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Effects of Chytrid and Carbaryl Exposure on Survival, Growth and Skin Peptide Defenses in Foothill Yellow-legged Frogs

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Environmental contaminants and disease may synergistically contribute to amphibian population declines. Sub-lethal levels of contaminants can suppress amphibian immune defenses and, thereby, may facilitate disease outbreaks. We conducted laboratory experiments on newly metamorphosed foothill yellow-legged frogs (*Rana boylii*) to determine whether sublethal exposure to the pesticide carbaryl would increase susceptibility to the pathogenic chytrid fungus *Batrachochytrium dendrobatidis* that is widely associated with amphibian declines. We examined the effect of carbaryl alone, chytrid alone, and interactions of the two on individual survival, growth, and antimicrobial skin defenses. We found no effect of chytrid, carbaryl, or their interaction on survival. However, chytrid infection reduced growth by approximately one-half. This is the first report of suppressed growth in post-metamorphic amphibians due to infection with chytrid. *Rana boylii* skin peptides strongly inhibited chytrid growth in vitro, which may explain why chytrid exposure did not result in significant mortality. Skin peptide defenses were significantly reduced after exposure to carbaryl suggesting that pesticides may inhibit this innate immune defense and increase susceptibility to disease.

Introduction

Both pesticide exposure and disease have emerged as possible explanations for worldwide amphibian declines. Particularly in western North America, pesticide drift has been suggested to be a primary cause of amphibian declines for nearly a decade (*1*−*3*), yet there has been surprisingly little ecotoxicological research on amphibian population declines. Disease is strongly implicated in declines worldwide, with the discovery of a previously unknown chytrid fungus (*Batrachochytrium dendrobatidis*, hereafter referred to as "chytrid") associated with field mortality in Australia, North, South, and Central America, and Europe. As the research and regulatory communities recognize that pesticides, disease, and other potential stressors may act synergistically to bring about population declines, experimental work on multiple stressors and their interactions has emerged as an important direction for amphibian decline studies.

In California, declines of four frog species (*Rana boylii*, *R. cascadae, R. draytonii*, and *R. muscosa*) are strongly associated with the amount of upwind pesticide use (*4*). Furthermore, for these four species and the Yosemite toad (*Bufo canorus*), upwind use of cholinesterase-inhibiting pesticides is more strongly associated with declines than other types of pesticides (*4*). However, documented field levels of pesticides are generally several orders of magnitude below lethal concentrations as determined in laboratory studies (*4*). Thus, if pesticides are contributing to declines, it is most likely not due to direct lethal effects, but rather through sublethal effects and interactions with other stressors (*5*). Recent studies by several research groups suggest that pesticides can inhibit immune defenses and increase disease susceptibility (6−10, reviewed in ref 11), suggesting that a pesticide−disease interaction may be an important factor in amphibian declines.

A disease that may interact with pesticides to promote declines in amphibian populations is chytridiomycosis (the disease caused by *B. dendrobatidis*). Amphibians with chytridiomycosis have been identified on six continents, and include at least 93 species (www.jcu.edu.au/school/phtm/PHTM/frogs/chyglob.htm). In California, chytrid has recently been found on a number of amphibian species, including *Rana boylii*. Two non-mutually exclusive explanations may account for the sudden increase in chytrid frequency. *Batrachochytrium dendrobatidis* may be a novel pathogen attacking a defenseless host (*12*). Alternatively, some aspect of the environment may have changed, increasing the susceptibility of amphibians to the disease (*13*). The possibility that the susceptibility of amphibian populations to the chytrid fungus has increased as a result of pesticide-induced immune suppression is consistent with field observations and the second explanation, but has yet to be studied empirically.

Our research was designed to examine the effects of pesticide and chytrid interactions on frogs. We conducted laboratory experiments to investigate whether low, sub-lethal doses of carbaryl, a common, current-use pesticide, affected foothill yellow-legged frog (*Rana boylii*) susceptibility to chytrid fungus. We exposed juvenile *R. boylii* to a single sublethal dose of carbaryl, followed by exposure to chytrid fungus. The animals were followed for 2 months to evaluate survival and growth. We also examined the effect of pesticide exposure on the production or release of antimicrobial skin peptides in our experimental *R. boylii* because skin peptides may be a key element of amphibian immune defenses against chytrid fungus (*14*−*17*). Finally, we examined the ability of *R. boylii* skin peptides to inhibit growth of chytrid cultures in vitro to assess their potential role in providing protection from chytridiomycosis for this declining amphibian species.

