

## TOXICITY OF TWO INSECTICIDES TO CALIFORNIA, USA, ANURANS AND ITS RELEVANCE TO DECLINING AMPHIBIAN POPULATIONS

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**Abstract**—Contaminants have been associated with population declines of several amphibian species in California (USA). Pesticides from the Central Valley of California are transported by winds into the Sierra Nevada Mountains and precipitate into wet meadows where amphibians breed. The present study examined the chronic toxicity of two of the insecticides most commonly used in the Central Valley and found in the mountains, chlorpyrifos and endosulfan, to larval Pacific treefrogs (*Pseudacris regilla*) and foothill yellow-legged frogs (*Rana boylei*) and discusses the implications of this toxicity to declining amphibian populations. Larvae were exposed to the pesticides from Gosner stages 25 to 26 through metamorphosis. The estimated median lethal concentration (LC50) for chlorpyrifos was 365 µg/L in *P. regilla* and 66.5 µg/L for *R. boylei*. Time to metamorphosis increased with concentration of chlorpyrifos in both species, and cholinesterase activity declined with exposure concentration in metamorphs of both species at Gosner stages 42 to 46. For endosulfan, the estimated LC50 was 15.6 µg/L for *P. regilla* and 0.55 µg/L for *R. boylei*. All *R. boylei* exposed to concentrations of greater than 0.8 µg/L died before they entered metamorphosis. *Pseudacris regilla* remains relatively abundant and is broadly distributed throughout California. In contrast, *R. boylei* is among the species experiencing severe population declines. The present study adds to the increasing evidence that pesticides are very harmful to amphibians living in areas that are miles from sources of pesticide application.

**Keywords**—Endosulfan   Chlorpyrifos   Amphibian population declines   *Rana boylei*   *Pseudacris regilla*

## INTRODUCTION

Amphibian populations around the world are experiencing severe declines, even extirpations [1,2]. Many of the most severely declining populations have montane distributions [3,4]. This may be because montane areas are especially susceptible to transport and deposition of organic contaminants [5]. In California (USA), population declines have been reported in several montane and submontane species [6–8]. Suggested causal mechanisms in these declines include contaminants [7], habitat destruction and diseases [9], and introduced predators [10].

Evidence is growing that insecticides are having negative effects on amphibian populations in the Sierra Nevada Mountains of California. The San Joaquin Valley, an intensely agricultural region, lies upwind of the more pristine montane habitats where amphibians are disappearing. Thousands of kilograms of active ingredient pesticides are sprayed on crops in this region annually. The most commonly used insecticides include the organochlorine endosulfan and the cholinesterase (ChE)-inhibiting organophosphorous insecticides, such as chlorpyrifos, diazinon, and malathion [11] (California Pesticide Information Portal, <http://www.cdpr.ca.gov/docs/pur/purmain.htm>). These insecticides are found in air, snow, and surface waters of national parks and other sites in the Sierra Nevada Mountains [12,13]. They also have been detected in amphibian tissues [14–16]. Whereas acute toxicity data exist for a few of these insecticides [17], the effects of long-term

or chronic exposure are less well known. Pesticides can have many adverse effects on amphibians, including decreased growth and developmental rates, increased incidence of external abnormalities, impaired reproductive potential, and death. They also can interact with other factors to alter mortality [18]. The purpose of the present study is to explore the chronic effects of two insecticides commonly found in the Sierra Nevada Mountains, chlorpyrifos and endosulfan, and to compare dose–response relationships found in the laboratory with insecticide data from the field.

