed the test animal. The minimum lethal concentration was then calculated as 4.8 mg/l (the midpoint between the lowest lethal level (5.8 mg/l) and the highest non-lethal level (3.8 mg/l)).

Peptide collection

Groups of 4-5 animals were weighed and placed in 50 ml of collection buffer (50 mM sodium chloride, 25 mM sodium acetate pH 7. 0) containing 200 μM norepinephrine. The animals remained in the collection buffer for 15 minutes. After removal of the animals, the buffer was acidified by adding 0.5 ml of trifluoroacetic acid (TFA), frozen, and shipped to the Rollins-Smith laboratory for further processing. Each sample was passed over two C-18 Sep-Pak cartridges connected in tandem, and peptides were eluted by addition of 70% acetonitrile, 29.9% water, 0.1% trifluoroacetic acid (TFA) (v/v/v). The eluted peptides were spun under vacuum to concentrate them to dryness.

Chytrid growth inhibition assays

We measured the effects of peptides on chytrid growth by plating 5×10^4 mature cells or 5×10^5 zoospores in a volume of 50 μl of H broth (10 g tryptone and 3.2 g glucose per 1 liter of glass distilled water) in replicates of five (occasionally 3 or 4 or 6) in a 96-well flat bottom microtiter plate (Costar 3596, Corning Inc., Corning NY, USA) with or without the addition of 50 μl serial dilutions of peptides in broth. The plates were covered, wrapped in plastic wrap to limit

moisture loss, and incubated on a laboratory bench at 23°C. To determine maximal growth (positive control for growth), some wells received 50 μl of broth without peptide. To determine the value for maximal inhibition (negative control for growth) some wells (on a separate plate) received 50 μl of broth containing 0.4% paraformaldehyde (PF). Growth of mature chytrid cells at 1-4 days was measured as increased optical density at 492 nm (OD_{492}) with an ELISA plate reader. For growth inhibition assays using zoospores, the incubation period was extended up to seven days.

Additional tables and figures

Table S1. ANCOVA for the effects of carbaryl and chytrid on the growth of *R. boylii* metamorphs. The dependent variable is log final mass, with log initial mass as a covariate.

Source	Mean Squared Error	df	F	*P*-value
Intercept	3.05	1	153.9	< 0.001
Log initial mass (covariate)	0.86	1	53.5	< 0.001
Chytrid	0.36	1	18.3	< 0.001
Carbaryl	0.01	1	0.5	0.51
Chytrid X Carbaryl	< 0.01	1	0.1	0.81
Error	0.02	19		
Total		24		

R Squared = .755 (Adjusted R Squared = .703)

Table S2. Growth inhibition of *B. dendrobatidis* zoospores or mature cells by skin peptides collected from postmetamorphic *R. boylii* at days 3, 7, and 18.

Treatment	Day of Sampling	MIC[a] against Zoospores (µg/ml)	MIC against Mature Chytrids (µg/ml)
Chytrid, No carbaryl	3	25	62.5
	7	50, >200	250, 500
	18	200	200, 500
No chytrid, No carbaryl	3	ND	50, 50
	7	ND	100, 100
	18	ND	500, 500
Chytrid, Carbaryl	3	<6.25, 12.5	ND
	7	12.5, 25	25, 500
	18	12.5, 500	ND
No chytrid, Carbaryl	3	25,25	ND
	7	50,50	ND
	18	25,200	ND

[a]Minimal inhibitory concentration (MIC) is the lowest concentration of peptides at which growth was not significantly greater than negative controls. ND = not determined.

Figure S1.