Materials and Methods

Choice of Species. We worked with foothill yellow-legged frogs because declines of this species are strongly associated

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with upwind pesticide use (*4*). Additionally, as a member of the *boylii* group of ranid frogs, *R. boylii* shares phylogenetic affinities with other declining ranid frogs in California, and our results should be relevant to declines of these species. We collected young *R. boylii* metamorphs from the Garcia River (Mendocino County, CA), October 6, 2002. See Supporting Information for details of animal care.

Chemicals and Determination of Pesticide Lethal Levels. We choose the pesticide carbaryl (1-naphthyl N-methyl carbamate) for our experiments because it is widely used in California and the United States. It is also a strong cholinesterase inhibitor and thus may be representative of cholinesterase-inhibiting pesticides (most organophosphate and carbamate pesticides). We used commercially available carbaryl (Monterey brand "7" manufactured for Lawn and Garden Products, Fresno CA). All other chemicals were purchased from Sigma Chemical (St. Louis, MO) unless otherwise indicated. We conducted "up−down" lethality tests to estimate the 24-hour minimum lethal concentration of carbaryl for *R. boylii* metamorphs (See Supporting Information for details).

Culture of Chytrid and Exposure of Frogs to the Pathogen. We used chytrid strain "LJR119" isolated from *R. muscosa* from the northern Sierra Nevada by Lara Rachowicz at the University of California, Berkeley. The chytrid was raised on 1% tryptone agar plates. Zoospores were washed off the plates by flushing with deionized water, counted using a hemocytometer counting chamber, and diluted to a concentration of 1.9×10^5 zoospores per mL. Animals were placed in one-liter beakers for 24 h and exposed to 9.4×10^6 zoospores per frog in a volume of 50 mL of water. Control animals were exposed to a comparable volume of water that was flushed from sterile agar plates.

Experimental Design. We tested for an effect of chytrid, pesticides, and their interaction on survival, individual growth, and quantity of skin peptide secretions. We used a two-way, full factorial design, with chytrid or no chytrid crossed with no carbaryl or a carbaryl dose of 0.48 mg/L. Animals were dosed with a pesticide solution made with one liter artificial pond water (25% Holtfreter's solution) and 1.06 mL "7" pesticide (41.2% Carbaryl active ingredient, 1.1 specific gravity), and then diluted 1000-fold. The resulting carbaryl dose (0.48 mg/L) was 10% of the estimated minimum lethal concentration (4.8 mg/l) from the "up−down" tests and thus represented a sublethal level. This level is lower than that which frogs might receive from direct agricultural runoff (*18*, *19*) but higher than likely exposure levels in remote montane locations. Beginning on day 0, frogs were exposed to pesticides for 24 h in one-liter jars containing 70 mL of the pesticide/artificial pond water solution. At the same time, the no-carbaryl control treatment animals were placed in identical jars with 70 mL of artificial pond water for 24 h. At the end of the exposure period all animals were returned to individual containers with clean water. Twenty-four h chytrid treatments began on day 3. Each animal was housed and treated separately, and therefore, each constituted an independent replicate. The experiment was run for 70 days. Animals were monitored daily, and any dead animals were preserved in 10% buffered formalin. At the end of the experiment, all surviving animals were weighed, then euthanized and preserved. An aborted attempt to collect blood samples on day 19 caused the unexpected death of five animals (four in the no pesticide, no chytrid treatment). These animals were excluded from all statistical analysis.

We tested for treatment effects of chytrid and carbaryl on survival with a chi square test on proportion surviving per treatment. First, to maximize power, we tested for an effect of each factor separately (i.e., carbaryl or chytrid exposure), by pooling treatments to produce a one-factor test. Then, both factors were tested together. Growth rates were analyzed using ANCOVA with log transformed final weight as the dependent variable and log transformed initial weight as a covariate. Unless otherwise noted, a p-value of ≤ 0.05 was considered statistically significant for all statistical analyses.