## MATERIALS AND METHODS

*Source and treatment of subjects*

Six egg masses of *Pseudacris regilla* were collected in May 2004 from coastal ponds at Point Reyes (CA, USA; 38°02.850'N, 122°47.780'W and 38°03.611'N, 122°48.495'W). Four *Rana boylei* egg masses were collected at Soda Creek in Lake County (CA, USA; 39°25.300'N, 122°58.700'W). These sites lie upwind of agricultural activities in the Central Valley and are not near any areas where pesticides are used in significant quantities. Eggs were shipped overnight to the Cooperative Wildlife Research Laboratory. Eggs were incubated in reconstituted, medium soft water [19] until they hatched and reached the free-swimming stage (Gosner stage 25 [20]), which occurred approximately 10 to 14 d after shipment. All tadpoles were kept together in the same aquarium so that larvae from different egg masses were mixed before assignment to treatment. Six tadpoles of a single species were placed into each 8-L, all-glass, chemically cleaned aquarium that had been randomly assigned to treatment and location within holding tanks; three replicate aquaria were used for each combination of species, chemical, and concentration plus solvent controls. Room temperature was

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kept at 21 to 22°C, and photoperiod was maintained at 12:12-h light:dark. Tadpoles were fed boiled, organic romaine lettuce and high-protein, flaked fish food ad libitum. The food had been analyzed previously for endosulfan and chlorpyrifos, and concentrations were less than the detection limits. The design was static renewal, with 100% replacement of water twice per week. Husbandry and treatment were approved by the Southern Illinois University Institutional Animal Care and Use Committee.

Each day, tadpoles were observed for unusual behavior, morbidity, and death. Death was determined by lack of response to gentle prodding or obvious discoloration. Dead animals were removed to prevent mold and bacterial buildup. At 10 and 30 d of exposure, *P. regilla* tadpoles were weighed to the nearest 0.001 g, individually measured for snout-vent length (SVL) to the nearest 0.1 mm with electronic calipers, and staged for development. *Rana boylei* tadpoles were treated similarly at 14, 34, and 54 d postinitiation; differences in interperiod duration for the two species were related to the longer developmental period of *R. boylei*. To avoid undue stress and prolonged exposure to air, tadpoles were staged to one of the following categories: Prelimb (denoted by absence of hind limbs to the unaided eye, corresponding to Gosner stages 25–26), limb bud (hind limb visible to naked eye but no clear joint formed, Gosner stages 27–34), hind limb (knee joint apparent, Gosner stages 35–41), metamorph (at least one forelimb present, Gosner stages 42–45), and juvenile (complete resorption of tail, Gosner stage 46). At Gosner stage 42, animals were individually measured, weighed, and placed in slanted jars with approximately 50 to 75 ml of water from their respective treatment until completion of metamorphosis. At that time, they were measured again, killed with tricaine methane sulfonate (MS-222 or Finquel; Sigma-Aldrich), and stored at –80°C until analysis. It takes 40 to 45 d after hatching for *P. regilla* and 80 to 85 d for *R. boylei* to complete metamorphosis.

#### Chemical treatment

Insecticides used in dosing were reagent-grade chlorpyrifos (purity, 99%; Chemical Abstracts Service no. 2921-88-2; Res-tek) and a 50:50 mixture of endosulfan I and endosulfan II (purity, 99%; Chemical Abstracts Service no. 115-29-7; Res-tek). Reagent grade was used instead of commercialized formulations, because pesticides in the Sierras are products of volatilization and subsequent deposition, not of direct agricultural application. They were dissolved in acetone to make stock solutions and kept at –20°C. Individual doses were made by pipetting prescribed amounts of stock into amber vials with sufficient acetone to make a total volume of 2 ml. Treatments consisted of controls (2 ml of reconstituted water only), acetone controls (2 ml of acetone), and 0.8, 3.12, 12.5, 50, and 200 µg/L of endosulfan or chlorpyrifos. Doses were added to the appropriate aquaria with each water change.

Water samples for analysis were taken within 12 h of a water change and adding pesticides to aquaria, but extraction was delayed. Extraction of insecticides in water to validate concentrations followed the method described by Belden et al. [21] using solid-phase extraction, gas chromatography (Hewlett-Packard model 5890), and nitrogen-phosphorus detection. Detection limits for the present study were 2.8 µg/L for chlorpyrifos and 1.5 µg/L for endosulfan. Thus, our lowest test concentrations were less than the detection limits. Measured concentrations averaged 61% of nominal for chlorpyrifos and 58% for endosulfan. All statistical analyses are conducted on nominal concentrations.

