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INVASIVE SPECIES

Effects of carbaryl on species interactions of the foothill yellow legged frog (*Rana boylii*) and the Pacific treefrog (*Pseudacris regilla*)

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Abstract Amphibian species worldwide are being confronted with novel anthropogenic stressors such as contaminants and invasive species. While much work has been done to examine these factors individually, less is known about how these stressors might interact. No studies to this point have examined the potentially synergistic impacts between these two stressors on a threatened amphibian species. We present the results from three separate laboratory studies focusing on two species of frogs, the Pacific tree frog (*Pseudacris regilla*) and a federal species of concern, the foothill yellow legged frog (*Rana boylii*). These experiments examine the toxicity of an insecticide, carbaryl, on each species, on their competitive interactions, and on their interaction with a non-native crayfish predator (*Pacifastacus leniusculus*). *R. boylii* were more susceptible to pesticide exposure than *P. regilla* and exposure reduced their ability to compete. This differential effect of the pesticide resulted in a remarkable increase in mortality (50%) for *R. boylii* with an invasive crayfish predator present while *P. regilla* exhibited no change. These results add to concerns over the utility of single species toxicity tests in determining safe levels for environmental exposure and advocate for the use of multiple species tests that focus on key species interactions.

Keywords Pesticide · Multiple stressors · Predation · Competition · Sub-lethal effects

Introduction

Amphibians are often deemed as species sensitive to environmental contaminants and it is argued that these contaminants are of critical importance in understanding habitat degradation (Hopkins, 2007). Yet, different amphibian species exhibit a wide variation in their sensitivity to the same chemical (Kerby et al., 2010; Weltje et al., 2013). These differences have been highlighted in a wide range of species and bring into question the usefulness of a single species model organism for determining safe levels of exposure. Nowhere is this of more significance than when attempting to determine safe levels for endangered and threatened species.

While several studies have improved our understanding of these declines, in many cases amphibians are still disappearing at a rapid rate without suitable explanation (Stuart et al., 2004). While there are several demonstrated reasons for declines such as

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habitat destruction and the disease chytridiomycosis (Collins & Storfer, 2004), others are examining the effects of combined stressors on amphibian populations (e.g., Kerby & Storfer, 2009; Distel & Boone 2011). These studies of multiple stressors are important in that they better simulate the actual experience of species dealing with simultaneous anthropogenic impacts.

Invasive species are known to impact many amphibian species (see review Kats & Ferrer, 2003). In particular, invasive crayfish have been demonstrated to impact several species in California (Gamradt & Kats, 1995) and worldwide (Nakata et al., 2005; Gherardi, 2006). The signal crayfish, *Pacifastacus leniusculus,* is native to the Northwest United States and the northernmost part of California. Likely through bait bucket introductions, this species has expanded its range throughout California and is known to be a problematic invasive species impacting stream communities (Pintor et al., 2009). Via field surveys stemming from that work, we have found introduced populations of crayfish co-occurring with several native amphibian species including the Pacific treefrog (*Pseudacris regilla*) and the threatened foothill yellow legged frog (*Rana boylii*).

In addition to invasive predators, the large use of pesticides in the Central Valley region is thought to be a contributor to amphibian declines (Sparling et al., 2001; Davidson et al., 2002; Davidson, 2004). However, when amphibian tadpoles are examined in toxicity tests at ecologically relevant concentrations, often no strong lethal effects are detected (i.e., Kerby et al., 2010). One criticism of these types of tests is that they often lack realistic physical structure or community complexity (Relyea & Hoverman, 2006). Examination of the effects of pesticide exposure on aquatic multi-species communities with predation or competition should provide a more accurate depiction of the impacts pesticides are having on amphibian populations. Interestingly, these studies sometimes find no ill effects on tadpoles (e.g., Relyea, 2004) while in others pesticides are found to be beneficial to tadpole growth and survival (e.g., Boone & Semlitsch, 2001).

Several studies have found that when tadpoles are confronted with multiple stressors simultaneously, pesticide exposed tadpoles suffer a significant reduction in survival. When exposed to an herbicide, salamander larvae then subsequently exposed to a sunfish predator exhibited significant reduced survival (Rohr unpublished data). Bridges (1999a) documented the same effects when tadpoles were exposed to an insecticide and then to newt predators. Relyea (2003) has shown that when exposed to the commonly used insecticide, carbaryl, and predatory cues of a newt, several amphibian species exhibit decreased survival despite not being actually preyed upon. Conversely, Bridges (1999a) found that when both tadpole prey and a newt predator are exposed simultaneously overall tadpole survival does not change. In a previous study (Kerby et al., 2012), we found that exposure to the insecticide diazinon altered the predator–prey interaction primarily resulting in net negative impacts of the pesticide on a predator. The incorporation of a predator that is less tolerant of pesticide exposure than its prey should result in reduced prey survival.

Sparling and Fellers (2009) elucidate that for two widely used insecticides, chlorpyrifos and endosulfan, *R. boylii* tadpoles are much more susceptible than *P. regilla* in terms of both mortality and in sub-lethal measures such as time to metamorphosis. Our study continues in this vein by examining differences in susceptibility to another popular pesticide carbaryl. In addition to doing a traditional toxicity study though, we also examine how sub-lethal levels of carbaryl alter interactions between the two species of tadpoles, the common Pacific treefrog (*P. regilla*) and the threatened foothill yellow legged frog (*R. boylii*). This is examined via competition and when confronted with an introduced predator, the signal crayfish (*P. leniusculus*).

We hypothesize that *R. boylii* will exhibit a higher susceptibility to carbaryl than *P. regilla.* We also hypothesize that the differential effects of carbaryl will lead to a decrease in both competitive ability and response to predator for *R. boylii* tadpoles.

Materials and methods

Tadpoles

Pseudacris regilla and *R. boylii* are known to co-occur and have competitive interactions in northern California streams (Kupferberg, 1997). *R. boylii* is a species of special concern in the state of California and its declines have been linked to pesticide use (Davidson, 2004). *P. regilla* is a ubiquitous species found throughout the west coast, and is exposed to pesticides throughout its range (Datta et al., 1998), but does not exhibit the same patterns of decline as *R. boylii* (Davidson, 2004). All organisms were collected under approved permitting from the California Department of Fish and Game. Given the protected status of *R. boylii* were only collected from sites that had large numbers of tadpoles present (>1,000) as per permit guidelines.