Evaluation of Carbaryl Exposure. To allow comparison between our carbaryl exposures and any future sampling of carbaryl in tissue of animals taken from the field, we exposed four additional animals that were not part of our main experiment to the pesticide treatment. Following the exposure, they were euthanized, frozen at −20 °C, and tested for carbaryl levels in body tissue. Testing was done by the Geochemical and Environmental Research Group (Texas A&M University, College Park, TX). Sample extracts of body tissue were purified by gel permeation chromatography and then analyzed using a gas chromatograph/mass spectrometer under selective ion monitoring.

Evaluation of Chytrid Infection. Chytrid infections were determined by histological examination of formalin-fixed frogs at the conclusion of the experiment. All frogs from each of the two chytrid treatments (total = 14 frogs), and two randomly selected frogs from each of the two non-chytrid treatments were tested for presence of chytrid infection. The sampling of the frogs in the chytrid treatments was intended to confirm chytrid infections. The sampling in the non-chytrid treatments was to check for accidental chytrid exposure and infection. Skin scraped from the superficial ventral integument was evaluated for chytrids using a standard wet mount preparation. As a further check, tissue from animals that were identified as chytrid-positive by skin scraping were embedded in paraffin, sectioned at five micrometer thickness, stained with hematoxylin and eosin, and examined for chytrid.

Evaluation of Skin Peptide Concentrations. To determine whether treatments would affect the quantity and composition of recoverable skin peptides, frogs were induced to secrete peptides at day 3 (after the pesticide treatment had been applied, but immediately preceding the application of the chytrid treatment), and again at days 7, 18, 33, and 49. Skin peptides were induced by immersion in 200 μM norepinephrine hydrochloride, following Woodhams et al. (*16*) (See Supporting Information for details). The total concentration of skin peptides recovered was determined by Micro BCA Assay (Pierce, Rockford, IL) following manufacturer's instructions, except that bradykinin (RPPGFSPFR) (Sigma Chemical, St. Louis, MO) was used to establish a standard curve. It was necessary to pool animals into groups of 4 or 5 to obtain a sufficient quantity of peptide for accurate measurement. After pooling, we had two peptide samples for each of the four treatments. Death of some animals prior to the day 33 sampling resulted in only one peptide sample in the no-carbaryl, no-chytrid treatment on day 33 and day 49. For day 3 peptide samples (in which there were only carbaryl and no carbaryl treatments), we tested differences in total recovered peptides using a t-test. For all subsequent days, we tested whether the pesticide and chytrid treatments affected total recovered peptides, and whether these effects changed over time using a repeated measures design. The pesticide treatment and chytrid treatment were considered fixed effects, and time (i.e., days 7, 18, 33, and 49) was treated as a continuous variable. In our repeated measures analysis, we were missing two data points, and thus used Satterthwaite's approximation to calculate the denominator degrees of freedom (*20*). The total recovered peptides for each group of animals was log transformed to meet normality assumptions. Our analyses were conducted with SAS statistical software (*21*).

Evaluation of Antimicrobial Peptide Defenses against Chytrid. Six new peptides with antimicrobial activity (brevinin-1BYa, brevinin-1BYb, brevinin-1BYc, ranatuerin-2BYa, ranatuerin-1BYb, and temporin-1BYa) were previously iso-

TABLE 1. Mortality and Growth of Juvenile *R. Boylii* Exposed to Chytrid and Carbaryl[a]

treatment	mortality dead/total (%)	growth mean (SD)
no carbaryl, no chytrid	1/5 (20.0)	0.73 (0.4)
carbaryl, no chytrid	2/10 (20.0)	0.80 (0.17)
no carbaryl, chytrid	1/7 (14.3)	0.32 (0.15)
carbaryl, chytrid	1/7 (14.3)	0.30 (0.23)
pooled treatments		
no chytrid	3/15 (20.0)	0.78 (0.25)
chytrid	2/14 (14.3)	0.31 (0.19)

[a] Growth is final weight (g) of surviving animals on day 70, minus initial weight.