For tissue analyses, metamorphs were freeze-dried and finely diced with acetone-rinsed, stainless-steel scissors in a Teflon® bowl. They were further dried with sodium sulfate and ultrasonically extracted three times with a 50:50 solution of hexane and dichloromethane. Samples were cleaned through a florisil column, reconcentrated, and analyzed on a gas chromatography/electron-capture detector. Detection limits in tissues were 10 ng/g.

Half of the metamorphs or juveniles exposed to chlorpyrifos were randomly selected from each concentration and analyzed for total ChE using a photometric method [22] modified for a 96-well microplate reader. Each sample was run in triplicate or until a coefficient of variation among replicates was less than 5%. The mean activity rate was then calculated for each animal and converted to µmol/g tissue/s for ChE activity.

#### Statistical analyses

Statistical analyses were conducted with SAS software (Ver 9.1.3; SAS Institute). For exposures that met a goodness-of-fit test, we used a logistic method to determine dose-response relationships. Data were analyzed on a per-tank basis using the aggregate function in SAS. For exposures that were statistically different from a goodness of fit, we used a 10% trimmed Spearman-Kärber (SK) method for determining median lethal concentration (LC50) [23]. We also provide trimmed SK estimates for the other exposures for consistency.

For other endpoints, such as growth and development, data were first checked for meeting the assumptions of parametric statistics. If necessary, they were log-transformed (continuous data) or arcsine-transformed (proportions) and rechecked for meeting assumptions. All endpoint data met the assumptions of parametric statistics either before or after transformation. Before inclusion of all treatments, specific comparisons of endpoints were made between actual and acetone controls. None of these comparisons were significant ( $p > 0.30$ ), and control groups were combined. For analyses of variance, mean values of endpoint data were determined for each aquarium, and these aquaria were considered to be the experimental units. For final measurements at metamorphosis, we also used an analysis of covariance (ANCOVA) with time to metamorphosis as the covariate to adjust for duration of growing periods. We used  $\alpha = 0.05$  to judge statistical significance.

## RESULTS

#### Survival

Survival estimates with chlorpyrifos differed between species. The chlorpyrifos exposure for *P. regilla* did not meet the goodness-of-fit test. Therefore, the SK LC50 for this species was 365 µg/L. For *R. boylei*, the logistic LC50 was 108.7 µg/L (95% confidence interval, 0–361.3 µg/L), and the SK LC50 was 66.5 µg/L. With analyses of variance on the number of animals dying by species and treatment, we found significant differences between species ( $F_{1,42} = 17.84$ ,  $p = 0.0002$ ) and concentration ( $F_{5,42} = 5.96$ ,  $p = 0.006$ ) but not in their interaction. Mortality was greater in *R. boylei* than in *P. regilla* and greater at 200 µg/L than at any other concentration. Mean time to death for both species did not differ statistically among treatments (Table 1).

For endosulfan, the SK LC50 for *P. regilla* was 15.6 µg/L. For *R. boylei*, the logistic LC50 was 0.233 µg/L (95% confidence interval, 0–0.528 µg/L), and the SK LC50 was 0.55 µg/L. The proportion of animals dying differed between

Table 1. Percentage survival and time to death for *Pseudacris regilla* and *Rana boylei* tadpoles exposed through metamorphosis to chlorpyrifos or endosulfan<sup>a</sup>

Dose ( $\mu\text{g/L}$ )	<i>n</i>	<i>Pseudacris regilla</i>		<i>Rana boylei</i>	
		% Survival	Days to death	% Survival	Days to death
Chlorpyrifos					
0	42	92.7 (9.1)	17.7 (7.4)	80.4 (19.5)	30.8 (22.6)
0.8	18	100		57.8 (15.4)	44.0 (12.1)
3.12	18	94.3 (9.8)	43.0	77.8 (19.2)	41.5 (4.9)
12.5	18	83.3 (28.9)	23.5 (27.6)	72.0 (19.0)	33.4 (16.2)
50	18	77.6 (9.4)	40.0 (22.0)	74.3 (12.5)	62.3 (26.0)
200	18	66.6 (16.5)	42.2 (13.9)	16.7 (28.9)	31.4 (8.3)
Endosulfan					
0	42	92.7 (9.1)	20.5 (7.8) A	80.4 (19.5)	35.3 (28.3) A
0.8	18	94.3 (0.8)	29.0 (21.7) A	11.1 (9.6)	33.6 (21.8) A
3.12	18	83.0 (0)	33.2 (23.1) A	0	14.3 (6.4) AB
12.5	18	17.8 (1.9)	23.6 (7.9) A	0	11.7 (5.3) B
50	18	0 C	14.3 (15.6) B	0	7.3 (2.2) B
200	18	0 C	15.5 (18.7) B	0	6.6 (0.6) B