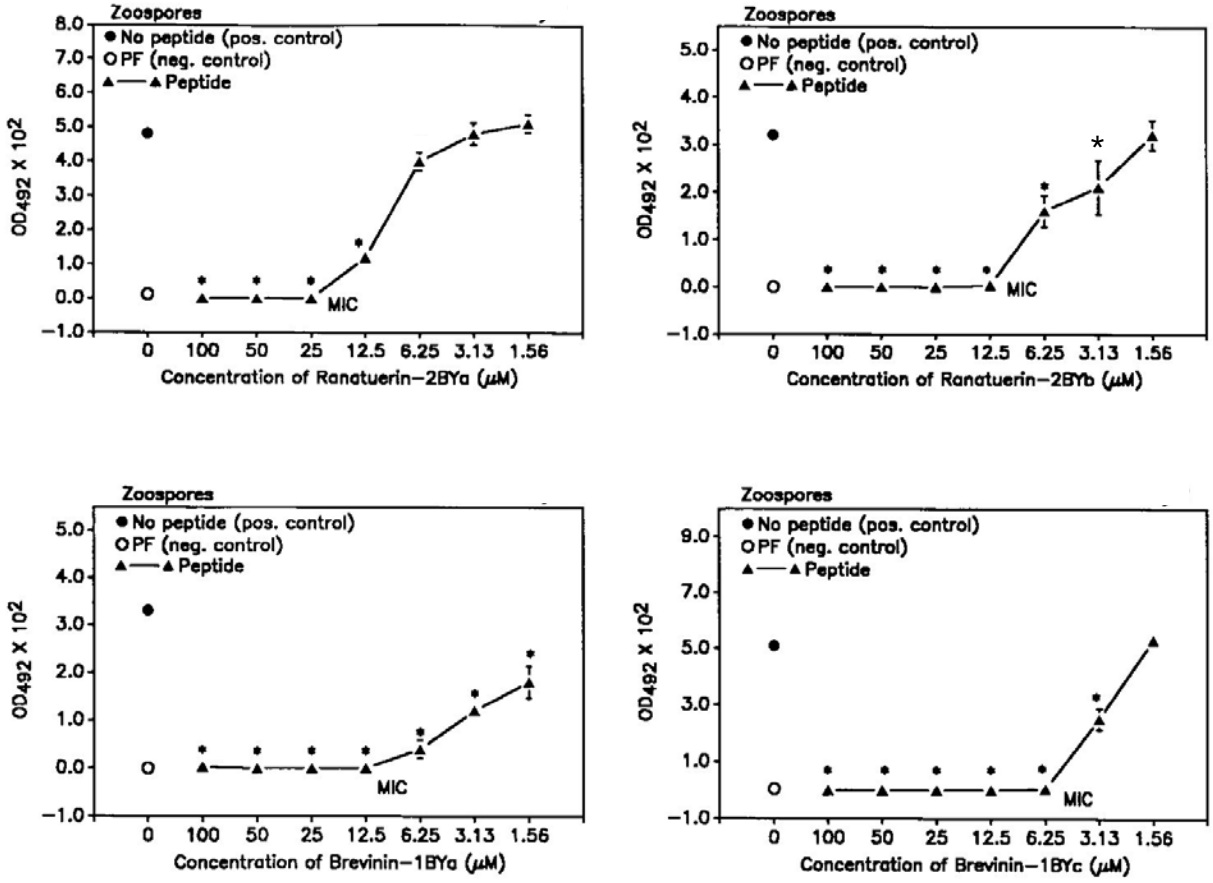

Figure S1. Growth inhibition of *B. dendrobatidis* zoospores at 7 days of culture by purified ranatuerin-2BYa, Ranatuerin-2BYb, brevinin-1BYa, and brevinin-1BYc. The y-axis indicates chytrid culture growth as measured by increased optical density at 492 nm. Each data point represents the mean ± SE of three to six replicate wells. If no error bar is shown, the SE was less than the diameter of the symbol. Pos. control wells show growth of cells in the absence of added peptides. Neg. control wells show lack of growth due to addition of 0.4% paraformaldehyde (PF). *Significantly less growth than positive controls (Dunnett's *t*-test, $p \leq 0.05$). MIC is the lowest concentration at which growth was not significantly greater than negative controls.