lated from the skin secretions of a mature adult male *R. boylii* (*22*). We tested four of these peptides in purified form for growth inhibition of chytrid zoospores. In addition to the purified peptides, we tested partially purified peptide mixtures collected from the skin secretions of our experimental animals for growth inhibition of mature chytrids and chytrid zoospores. Growth inhibition assays were conducted as previously described (*14*, *23*, *24*) (See Supporting Information for details). Chytrid growth with peptides was compared to positive controls (no peptides) and negative controls (0.4% paraformaldehyde). We were specifically interested in testing if chytrid growth with each of the different concentrations of skin peptides was significantly different from the chytrid growth observed in the positive control. To accomplish this, we conducted an ANOVA on chytrid culture growth, where each concentration of skin peptides was a treatment (replicated 3−6 times). We tested whether the chytrid growth observed in these different treatments was significantly lower than the chytrid growth observed in the positive control with Dunnett's *t*-test, which controls the error rate for multiple comparisons between a single control treatment and additional treatments. The minimal inhibitory concentration (MIC) was determined as the lowest concentration at which chytrid growth was not significantly greater than that observed for negative controls.

Results

Carbaryl Body Levels and Chytrid Infection. Of the four frogs tested for carbaryl in body tissue, one had 0.9 μg/gbw carbaryl (μg carbaryl/g frog body weight), one had 0.6 μg/gbw carbaryl, and two had levels below the 0.1 μg/gbw detection limits. Of the 18 frogs tested for chytrid infection, only one was found to have detectable chytrid infection. The animal was in the carbaryl, chytrid treatment, and had died 3 days after the initiation of chytrid exposure. Histological examination showed heavy chytrid infection. The lack of chytrid-positive skin scrapes from all animals surviving more than 3 days after chytrid exposure may be due to infected animals clearing their infections in the course of the experiment (see below).

Survival. There was low mortality (14−20% mortality) across all treatments (Table 1), with no significant effect of treatment on survival (Pearson Chi-square, $p = 0.98$). There was no significant effect on survival of either chytrid or pesticide treatments tested separately (pooled chytrid treatments, Chi-square, $p = 0.68$; pooled carbaryl treatments, Chi-square, $p = 0.95$). Mortality was actually somewhat lower in the chytrid treatments (Table 1).

Growth. The overall ANCOVA testing for treatment effects on final mass was significant (Supporting Information, Table S1). There was no pesticide treatment effect on final mass (ANCOVA, df = 1, $F = 0.5$, $p = 0.51$). However, chytrid treatment had a significant negative effect on final mass (ANCOVA, df = 1, $F = 18.3$, $p < 0.001$). *Rana boylii* exposed to chytrid grew only 40% as much as individuals not exposed

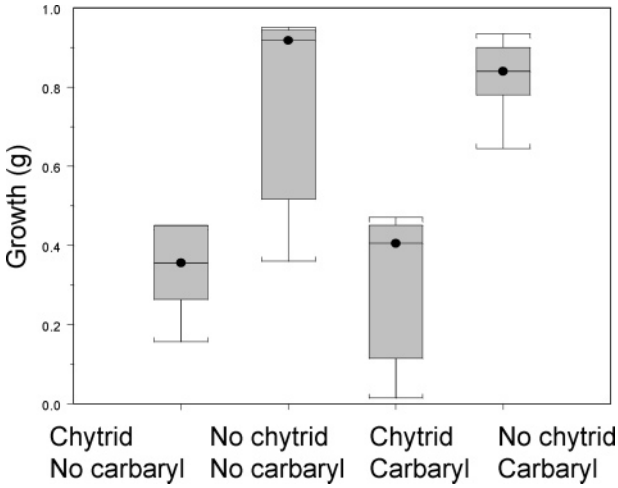

FIGURE 1. Growth of individual *R. boylii* metamorphs as a function of pesticide and chytrid exposure. Growth was calculated as (final weight minus initial weight).

to chytrid (Table 1, Figure 1). Initial mass was positively related to final mass, and there were no significant interaction between chytrid and carbaryl exposure on growth.