For experiments one and two, Pacific treefrog, *P. regilla*; and foothill yellow legged frog, *R. boylii* individuals were collected as egg masses (Yolo and Nevada County, CA, USA, respectively) and raised on ad libitum Tetramin fish food (Tetra, Blacksburg, VA, USA) in controlled environmental chambers (light dark cycle 16:8, 14 degrees C). Pacific treefrog and foothill yellow legged tadpoles used in these experiments were between Gosner stages 26–29 (Gosner, 1960). For the third experiment, *P. regilla* tadpoles (Gosner stage 26–30) were collected from a natural reserve area on the UC Davis campus (Yolo county, CA) and *R. boylii* tadpoles (Gosner stage 26–30) were collected from Oregon Creek (Nevada county, CA). Neither location contained predatory crayfish. Species were held separately in 37 l aquaria at natural densities ($\sim 0.05/\text{cm}^2$) in 16 ° C temperature controlled chambers for one week. Photoperiod was set according to local seasonal pattern (15:9 day/night) and each aquarium contained aerated dechlorinated water. Prior to the experiment, tadpoles were fed ad libitum with commercial algal disks (Hartz Mountain Corporation).

Crayfish

Signal crayfish (*P. leniusculus*) are native to the northernmost part of California, but have spread south in many California streams via release from fisherman's bait buckets or escape from aquaculture facilities. The increased range of this crayfish now overlaps in several areas with the current and historical range of *R. boylii*. Overall, little research has been done to examine the predatory effects of *P. leniusculus* on California amphibian species but it is thought that these crayfish are might be significantly reducing *R. boylii* abundances (Maria Ellis, personal communication). Studies on another invasive crayfish, *Procambarus clarkii,* exhibit a significant predatory impact to *P. regilla* tadpole numbers (Kerby et al., 2005).

Signal crayfish (Carapace length 4.5–5.5 cm) were collected from the American River (Sacramento County, CA) using dip nets. Individuals were held in 37 l aquaria (as above) for the 2 days prior to the experiment. Crayfish were fed Tetramin fish flake food (Spectrum Brands, Inc.) ad libitum and were moved to plastic cups (see below) 2 h prior to the experiment.

Pesticide

The pesticide carbaryl (99% purity) was obtained from the Chemservice (Westchester, PA). This insecticide is a carbamate which acts as a neurotoxin in both insects and amphibians. For all three experiments, we created stock solutions by diluting the active ingredient in methanol to a concentration of 1.25 g/l (verified via GC/MS by an independent laboratory). Subsequent dilutions for each experimental concentration were done in distilled water. Control treatments received equivalent aliquots of methanol only. In the toxicity study, a water only control was also used but due to no difference in results these treatments were pooled. Subsequent studies utilized solvent controls only. A previous study also exhibited no observed effect of methanol solvent on mortality or behavior of amphibian species (Kerby et al., 2012). Carbaryl has a half-life of 10 days in measured pH conditions (8.0–8.2) and so experiments longer than 7 days underwent static renewal water changes. Carbaryl has been found in water bodies at concentrations up to 4.8 mg/l (Norris et al., 1983).

Experiment 1: carbaryl toxicity

Experimental design

We tested species responses to carbaryl in three concentrations (50, 500, 5000 µg/l) with two control treatments (dechlorinated water, methanol solvent added) for a total of five treatments. To administer each treatment (50, 500, 5000 µg/l), we diluted our stock, respectively, to administer 1 ml aliquots into 250 ml of well water in 500 ml glass jars. Each jar held one tadpole. A subset of two jars from each treatment was verified for final concentration. The design was a static water test without renewal with 20 replicates of each treatment. For solvent controls, 100 µl of methanol or filtered well water was added to the respective jars. Treatments were randomly arrayed in a controlled environment chamber at 14°C in a 16L:8D photoperiod.

Each trial ran for a total of 4 days. Surviving tadpoles were euthanized in MS-222.

Toxicity analyses

While the experiment was designed primarily to measure differences in behavior, we are able to obtain an estimate of LC_{50} values. Survival data were used from hour 72 as in traditional LC_{50} tests, although the experiment continued for another 8 h to obtain additional behavioral data (day 4). We examined survival data using a probit analysis. Statistical analysis was performed using the software R v.2.0.12.

Behavioral observations

To serve as refuge in each jar, we utilized a tinted plexiglass plate (4 × 5 cm) on two diagonally placed flattened glass beads (1.5 cm diameter). Tadpoles could be seen through the plate and monitored for condition (sensu Kerby & Kats, 1998). Behavioral observations were made on each of the 280 jars once an hour, 11 times per day (9 a.m. to 8 p.m.) over 4 days. At the end of the third day, Tetramin food was added to each jar. During each observation, each tadpole was spot checked (viewed at a single moment) to determine its position (in refuge, in open), condition (no change, ill, dead), and movement (yes, no) of tadpoles. Position in open is defined as no part of the tadpole under the refuge plate. Condition was defined as ill if tadpoles were discolored or had curled tails. Movement was defined as if tadpoles changed position in any direction. Previous observations determined that these are visible characteristics that often precede death from pesticide exposure. The behavioral measures analyzed were (1) refuge use = number out in the open/total number alive and not ill or dead, and (2) activity = number moving out in the open/total number out in the open. After the final observation each day, every tadpole was carefully examined to determine and record mortality.

Behavior statistical analyses

We performed statistical analyses on the behavior of tadpoles at differing concentrations over the 4-day period. To deal with dead individuals, behavior was analyzed as arc sine transformed proportion (number exhibiting behavior/total alive). Due to large mortality at high concentrations beyond the first day, we present the behavioral data at the 24 h mark. These data were examined via a two-way ANOVA (species x concentration). Planned comparisons between the two control groups within each species exhibited no significant differences, so these were pooled into a single control group that was then examined to pesticide treatments via Dunnett planned comparisons.

Experiment 2: competition

Experimental procedure

We used a two-factor design to determine any effects of carbaryl exposure on competitive interactions between tadpole species. Treatments consisted of either eight tadpoles of a single species (8 *P. regilla* or 8 *R. boylii*) or a combined treatment with four tadpoles of each species. Each of these treatments was replicated five times in either carbaryl (50 µg/l) or no carbaryl treatments. Based on Experiment 1, we selected the concentration that induces behavioral effects, but is well below the lethal concentration (LC_{50}: ~600 µg/l) for the more sensitive *R. boylii* species. Due to the use of a threatened species, *R. boylii,* we were unable to collect enough animals to include treatments that directly measured intraspecific competition (i.e., 4 tadpoles of each species alone).