<sup>a</sup> Values are presented as the mean (standard deviation). Values with the same uppercase letters for a column cannot be distinguished at  $p = 0.05$  based on Tukey's honestly significant difference test.

species ( $F_{1,42} = 68.54$ ,  $p < 0.0001$ ), concentration ( $F_{5,42} = 68.76$ ,  $p < 0.0001$ ), and interaction between the two terms ( $F_{5,42} = 13.32$ ,  $p < 0.0001$ ). For *P. regilla*, mean time of death varied significantly among treatments ( $F_{5,10} = 4.57$ ,  $p = 0.0199$ ), with animals in the 50 or 200  $\mu\text{g/L}$  treatment dying more quickly than those in other treatments. *Rana boylei* also showed significant differences among concentrations in mean time to death ( $F_{5,10} = 7.61$ ,  $p = 0.0034$ ). Larvae in the control or 0.8  $\mu\text{g/L}$  group survived three- to fivefold longer than those at concentrations greater than 3.0  $\mu\text{g/L}$ , and the trend was negative between exposure concentration and mean time to death (Table 1). No differences were found between species in time to death.

#### Effects on development and growth

Chlorpyrifos did not affect the body mass or SVL of *P. regilla* tadpoles at either 10 or 30 d of exposure. Nor did it affect their rate of early development, as measured by the percentage of tadpoles with well-developed hind limbs at 30 d of exposure (Table 2). Similarly, chlorpyrifos had no effect on body mass or SVL of *R. boylei* during the first 14 d of exposure. By 34 d, however, a significant difference was found in SVL (Table 3) because of concentration ( $F_{5,21} = 3.35$ ,  $p = 0.029$ ); tadpoles exposed to 200  $\mu\text{g/L}$  were shorter than controls. No differences were detected in body mass at this time ( $p = 0.3152$ ). At 54 d, body mass ( $F_{5,19} = 3.46$ ,  $p = 0.030$ ) varied among treatments, with tadpoles in the 200  $\mu\text{g/L}$  treatment weighing less than those at all other treatments except 50  $\mu\text{g/L}$ , but no significant differences were found in SVL at that time. *Pseudacris regilla* tadpoles took an increasingly longer time to reach metamorphosis as the concentration of chlorpyrifos increased ( $F_{5,15} = 5.46$ ,  $p = 0.005$ ) (Table 4). Tadpoles at 200  $\mu\text{g/L}$  took approximately 7 d longer to reach Gosner stage 42 than did those exposed to lower concentrations. Based on ANCOVA at metamorphosis, a difference was found in body mass ( $F_{3,31} = 27.44$ ,  $p = 0.0001$ ) because of treatment and across time ( $F_{1,31} = 9.40$ ,  $p = 0.0057$ ), but no difference was found in their interaction. The SVL was not statistically different as a result of treatment or the interaction term, but it did increase with time to metamorphosis ( $F_{1,45} =$

19.63,  $p < 0.0001$ ). Time to metamorphosis also increased with chlorpyrifos concentration in *R. boylei* ( $F_{5,73} = 4.28$ ,  $p = 0.016$ ). Significant time effects were found in ANCOVAs for body mass ( $F_{1,73} = 385.05$ ,  $p < 0.0001$ ) and SVL ( $F_{1,73} = 454.98$ ,  $p < 0.0001$ ), but no significant concentration or interaction effects were detected.