Antimicrobial Peptide Defenses against Chytrid. Skin peptides collected from control (no carbaryl, no chytrid) animals inhibited growth of zoospores (Figure 2a) and mature chytrids (Figure 2b) at concentrations of 6.25 μg/mL and above (Figure 2; Zoospores: ANOVA, df = 8, 35, 12.6, $F = 171$, $p < 0.0001$; Mature Chytrids: ANOVA, df = 8, 36, 12.6, $F = 95.28$, $p < 0.0001$). The MIC against mature chytrid cells was 50 μg/mL (Figure 2b) and against zoospores was 25 μg/mL (Figure 2a). These observations suggest that *R. boylii* secretes potent antifungal peptides. All four of the purified peptides previously isolated from *R. boylii* significantly inhibited growth of zoospores at concentrations above 6.25 μM (Supporting Information, Figure S1). The MICs were 6 μM for brevinin-1BYc; 12.5 μM for brevinin-1BYa and ranatuerin-2BYb; and 25 μM for ranatuerin-2BYa (Figure S1). Thus, all four peptides were potent inhibitors of growth of chytrid, as was the cocktail of total skin peptides from wild-caught frogs.

Effects of Carbaryl and Chytrid on Skin Peptides. The concentration of total skin peptides was estimated by Micro BCA Assay using bradykinin as a standard. Because the mixture of skin peptides is diverse and contains peptides with variable numbers of the amino acids cysteine, cystine, tryptophan, and tyrosine, the Micro BCA Assay is not a precise measure. However, it is the most sensitive measure of total peptides recovered under different treatment protocols. Using this assay, total peptide concentrations recovered at day 3 in frogs not exposed to carbaryl was more than 5 times greater than in frogs exposed to carbaryl (*t*-test on peptide concentrations at day 3: df = 6, $t = 2.797$, $p < 0.031$) (Figure 3). The repeated measures analysis of peptide concentration over time (days 7, 18, 33, and 49) found no effect of carbaryl treatments on skin peptides of frogs through the remainder of the experiment (ANOVA, df = 1, 21.3, $F = 2.56$, $p < 0.12$). There was no significant effect of chytrid treatment (ANOVA, df = 1, 21.3, $F = 0.92$, $p < 0.35$) on peptide concentrations, but the peptide concentrations steadily increased through the duration of the experiment (ANOVA, df = 1, 20, $F = 35.38$, $p < 0.001$) (Figure 3). None of the interactions were significant (day *X* pesticide: ANOVA, df = 1, 20, $F = 1.55$, $p < 0.23$; day *X* chytrid ANOVA, df = 1, 20, $F = 0.77$, $p < 0.39$; pesticide *X* chytrid *X* day: ANOVA, df = 1, 20, $F = 0.17$, $p < 0.68$).

The ability of skin peptides from experimental and control groups of frogs to inhibit chytrid growth in vitro was assessed