Experiments were conducted in 37 l aquaria filled with 10 l of filtered well water. A 10 cm × 10 cm semitransparent refuge plate (as above) made of tinted plexiglass set on 1-cm-tall glass beads was used to provide a place of cover for tadpoles. Prior to the experiment, mass and developmental stage was recorded for all tadpoles (*R. boylii:* all stage 26, mass $\bar{x} = 0.0425$ g, SE = 0.0009, *P. regilla:* stage $\bar{x} = 26.12$, SE = 0.03, mass $\bar{x} = 0.102$ g, SE = 0.0047) and individuals were randomly assigned to treatments. Tadpoles were fed 0.2 g of fish flakes daily and kept in lighting and temperature conditions as above. The water in each of the tanks was completely changed on day 7 and all tadpoles were again measured (stage and mass). The pH of the water was tested at the beginning, middle, and end of the experiment and was not different either over time or between treatments (pH $\bar{x} = 8.01$ SE = 0.20). Pesticide applications were reapplied immediately following the water change. Tadpole mortality was low overall (7%) and dead tadpoles were not replaced to maintain consistency in tadpole cumulative pesticide exposure. At the end of the 2 week experiment, tadpoles were again measured (stage and mass) and then euthanized with MS-222. Tadpole development was examined using a Gosner stage table (Gosner, 1960). To reduce error, instead of measuring the mass of each tadpole individually, each species within a tank was measured collectively and subsequently divided by the number measured. Tadpoles were placed as a group on a paper towel to remove excess water and then measured. Growth and development data were analyzed using 2-way ANOVAs (species composition × carbaryl presence). Multiple comparisons between pairs of treatments were performed using a Tukey test.

Behavioral data collection and analysis

In addition to data on mortality, development and growth, we conducted behavioral observations to examine any possible adverse effects of carbaryl exposure on feeding behavior. We did 8 hourly observations on four separate days: immediately following pesticide application (Days 1 and 8) and 5 days following pesticide application (Days 6 and 13). Feeding was defined as a tadpole eating flakes off of the tank bottom and recorded as yes/no data. Hourly observations were averaged over each day and statistically analyzed using a two-way ANOVA (species composition x pesticide presence) repeated measures test. Any proportion data were arc sine transformed for use in analysis.

Experiment 3: invasive predator

Experimental procedure

We examined the effects of the pesticide carbaryl on both amphibian species in the presence of a non-native crayfish predator. A 2 × 2 factorial design was used with two pesticide treatments (solvent control, 50 µg/l) and two predator treatments (crayfish, no crayfish). As in Experiment 2, we selected a concentration that induces behavioral effects, but is well below the lethal concentration for the more sensitive *R. boylii* species. Each of the six replicates consisted of five individuals of each amphibian species (Gosner stage 26–30) for a total of ten larvae per tank. In predator treatments, a single crayfish was placed in a 37 l aquarium tank approximately 5 min following tadpole addition. Crayfish predators were kept in plastic cups (10 cm diameter × 7 cm tall with 2 cm × 2 cm squares cut on opposite sides covered with 0.08 cm mesh screening) for the first 3 days of the experiment, and then released on the morning of day four. This was done to examine any synergistic effects of pesticide with predator cue alone (sensu Relyea & Mills, 2001). Tanks were dosed with pesticide or control aliquots 5 min after predators were added to cups. Empty plastic cups were included in no predator control treatments and all cups were removed from tanks following predator releases on day four. Crayfish were then allowed to roam freely until termination of the experiment at the end of day seven. We included refuge plates (as above) to provide an area for tadpoles to escape predation.

Behavioral data collection and analysis

Both tadpole species were observed hourly (9 a.m. to 4 p.m.) over 7 days and the total number alive was recorded during each observation. In addition, we observed the activity and location of tadpoles. Activity was measured as the total number of tadpoles in a tank moving over a 30 s period. Movement is defined as physical displacement of greater than 1 cm and not simply moving their tail in place. The number of tadpoles in several locations throughout the tank (refuge, surface, edge, open) were also recorded over the 30 s period. Tadpoles were considered in refugia if more than 2/3 of their body was under the refuge plate. Tadpoles with any part of their body touching the surface of the water were categorized as on the surface. Finally, crayfish activity was recorded categorically as yes/no at each hourly observation. Movement was recorded as physical displacement of greater than 1 cm (walking or swimming) and not solely as the movement of legs in a single place (as is typical when foraging).

Tadpole survivorship was examined using a two-way ANOVA on the final percentage that survived (arcsine square root transformation). Behavioral variables were tested using two-way repeated measure ANOVAs averaging measures of the days prior (1–3) and subsequent (4–7) to crayfish predator release. When there were significant time effect differences, we subsequently performed separate ANOVAs on the first 3 days (pre-crayfish release) and the last 4 days (crayfish released) to differentiate differences between treatments in each context. Crayfish activity was examined using a one-way ANOVA.

Results

Experiment 1: toxicity

Carbaryl was more lethal to the threatened species, *R. boylii,* than to *P. regilla.* The estimated 72 h LC_{50} value for *R. boylii* is 585 μg/l ± 229 and for *P. regilla is* 3006 μg/l ± 955. While the values are based upon approximations, the 95% confidence intervals for the two estimates do not overlap. Sub-lethal estimates for overall activity after 24 h values followed the same pattern with *R. boylii* reducing activity with increasing concentration while *P. regilla* showed no effects (Pesticide: $F_{3,169} = 6.24, P > 0.001$, Species: $F_{1,169} = 8.92, P = 0.003$, Interaction: $F_{3,169} = 0.67, P = 0.57$; Fig. 1). Both species exhibited a very high survival rate at the lowest concentration of 50 μg/l (95%) and so this level was used in subsequent experiments to determine potential interactions of sub-lethal concentration with species interactions.

Experiment 2: competition

Survival

There was no significant effect of either pesticide or species composition on survival of either *P. regilla* (Pesticide: $F_{1,16} = 0.04, P = 0.84$, Species: $F_{1,16} = 2.0, P = 0.18$, Interaction: $F_{1,16} = 0.37, P = 0.55$) or *R. boylii* tadpoles (Pesticide: $F_{1,16} = 0.04, P = 0.84$, Species: $F_{1,16} = 0.36, P = 0.56$. Interaction: $F_{1,16} = 0.04, P = 0.84$). 93% of *P. regilla* and 92% of the *R. boylii* tadpoles survived in pesticide-exposed treatments.

Development

Carbaryl presence slightly reduced developmental rate in *R. boylii* tadpoles ($F_{1,16} = 4.93, P = 0.041$; Fig. 2). *P. regilla* alone did not reduce development of *R. boylii* ($F_{1,16} = 0.018, P = 0.89$). There was no interactive effect of pesticide and *P. regilla* on development ($F_{1,16} = 1.70, P = 0.21$).

Conversely, the *P. regilla* tadpoles developed further in the presence of *R. boylii* tadpoles ($F_{1,16} = 14.15, P = 0.002$; Fig. 2), and suffered no developmental impacts from carbaryl presence alone ($F_{1,16} = 1.55, P = 0.23$). A near significant interaction effect ($F_{1,16} = 4.12, P = 0.06$) was detected. *P. regilla* tadpoles in the combined carbaryl and *R. boylii* treatment developed more than both the *P. regilla* only treatments (Tukey comparison with pesticide, $q_{24,6} = 17.75, P < 0.001$; Tukey comparison without pesticide, $q_{24,6} = 15.35, P < 0.001$) and the *R. boylii* treatment with no pesticide (Tukey comparison, $q_{24,6} = 10.04, P < 0.001$). *R. boylii* tadpoles did not develop as rapidly as *P. regilla* tadpoles across all treatments (0.49 vs. 3.33 Gosner stages over 2 weeks; ANOVA $F_{1,38} = 88.83, P = 0.001$).