Endosulfan significantly affected growth and development rates in both species. For *P. regilla*, body mass ( $F_{5,11} = 38.42$ ,  $p < 0.0001$ ) and SVL ( $F_{5,11} = 26.72$ ,  $p < 0.0001$ ) (Table 2) differed among treatments after 10 d of exposure. Tadpoles exposed at 12.5 to 200  $\mu\text{g/L}$  were lighter and smaller than those exposed at lower concentrations. At 30 d, all animals at 50 and 200  $\mu\text{g/L}$  had died, but differences were still present among existing treatments for body mass ( $F_{3,11} = 17.34$ ,  $p = 0.0002$ ) and SVL ( $F_{3,11} = 30.98$ ,  $p < 0.0001$ ); survivors at 12.5  $\mu\text{g/L}$  were lighter and smaller than those at other exposure concentrations. At this time, fewer tadpoles had reached the hind limb stage at 12.5  $\mu\text{g/L}$  than at other concentrations ( $F_{3,11} = 9.24$ ,  $p = 0.002$ ). For *R. boylei*, significant differences were found among treatments in body mass ( $F_{4,19} = 7.52$ ,  $p = 0.0016$ ) and SVL ( $F_{4,19} = 5.02$ ,  $p = 0.009$ ) after 14 d (Table 3). At 34 d and beyond, survivors were only found at control and 0.8  $\mu\text{g/L}$  concentrations. Body mass did not differ between these two treatments, but tadpoles exposed to 0.8  $\mu\text{g/L}$  were 58% lighter than controls at 54 d ( $F_{1,8} = 9.26$ ,  $p = 0.0188$ ).

For animals entering Gosner stage 42, time to metamorphosis did not differ across treatments for *P. regilla* or *R. boylei* exposed to endosulfan (Table 4). An ANCOVA on *P. regilla* body mass with time to metamorphosis as covariate revealed significant effects of treatment ( $F_{3,31} = 27.14$ ,  $p < 0.0001$ ) and time ( $F_{1,31} = 9.40$ ,  $p = 0.006$ ), but their interaction was nonsignificant. For SVL, only the time effect was significant ( $F_{1,31} = 46.69$ ,  $p < 0.0001$ ).

Tadpoles of both species quickly developed distinct, right-angle bends in their bodies just below the head when exposed to endosulfan concentrations of greater than 3  $\mu\text{g/L}$ . The incidence of these abnormalities increased with both concentration and time and occurred in 100% of tadpoles exposed to endosulfan at 50  $\mu\text{g/L}$  or greater within a couple of days.

Table 2. Growth, snout–vent length (SVL), and development (% hind limb development) of *Pseudacris regilla* tadpoles exposed to chlorpyrifos or endosulfan<sup>a</sup>

Dose (µg/L)	Day 10			Day 30		
	Body mass (g)	SVL (mm)	% Hind limb	Body mass (g)	SVL (mm)	% Hind limb
<b>Chlorpyrifos</b>						
0	0.24 (0.04)	10.8 (0.8)	0	0.45 (0.05)	14.4 (0.4)	92.8 (8.9)
0.8	0.28 (0.12)	11.2 (1.3)	0	0.51 (0.05)	14.8 (1.0)	83.3 (16.7)
3.12	0.18 (0.03)	10.6 (0.4)	0	0.47 (0.07)	14.2 (0.4)	83.3 (28.8)
12.5	0.16 (0.09)	9.8 (2.7)	0	0.27 (0.18)	11.5 (3.6)	55.5 (50.9)
50	0.18 (0.08)	10.5 (1.6)	0	0.39 (0.09)	13.6 (1.3)	86.7 (23.1)
200	0.14 (0.05)	9.6 (1.7)	0	0.36 (0.09)	12.9 (1.6)	76.7 (25.1)
<b>Endosulfan</b>						
0	0.24 (0.04) A	10.8 (0.8) A	0	0.45 (0.05) A	14.5 (0.4) A	92.8 (8.9) A
0.8	0.20 (0.004) A	10.8 (0.2) A	0	0.36 (0.05) A	13.9 (0.6) A	94.4 (9.6) A
3.12	0.20 (0.5) A	11.0 (1.1) A	0	0.35 (0.10) A	13.4 (1.7) A	72.2 (48.1) A
12.5	0.07 (0.19) C	7.4 B	0	0.07 (0.06) B	7.6 (1.4) B	0 B
50	0.039 C	6.6 BC	0			
200	0.032 C	4.7 C	0			

<sup>a</sup> Values are presented as the mean (standard deviation). Values with the same uppercase letter for a chemical and column cannot be distinguished at  $p < 0.05$ .