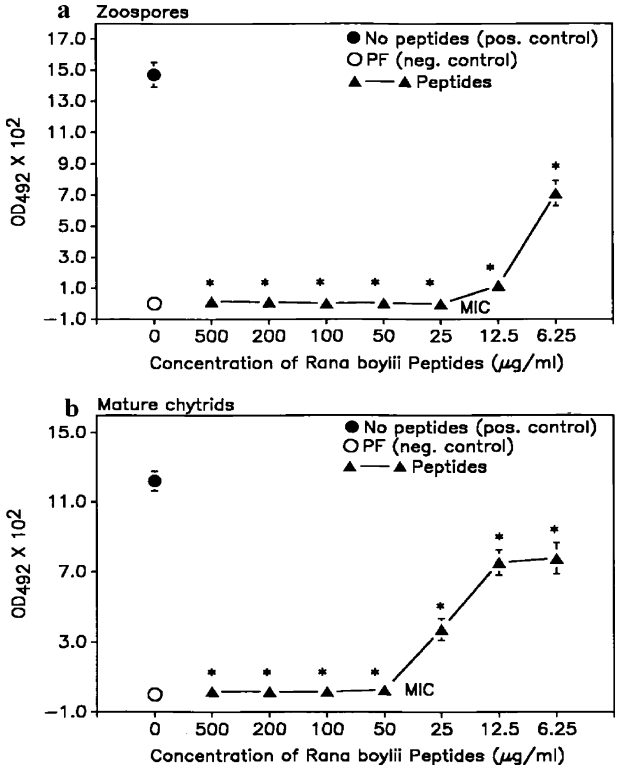

FIGURE 2. Growth inhibition of chytrid by *R. boylii* skin peptides. The *y*-axis indicates chytrid culture growth as measured by increased optical density at 492 nm. Top panel: zoospores after 7 days of culture. Bottom panel: Mature chytrid cells after 4 days of culture. In both cases, incubation occurred in the presence of skin secretions from experimental animals that had not been exposed to chytrid or carbaryl. Each data point represents the mean ± standard error (SE) of three to six replicate wells. If no error bar is shown, the SE was less than the diameter of the symbol. Positive control wells show growth of cells in the absence of added peptides. Negative control wells show lack of growth due to addition of 0.4% paraformaldehyde (PF). Asterisk means 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 in negative controls.

for samples obtained at days 3, 7, and 18. Although carbaryl exposure affected the amount of recoverable skin peptides on day 3, it did not appear to affect the ability of peptides to inhibit chytrid growth in vitro. Regardless of whether the frogs had been treated with carbaryl or not, all of the peptide preparations inhibited in vitro chytrid growth. The MICs for day 3 samples were 12.5−25 μg/mL for all groups tested against chytrid zoospores and 50−60 μg/mL against mature chytrids (Table S2). For samples collected at day 7, MICs were about 12.5−50 μg/mL against zoospores and 25−500 μg/mL against mature chytrids. Samples collected at day 18 were slightly less potent than those collected at the earlier time points. The MICs against zoospores ranged from 12.5−500 μg/mL (Table S2).

Discussion

Our experimental results indicate that wild-caught, postmetamorphic juveniles of *R. boylii* may be well-protected against chytridiomycosis-induced mortality. Exposure to chytrid resulted in dramatically reduced growth (Figure 1) but had no effect on mortality. Previous studies found that tadpoles of Gray treefrogs (*Hyla chrysoscelis*) and Fowler's toads (*Bufo fowleri*) exposed to chytrids had reduced growth and smaller weight at metamorphosis (*25*). This, however, is the first report of suppressed development in postmetamorphic frogs due to infection with chytrids.

Rana boylii's ability to fend off the lethal consequences of chytrid infection may be due to a strong complement of antimicrobial skin peptides. We found that *R. boylii* produces at least four peptides that are individually capable of suppressing chytrid growth in vitro. Exposure to carbaryl decreased recoverable peptide levels for at least 3 days. Decreased amounts of antimicrobial peptides could potentially increase a frog's susceptibility to chytrid. Thus we found a plausible mechanism whereby sublethal pesticide exposure suppresses immune defenses, and therefore, it may interact with disease to cause amphibian population declines. However, in our experiments we found no interaction between chytrid and pesticide exposure on mortality or growth suggesting that under these laboratory conditions, reduced skin peptide levels did not increase the effects of chytrid infections on *R. boylii*. These results raise a number of questions.

Why Did Exposure of *R. boylii* to Chytrid Fail to Induce Mortality? There have been a limited number of published studies describing experimental infections of amphibians with chytrid, and they demonstrate a wide range of susceptibilities among anuran species and life history stages (*26*−*28*). In many cases, exposure to 10^3 chytrid zoospores per frog was sufficient to cause high mortality. In the only other work on *R. boylii*, Elizabeth Davidson et al. (*29*) found that exposure of postmetamorphic juveniles to a dose of 8.5×10^3 or 8.5×10^2 zoospores per mL of water did not result in significantly greater mortality than in controls. Furthermore, animals that were initially infected with chytrid based on examination of sloughed skin had no identifiable infection determined by histology at later times, suggesting that *R. boylii* may be able to eliminate infections.

Based on experimental work with other anuran species, the zoospore exposure used in the current study (9.4×10^6 zoospores per frog) should have been sufficient to induce disease and death if *R. boylii* juveniles were highly susceptible to chytrid-induced mortality. Our finding of heavy chytrid infection only in the single animal that died early, along with dramatically reduced growth rates for chytrid-exposed animals suggests that all chytrid-exposed animals were infected, but that most individuals were able to control the infection, resulting in chytrid-negative skin scrapes two months after exposure. These observations suggest that *R. boylii* has effective defenses that prevent death (but not reduced growth) from chytridiomycosis, at least under our laboratory conditions.