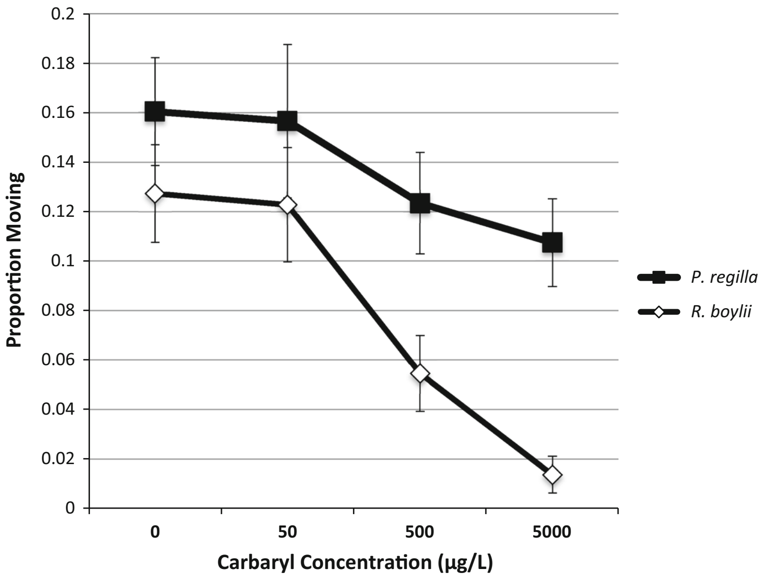


Fig. 1 Difference in movement between the two species, *P. regilla* and *R. boylii* when exposed to increasing concentrations of carbaryl. Higher concentrations of carbaryl reduced movement of *R boylii* significantly more than *P. regilla*

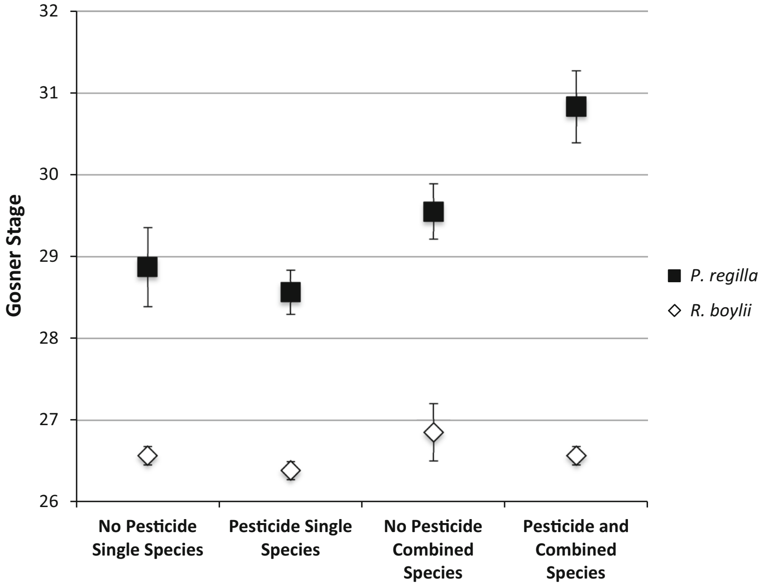


Fig. 2 Difference in development between the two species, *P. regilla* and *R. boylii* when exposed to increasing concentrations of carbaryl. *R. boylii* exhibits slight reduction in development when exposed to carbaryl. *P. regilla* exhibits a marked increase in development when combined with *R. boylii*

Growth

Following the same pattern, but far more pronounced, the mass of *R. boylii* tadpoles was reduced in the presence of *P. regilla* tadpoles ($F_{1,16} = 4.93$, $P = 0.041$; Fig. 3). No effect of carbaryl ($F_{1,16} = 1.54$, $P = 0.23$) or interaction effect of both factors ($F_{1,16} = 0.42$, $P = 0.52$) was detected for growth despite detected differences in development. *R. boylii* in the combined treatment of carbaryl and *P. regilla* did exhibit a reduction in mass when compared to *R. boylii* only with carbaryl (Tukey comparison, $q_{24,6} = 5.04$, $P < 0.05$) and without carbaryl (Tukey comparison, $q_{24,6} = 6.13$, $P < 0.01$; Fig. 3). The combined stressors result in the least growth overall, similar to the pattern observed with developmental rate.

Conversely, the presence of *R. boylii* increased *P. regilla* mass by 77% ($F_{1,16} = 33.23$, $P = 0.001$; Fig. 3). We detected no main effect of carbaryl ($F_{1,16} = 0.004$, $P = 0.95$) or interaction effect between the factors ($F_{1,16} = 0.01$, $P = 0.92$). Across all treatments, *P. regilla* also grew more than *R. boylii* (0.10 g vs. 0.037 g per tadpole; ANOVA $F_{1,38} = 52.70$, $P = 0.001$).

Feeding

For *R. boylii,* there was no main effect of carbaryl alone ($F_{1,15} = 0.035$, $P = 0.85$) but rather an interactive effect of species composition and carbaryl ($F_{1,15} = 4.70$, $P = 0.047$). In the absence of carbaryl, *R. boylii* tadpoles fed at nearly twice as high a rate when held with *P. regilla* tadpoles as opposed to controls, yet in the presence of carbaryl, they fed similarly (Multiple comparison, $t = 1.95$, df = 15, $P = 0.035$; Fig. 4).

The presence of carbaryl or species composition had no effect on feeding in *P. regilla* tadpoles (Pesticide; $F_{1,15} = 3.42$, $P = 0.084$; Species; $F_{1,15} = 1.08$, $P = 0.32$; Interaction; $F_{1,15} = 0.35$, $P = 0.57$). Overall, *P. regilla* tadpoles fed less frequently than *R. boylii* tadpoles (23.5 vs. 34.7%; $F_{1,15} = 11.48$, $P = 0.004$).