Rana boylii appears to have robust antimicrobial peptide defenses that may protect postmetamorphic juveniles from infection. Six antimicrobial peptides have previously been isolated from this species (*22*), and the four individual peptides that we tested in this study against zoospores of chytrid were very effective inhibitors of growth (MICs of 6−25 μM, Figure S1). Overall, the skin peptides that we collected from our study animals were also quite effective in growth inhibition of chytrid (Figure 2 and Table S2).

To evaluate whether frogs actually produce a sufficient quantity of skin peptides to deter chytrids, we compared peptide concentrations found to be effective in our chytrid growth assays to the estimated total skin peptides produced per cm^2 of surface area for an individual frog. Each untreated control frog produced about 230 μg/gbw of peptides as determined by Micro BCA assay. A frog weighing about 1.6 g (an average weight for animals early in the study) has an estimated 12.9 cm^2 of surface area (*30*). Thus, they produced about 28.6 μg/cm^2. If the mucous layer is 500 μm thick, then the fluid over 1 cm^2 of skin is equal to 50 μL, resulting in about 28.6 μg/50 μL of mucous. This amount is equivalent to 572 μg/mL of mucous. Our experiments showed that the MICs for skin peptides from *R. boylii* against chytrid were about 25−500 μg/mL, suggesting that *R. boylii* has excellent

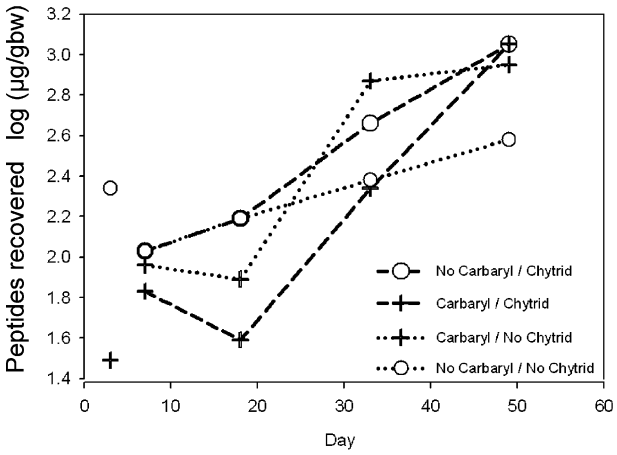

FIGURE 3. Mean skin peptides (log μg/grams body weight) recovered from frogs by treatment by day. Day 3 peptide collections preceded the start of the chytrid treatment, and therefore, peptide concentrations for day 3 were for carbaryl or no-carbaryl treatments with four samples each. On day 3, peptide concentrations were significantly less for carbaryl-exposed animals then non-exposed animals (*t*-test). For days 7 on, each data point represents the mean of two samples. However, death of some animals prior to the day 33 sampling resulted in only one sample in the no-carbaryl, no-chytrid treatment on day 33 and day 49. A repeated measures ANOVA for days 7−49 indicated a significant effect of time, but no significant chytrid, carbaryl, or interaction effects.

peptide defenses against chytrid zoospores. Taken together, these results imply that *R. boylii,* with a normal complement of skin peptides, may not be very susceptible to chytridiomycosis. Whether this represents a species-wide response or a more localized resistance that evolved locally in north-coastal California is an open question. It is also possible that *R. boylii* may be more susceptible to different strains of chytrid than the strain we used, which was isolated from *R. muscosa* from the Sierra Nevada.