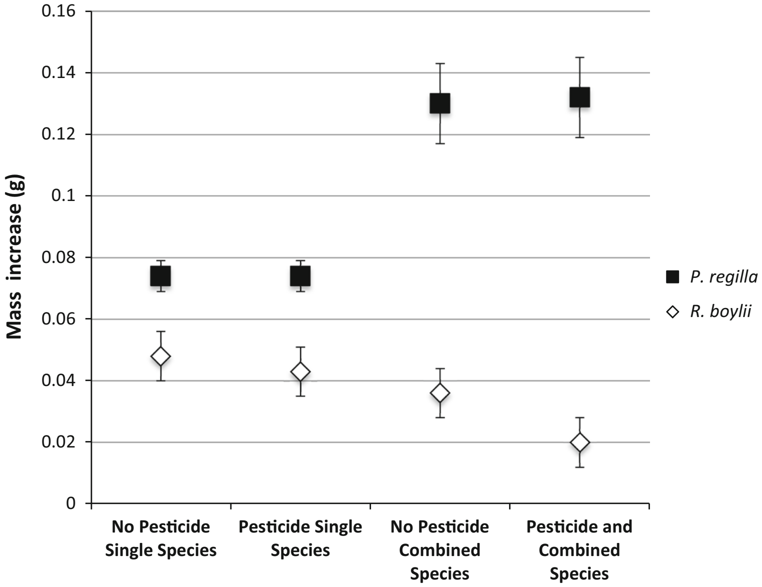
Experiment 3: predation

Tadpole survival

The exposure to combined predator cue (before predator release) and the insecticide carbaryl did not result in any mortality for either species over the first 3 days. Following the release of the crayfish predators from their cups, substantial differences existed between the two species.

Rana boylii tadpoles suffered significant mortality under the combined predator and pesticide treatments (Fig. 5). Main effects of both predator ($F_{1,19} = 15.35$,

Fig. 3 Difference in growth (increase in mass) between the two species, *P. regilla* and *R. boylii* when exposed to increasing concentrations of carbaryl. In combined species treatments, *R. boylii* significantly decreased growth while *P. regilla* significantly increased growth



$P = 0.001$), and pesticide exhibited reduced tadpole survival ($F_{1,19} = 11.77$, $P = 0.003$). Yet, a significant interaction effect was also detected (Predator x Pesticide interaction effect; $F_{1,19} = 4.87$, $P = 0.040$) caused by a large decrease in survival in the pesticide and predator combined treatment. Contrary to this, *P. regilla* tadpoles exhibited low mortality across all the treatments with no differences among treatments (Fig. 6; Predator- $F_{1,20} = 2.27$, $P = 0.15$; Pesticide- $F_{1,20} = 0.61$, $P = 0.45$; Predator x Pesticide interaction- $F_{1,20} = 0.44$, $P = 0.47$). The predatory crayfish suffered no mortality in the experiment and exhibited no differences in movement between pesticide treatments ($F_{1,10} = 2.25$, $P = 0.16$).

Tadpole behavior

Activity *Rana boylii* activity was reduced overall following predator release on day 4 ($F_{1,20} = 21.62$, $P = 0.001$). No overall effects of pesticide ($F_{1,20} = 3.12$, $P = 0.093$), predator ($F_{1,20} = 0.18$, $P = 0.68$), or interaction effect ($F_{1,20} = 1.01$, $P = 0.33$) were detected in the larger model; therefore, we subsequently partitioned out behavioral data into analyses of days 1–3 and of days 4–7 to better differentiate effects.

There was an effect of predator release to increase *P. regilla* activity overall ($F_{1,20} = 118.68$, $P = 0.001$). There were no main effects for either predator treatment ($F_{1,20} = 1.90$, $P = 0.18$) or pesticide presence ($F_{120} = 0.051$, $P = 0.82$), but there was an interaction effect ($F_{1,20} = 4.82$, $P = 0.04$) in the overall model where the combined effect of predator and pesticide reduced overall activity (as compared to each on their own resulting in an increased activity).

Pre-predator release (days 1–3)

R. boylii tadpoles reduced their activity in the presence of pesticide ($F_{1,20} = 5.13$, $P = 0.035$), but exhibited no effect due to predator cues ($F_{1,20} = 0.19$, $P = 0.67$). The combined treatment of predator cue and pesticide resulted in the lowest activity overall despite only detecting a nearly significant interaction effect ($F_{1,20} = 3.40$, $P = 0.080$; Fig. 6).

Pseudacris regilla activity was not influenced by pesticides ($F_{1,20} = 0.043$, $P = 0.84$), predator cue ($F_{1,20} = 0.001$, $P = 0.89$), or by an interaction between the two ($F_{1,20} = 0.99$, $P = 0.33$; Fig. 6).

Post-predator release (days 4–7)

We observed no effects of pesticide ($F_{1,19} = 1.18$, $P = 0.29$), predator ($F_{1,19} = 0.12$, $P = 0.73$), or interaction between the two ($F_{1,20} = 0.031$, $P = 0.86$) on *R. boylii* activity. We note that due to prey being consumed in the experiment, the power to detect differences was reduced.

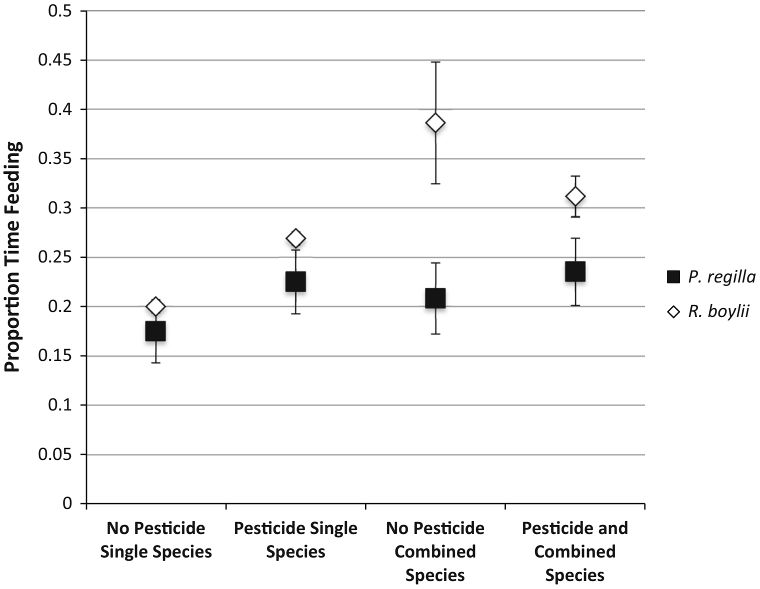


Fig. 4 Difference in feeding rate between the two species, *P. regilla* and *R. boylii* when exposed to competition and carbaryl. *P. regilla* fed less frequently than *R. boylii* overall. *R. boylii* fed more frequently while in the presence of *P. regilla*