Why was There No Apparent Interaction of Carbaryl and Chytrid to Increase Mortality? Our analysis of total peptide recovery following carbaryl treatment demonstrates that the concentration of norepinephrine-induced skin peptides was dramatically reduced in comparison with controls at day 3 (Figure 3), providing a mechanism by which carbaryl and chytrid might interact synergistically. However, there was no increased mortality in the carbaryl-treated frogs exposed to chytrids. One reasonable possibility is that the skin peptide concentrations that remain following carbaryl treatment (approximately 45 μg/gbw), although much reduced compared to controls, are sufficient to limit infection. Using the same calculations as above, 45 μg/gbw of skin peptide translates to a concentration of peptides in the mucous layer of about 70 μg/mL. In our in vitro experiments, zoospores were significantly inhibited by concentrations of skin peptides at 12.5 μg/mL or higher, although the greatest quantitative change in zoospore inhibition occurred between 50 and 100 μg/mL. Thus, while the amount of total recovered peptides in carbaryl-treated frogs was significantly reduced, it still may have been sufficient to inhibit zoospore colonization. It is also possible that a one-time, as opposed to continuing, carbaryl exposure allowed frogs to recover peptide defenses in time to defend against the effects of an initial chytrid infection. In the field, animals may receive multiple exposures to carbaryl or other pesticides. Finally, it is possible that while carbaryl suppressed skin peptide levels, other aspects of the immune system may have protected *R. boylii* against chytrid infection.

What is the Mechanism for Carbaryl Effects on Skin Peptides? Discharge of skin peptides is dependent on the natural release of epinephrine or norepinephrine at sympathetic nerve terminals innervating the granular glands of the skin (*31*, *32*). We speculate that carbaryl mimics the effects of norepinephrine on the sympathetic nervous system. Evidence to support this hypothesis comes from the similar effects of norepinephrine and carbaryl on short circuit current (SCC) in frog skin. In the semiaquatic frog *Leptodactylus chaquensis,* bathing isolated frog skin in a relatively low concentration of norepinephrine (12 μM) induces increased SCC due to secretion of chloride ions (Cl^-) (*33*). Carbaryl has been reported to have very similar effects on the SCC across the skin of *Rana esculenta* (*34*, *35*). Thus, it is possible that carbaryl mimics norepinephrine-induced skin peptide discharge. Alternatively, carbaryl may act as a nonspecific stressor that activates an alarm response of the sympathetic nervous system and discharge of skin peptides. In either case, the reduced concentrations of skin peptides detected in postmetamorphic *R. boylii* 3 days after carbaryl exposure are consistent with the interpretation that peptides had already been discharged at day zero when the carbaryl was applied, and that levels had not yet fully recovered.

In summary, our research demonstrated that sublethal carbaryl exposure inhibited peptide defenses in postmetamorphic juvenile *R. boylii*, and that these same peptides have a strong inhibitory effect on chytrid in culture. Although carbaryl exposure significantly reduced skin peptide levels, the frogs were still able to defend against chytrid infection, and we did not detect an interaction between carbaryl exposure and chytrid infection leading to increased mortality. Thus, for this well-defended anuran species, the reduced production of skin peptides after a single exposure to carbaryl was apparently still sufficient to ward off the lethal effects of chytrid infection. Although chytrid infection did not result in increased mortality, it strongly inhibited growth of postmetamorphic *R. boylii*, demonstrating that chytrid exposure can have negative effects on *R. boylii* populations even if it is not through direct mortality. The consequences of reduced growth have yet to be determined in *R. boylii*; however, size at and time to sexual maturity are key life-history parameter in anurans (*36*, *37*) that may well be reduced due to chytrid infection in this declining species. Given the clear potential for an interaction between pesticide exposure and disease due to reduced skin peptide production, we encourage additional experimental study of this potential interaction in less well-defended amphibian species. We need to better understand the effects of multiple and lower pesticide exposures on amphibian immune defenses for a range of current use pesticides. Furthermore, we need a better understanding of how impaired immune defenses, including reduced skin peptide levels, affect susceptibility to and the outcome of chytridiomycosis. Last, we encourage comparative field studies to examine the association between pesticide exposure, skin peptide concentrations, and extent of chytrid infection across multiple sites with different exposure histories.

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Supporting Information Available

Details of methods for animal care, determining lethal carbaryl levels, skin peptide collection, and chytrid growth inhibition assays are shown. In addition, there is an ANOVA table for treatment affects on frog growth, a table on chytrid growth inhibition assays by treatment, and a figure showing chytrid growth inhibition for purified skin peptides. This material is available free of charge via the Internet at http://pubs.acs.org.

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