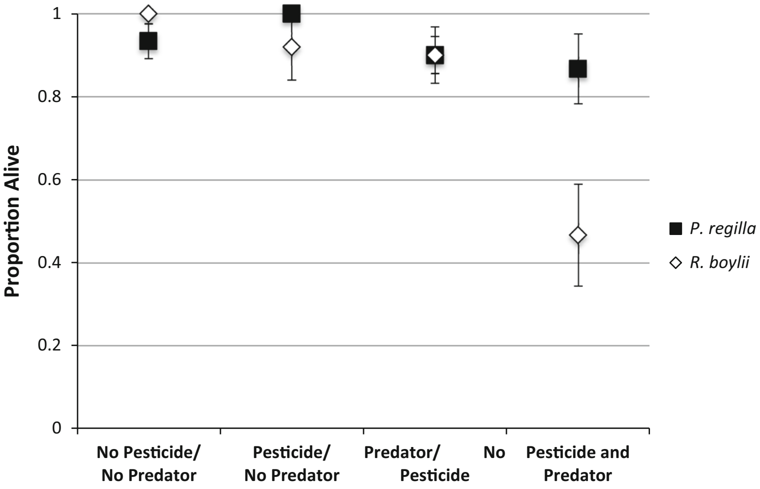


Fig. 5 Percent survival of *R. boylii* and *P. regilla* tadpoles in predation experiment. The combined treatment of carbaryl and crayfish predator exhibits a large reduction in survival for *R. boylii* tadpoles while having no effect on *P. regilla* tadpoles

After the predator was released, *P. regilla* tadpoles exhibited lower activity in the combined presence of pesticide and predators than in either treatment alone (Interaction effect; $F_{1,20} = 7.58$, $P = 0.012$). A main effect of predator was detected ($F_{1,20} = 4.75$, $P = 0.041$), but no main effect of pesticide was detected ($F_{1,20} = 0.044$, $P = 0.84$). Despite this pesticide, only treatments exhibited a consistently higher activity over the final 4 days, while the combined treatments of carbaryl and predator were consistently lower.

Surface and refuge use

R. boylii surface use increased following predator release ($F_{1,20} = 160.64$, $P = 0.001$). Predator presence increased surface use overall ($F_{1,20} = 7.86$, $P = 0.011$), and there were no effects of pesticide presence ($F_{1,20} = 2.93$, $P = 0.10$) or of an interaction effect ($F_{1,20} = 0.72$, $P = 0.41$) in the overall model.

Predator release resulted in an overall increase in *P. regilla* surface use to escape predation of the benthic crayfish ($F_{1,20} = 109.46$, $P = 0.001$). In the overall

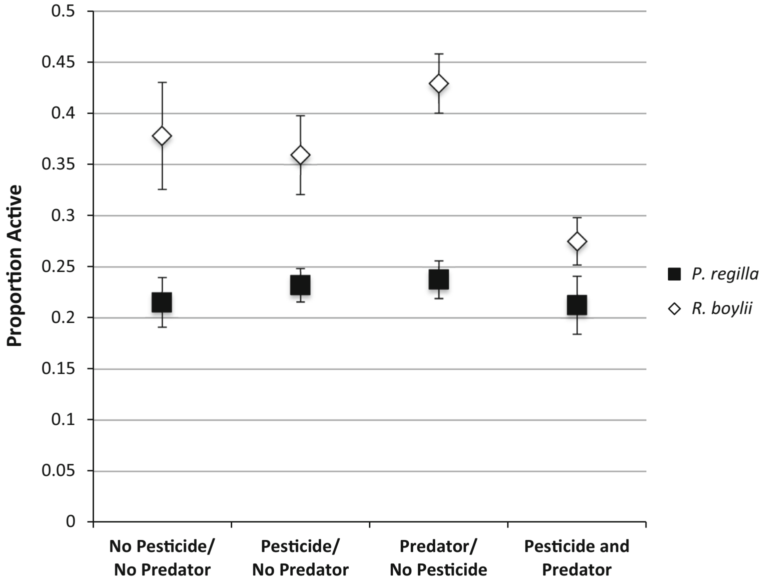


Fig. 6 Average activity of *P. regilla* and *R. boylii* in days 1-3 of the predation experiment. No significant change in activity for *P. regilla* was detected. *R. boylii* significantly reduced activity in the presence of carbaryl, although this effect is driven primarily by the combined treatment

model, there was no main effects of predator ($F_{1,20} = 1.23$, $P = 0.28$), pesticide ($F_{1,20} = 1.10$, $P = 0.31$), or of an interaction effect ($F_{1,20} = 0.19$, $P = 0.67$) between the two.

Pre-predator release (days 1–3)

*Rana boylii t*adpoles were found on the surface less in pesticide treatments ($F_{1,20} = 4.66$, $P = 0.043$). There was no effect of predator cues ($F_{1,20} = 0.021, P = 0.88$) or interaction effect ($F_{1,20} = 0.35$, $P = 0.56$). Pesticide presence increased *R. boylii* refuge use in tadpoles ($F_{1,19} = 4.40, P = 0.049$), while predator cue presence ($F_{1,19} = 0.22$, $P = 0.64$) and the interaction effect ($F_{1,19} = 0.20$, $P = 0.66$) were not significant. There was a time effect ($F_{2.38} = 12.77, P = 0.001$) with refuge use slowly increasing over time but exhibited no time interaction effects with pesticide ($F_{2.38} = 1.10$, $P = 0.34$), predator ($F_{3.38} = 0.12$, $P = 0.88$), or the three way interaction of time by pesticide by predator ($F_{2.38} = 1.00, P = 0.38$).

Pseudacris regilla tadpoles exhibited no surface use change in response to predator cues ($F_{1,20} = 2.18$, $P = 0.16$) or pesticide presence ($F_{1,20} = 1.24$, $P = 0.28$) or to the interaction of both ($F_{1,20} = 0.18$, $P = 0.67$). For refuge use, no main effects of pesticide ($F_{1,19} = 0.038$, $P = 0.85$), predator cue presence ($F_{1,19} = 0.76$, $P = 0.39$), or interaction effects ($F_{1,19} = 0.47$, $P = 0.50$) were detected. Tadpoles did reduce refuge use over time ($F_{2,38} = 18.33, P = 0.001$) but exhibited no interaction effects of time with pesticide ($F_{2.38} = 0.12$, $P = 0.89$), predator ($F_{2.38} = 1.06$, $p = 0.36$), or the three way interaction of time by pesticide by predator ($F_{2.38} = 2.016, P = 0.15$).

Post-predator release (days 4–7)

In contrast days 1-3, *R. boylii* at the surface increased in the presence of a predator ($F_{1,20} = 11.96$, $P = 0.002$). Interestingly, exposure to pesticide *increased* surface use in later days ($F_{1,20} = 9.66$, $P = 0.006$). We found no interaction effects between pesticide and predator ($F_{1,20} = 0.65$, $P = 0.43$) although the combined treatment exhibited consistently higher surface use (Fig. 7).

When predators were released, *R. boylii* tadpoles decreased their use of refuge in response to predators ($F_{1,19} = 15.06$, $P = 0.001$). Tadpoles also decreased refuge use in the presence of pesticides ($F_{1,19} = 4.26$, $P = 0.053$) although no interaction effect was detected ($F_{1,19} = 0.001, P = 0.99$). Tadpoles also decreased their use of refuge over time and then rapidly increased their refuge use on day 7 ($F_{3.57} = 17.69$, $P = 0.001$). No interaction effects of time with pesticide ($F_{3.57} = 0.97$, $P = 0.42$), predator ($F_{3.57} = 0.57, P = 0.64$), or the three way interaction were detected ($F_{3.57} = 0.42, P = 0.74$).

*Pseudacris regilla t*adpoles increased surface use in the presence of predators ($F_{1,20} = 13.09, P = 0.002$)

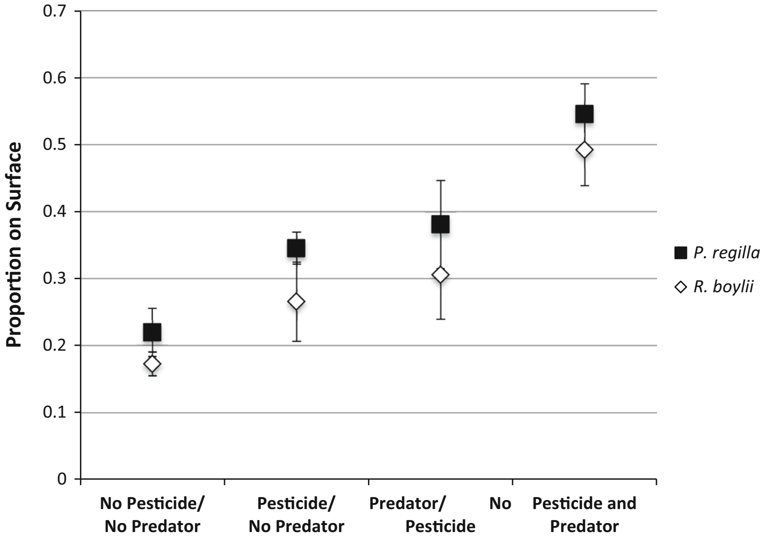


Fig. 7 Average surface use of *P. regilla* and *R. boylii* in days 4–7 (after release of predator) of the predation experiment. Both species responded similarly with increased surface use to escape predation. Both species also increased surface use in response to pesticide exposure

and also in the presence of pesticide ($F_{1,20} = 9.64$, $P = 0.006$) although no interaction effect was detected ($F_{1,20} = 0.10$, $P = 0.76$). The increase in surface use by *P. regilla* tadpoles in predator treatments is again an appropriate anti-predatory response for a benthic crayfish predator. In particular, the combined treatment of carbaryl and crayfish resulted in consistently higher surface use over the last 4 days (Fig. 7).

P seudacris regilla tadpoles did not change their use of refuge with respect to predators ($F_{1,19} = 1.49, P = 0.24$), but did decrease use with respect to pesticide presence ($F_{1,19} = 7.80$, $P = 0.012$). When examining days 4, 5, and 6 only, it appears that refuge use is decreased in all treatments compared to the control. There was no interaction effect of pesticide and predator detected ($F_{1,19} = 0.58$, $P = 0.45$), but tadpoles decreased refuge use over time in general ($F_{1,19} = 10.92$, $P = 0.001$). There were no interaction effects of time with pesticide ($F_{3,57} = 0.37$, $P = 0.77$), predator ($F_{3,57} = 2.42$, $P = 0.075$), or the three way interaction of time by pesticide by predator ($F_{3,57} = 0.073$, $P = 0.97$).

Discussion

As indicated in a previous study (Sparling & Fellers, 2009), the species of special concern, *R. boylii,* is more sensitive to insecticide exposure than the more commonly found *P. regilla*. In all three of our experiments, *R. boylii* exhibited negative effects when exposed to carbaryl. Moreover, at low concentrations this differential effect on the species had important outcomes for both competitive interactions between the species with an invasive crayfish predator. Perhaps most shocking is the dramatic decrease in survival for *R. boylii* when a "non-lethal" concentration of carbaryl was combined with an invasive predator resulting in what amounts to an order of magnitude decrease in an LC_{50} value (Fig. 5).

Toxicity test

In the acute toxicity test, *R. boylii* were found to be over an order of magnitude more sensitive than *P. regilla* in terms of LC_{50} estimates. In addition, higher concentrations of carbaryl resulted in a significant decrease in activity (Fig. 1). These results suggest that lower concentrations had little effects on both species, which is typical of many single species toxicity tests (Kerby et al., 2010). Interestingly, these interpretations were somewhat different when examined in a multiple species scenario.

Competition

Understanding how pesticides interact with aquatic communities is an important challenge for applied ecology (Relyea & Hoverman, 2006; Rohr et al., 2006). Previous studies examined how differences in predator susceptibility alter competitive outcomes in two species of tadpoles (Werner, 1991; Werner & Anholt, 1997). In this same way, differences in pesticide susceptibility should alter interactions between competitors (Rohr et al., 2006). This study exhibits how pesticides altered competitive interactions between species in a community, creating an advantage for one species and a corresponding disadvantage for the other. In this case, one can see that the combined presence of carbaryl and *P. regilla* shut down both the development (Fig. 2) and the growth (Fig. 3) of the threatened species *R. boylii.* This effect then indirectly benefits the development in *P. regilla* potentially via reduced performance by *R. boylii*.

The design of our competition experiment focused on the relative impact of inter vs. intra-specific competition. Other studies have demonstrated species differences in tadpoles exposed to carbaryl that alter competitive outcomes (e.g., Bulen & Distel, 2011; Distel & Boone, 2011). A previous study on competition between these two species did not find a strong inter-specific competitive effect, although the finding was based on tadpole mortality and not sub-lethal measures (Kupferberg, 1997). In concordance with this, we did not find any significant difference in mortality due to competitive interactions. However, we found interesting sub-lethal differences between *R. boylii* and *P. regilla* when competing interspecifically. When in the presence of *R. boylii, P. regilla* tadpoles fare better than with conspecifics regardless of pesticide presence (Fig. 4). Interestingly, *P. regilla* did not significantly alter its feeding rate in the presence of *R. boylii*, but did significantly increase growth and development. Feeding rates increased for *R. boylii* in the presence of *P. regilla*, and yet resulted in significantly reduced growth. This might be because *P. regilla* were more efficient foragers in the experiment and thus able to consume more food than *R. boylii.* Our measure of feeding rate focuses on the number of tadpoles feeding at four particular days. This does not allow us to measure the relative amount of food consumed over the entire experiment and might be misleading. We did not detect an overall delayed developmental response in *R. boylii* due to competitor presence, but this might simply be because *R. boylii* development is relatively slower and an effect might have been observed if the experiment was run longer.

Predation

The combined stressors of the pesticide carbaryl with an invasive crayfish predator had strikingly lethal consequences for the threatened species, *R. boylii,* while having no effects on survival for its competitor, *P. regilla.* The increased susceptibility to predation resulted in a dramatic decrease in survival to 50% for *R. boylii* tadpoles despite nearly no mortality in pesticide or predator treatments alone (Fig. 5). In terms of evaluating a “safe” pesticide exposure for *R. boylii* tadpoles, we see that the LC_{50} estimate from experiment 1 for carbaryl of nearly 600 µg/l in essence becomes an order of magnitude lower (50 µg/l) when combined with a crayfish predator. That is, the concentration at which 50% of the animals die becomes only 50 µg/l in the presence of the invasive predator. The key revelation of this study is that the examination of the stressors separately might conclude that alone neither the insecticide carbaryl or an invasive crayfish would cause significant mortality for either amphibian species. Yet, the combination of these effects yields a dramatic reduction in survival for *R. boylii.*

Multiple stressors are known to increase the effects of pesticides on some amphibians (Sih et al., 2004). Newt predator cues alone significantly increase the lethality of pesticides to several species of tadpoles (Relyea & Mills, 2001; Relyea, 2003). In contrast, we found no lethal effects of combined carbaryl and crayfish cues during the pre-crayfish release exposure, although we note that this is done over a significantly shorter time period and at a lower pesticide concentration than the Relyea studies (3 days vs. 2 weeks). The lethal effects we observed in this study were due to predation rather than tadpoles dying due to the combined stress of carbaryl and predatory cue. During behavioral observations, we, at no time, saw crayfish consuming dead tadpoles but rather live, although somewhat incapacitated, ones. In this sense, the decreased survival of *R. boylii* tadpoles was due primarily to their increased susceptibility to predation. This phenomenon of increased susceptibility to predation has been noted in other organisms, such as rainbow trout (Little et al., 1990) and *Daphnia* (Dodson et al., 1995), but not in tadpoles, and particularly not with a threatened species. Bridges (1997) recorded a reduction in swimming speed with carbaryl exposure but did not measure predatory effects in that study. In a separate study, Bridges (1999a) examined the effects of a newt predator on tadpole prey and found significant reduction in survival when prey were exposed and the predator was not (albeit at a significantly higher concentration, 2.5 mg/l). She tested the effects when both were exposed, but did not find a significant increase in predation due to the negative impacts of the pesticide on predators. In our study, we found no ill effects of pesticide exposure on the crayfish predator or on *P. regilla* tadpoles. There were also no lethal effects of the pesticide alone on *R. boylii* tadpoles. Rather, the pesticide carbaryl somehow altered the *R. boylii* tadpoles into becoming more susceptible to predation by crayfish.

When we examined both species behavior over the last 4 days, we in fact see that they behave quite similarly. Interestingly, refuge use decreased in the presence of predators for both species. Since crayfish are benthic predators, tadpoles responded by using the surface more (Fig. 7), and hence used refuge on the tank bottom less. Yet, in treatments with *P. regilla*, *R. boylii* tadpoles were significantly less active than predator control treatments, a common anti-predatory behavior for many tadpoles species. These post-crayfish release observations seem to suggest behavioral responses to predation events rather than a key insight into the alteration of behaviors by the pesticide (e.g., carbaryl reduces surface use and hence results in higher predation for carbaryl/crayfish treatments). To examine the effect of carbaryl, it is also informative to examine the behavioral data from days 1–3 prior to predator release. Since both species did not respond to the predatory cues prior to release, it is likely that tadpoles are not responding to predator cues, but rather to cues of predation (i.e., crushed tadpoles). Therefore, once *R. boylii* tadpoles were being consumed, both species then began exhibiting anti-predatory responses (around day 5). If we examine the effects of carbaryl on the two species behavior prior to crayfish release though, we can see that the pesticide alters surface use, refuge use, and activity in *R. boylii* tadpoles. The decrease in surface use and activity likely represents tadpoles that are adversely affected by the pesticide and therefore more easily preyed upon. Interestingly, there were no significant effects of pesticide on *P. regilla* tadpole behavior in days 1–3 suggesting that these tadpoles are not as susceptible to carbaryl exposure. This is in congruence with lethality data as well where *P. regilla* tadpoles are more resilient at an order of magnitude higher concentrations.

A clear negative effect of carbaryl on *P. regilla* was not detected in this study as is supported by other work on the species (e.g., Sparling et al., 2001). Due to its widespread occurrence, studies have suggested using the species as a surrogate for studying threatened species (Sparling et al., 2001). Using a surrogate species has many dangers on its own (see Caro et al., 2005), but can be particularly dangerous when dealing with contaminants. Given the variation in impacts of pesticides on amphibians (Bridges & Semlitsch, 2000; Kerby et al., 2010), extreme caution should be used when generalizing impacts (or lack thereof) from one species to another. Furthermore, this study exhibits that these impacts might not be revealed in single species toxicity tests, but only when combined with other organisms. Further study including predators and a surrounding community can change dynamics for the different species involved. Some larger mesocosm studies have suggested that the presence of pesticide indirectly benefits tadpoles via the removal of invertebrate competitors (Boone et al., 2004) or through the removal of a dangerous pathogen (Brown et al., 2013). In this case we show that while beneficial to one species, it might be detrimental to another and therefore each case should be considered on its own.

Conclusion

The field of behavioral ecology emphasizes the important role that behavior has for explaining population and community dynamics (Schmitz et al., 1997; Luttbeg & Kerby, 2005). A change in behavior often results in non-adaptive results, reduction of growth, feeding, mating opportunities, and sometimes death. A change in anti-predatory behavior can be particularly important because it is directly linked to survival. For example, as noted above, whether a species increases or decreases its activity in response to a given pesticide can explain how exposure to that pesticide influences relative susceptibility to predators. In addition, the behavioral ecotoxicological assays themselves can be done in containers with multiple species present. For example, when competing prey species are combined with one another and/or with a predator, the effects of carbaryl at a low concentration for longer periods of time may be either eliminated or magnified. Regardless, the inclusion of ecologically important behaviors into current sublethal experiments should be encouraged. Several of the sub-lethal effects that ecotoxicologists currently measure may themselves be caused by modification of behaviors (e.g., reduction in growth due to reduced feeding). In ecotoxicology, it should not only be important to understand the chemical mechanism that causes contaminants to effect species, but also behavioral mechanisms that affect the species ecology.

The field of ecotoxicology can borrow methods and insights from behavioral ecology as to what behaviors are important to monitor in certain species. If behavioral alterations are detected, further study in a field simulated experiment may improve understanding. Changes in the behaviors of prey may depend entirely on changes in the behavior of predators as well. Bridges (1999b) found that depending on the particular organisms exposed to pesticide, different outcomes occurred between predators and prey. While these sometimes seem like subtle differences, a past review in ecology suggests that the role of behavior (trait mediated effects) may be more important than actual mortality (density mediated effects) (Preisser et al., 2005). Understanding how contaminants alter these interactions may be complex, but also may provide the answer to as of yet unsolved questions—such as clear mechanisms for amphibian decline in certain areas.

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