### ChE and residues

In both species, ChE activity declined significantly as concentration increased (Fig. 1). In *P. regilla*, the regression between ChE activity and treatment was

$$\text{ChE} = 2.379 - 0.0030(\text{concentration}),$$

$$r^2 = 0.4424, p < 0.0001$$

and for *R. boylei*, it was

$$\text{ChE} = 1.593 - 0.0051(\text{concentration}),$$

$$r^2 = 0.3332, p < 0.0001$$

Tissue concentrations of chlorpyrifos increased with exposure concentrations. The best fit obtained (as determined by  $r^2$ ) between water and tissue concentrations of insecticides for *P. regilla* was defined as

$$[\text{tissue}] = 6.922 + 5.568[\text{water}],$$

$$r^2 = 0.964, p < 0.0001$$

where [tissue] is the concentration of chlorpyrifos in whole-body metamorphs (ng/g dry wt) and [water] is the nominal

concentration in water (µg/L). The mean concentration of chlorpyrifos in *P. regilla* exposed to 200 µg/L was 588 ng/g dry weight. Tadpoles typically are 85 to 90% water [24], so the wet-weight concentration would be approximately 65 ng/g. For *R. boylei* and chlorpyrifos, the best fit was defined as

$$\log_{10}[\text{tissue}] = 1.0117 + 1.3979 \log_{10}[\text{water}],$$

$$r^2 = 0.819, p < 0.0001$$

The mean concentration of chlorpyrifos at the 200 µg/L exposure in this species was 9,899 ng/g dry weight (~1,089 ng/g wet wt). The difference between the two species could be related to the longer duration of development for *R. boylei*.

Insufficient numbers of *R. boylei* reached metamorphosis in the endosulfan treatment to provide a valid analysis of tissue concentrations. For *P. regilla*, however, sample sizes were small, but we were able to obtain sufficient biomass to conduct a preliminary analysis (Fig. 2). Clear evidence of bioconcentration was found, especially with exposure concentrations of 12.5 µg/L or greater, where bioconcentration factors ranged from 247 to 283. Each of the three components of endosulfan (endosulfan I, endosulfan II, and endosulfan sulfate) had a

Table 3. Body mass and snout–vent length (SVL) for *Rana boylei* tadpoles exposed through metamorphosis to chlorpyrifos and endosulfan<sup>a</sup>

Dose (µg/L)	Day 14		Day 34		Day 54	
	Body mass (g)	SVL (mm)	Body mass (g)	SVL (mm)	Body mass (g)	SVL (mm)
<b>Chlorpyrifos</b>						
0	0.079 (0.025)	7.74 (0.65)	0.197 (0.087)	11.19 (1.51) A	0.515 (0.133) A	14.59 (1.72)
0.8	0.069 (0.008)	7.71 (0.64)	0.194 (0.072)	10.86 (0.92) AB	0.542 (0.129) A	13.81 (1.49)
3.12	0.082 (0.033)	7.00 (0.13)	0.218 (0.031)	11.3 (0.17) AB	0.585 (0.178) A	14.56 (1.65)
12.5	0.059 (0.017)	7.41 (0.67)	0.144 (0.061)	9.74 (1.44) AB	0.565 (0.176) A	14.13 (1.73)
50	0.057 (0.015)	7.04 (0.58)	0.159 (0.050)	9.53 (0.42) AB	0.410 (0.018) AB	13.48 (1.13)
200	0.055 (0.028)	7.60 (0.44)	0.099 (0.056)	8.74 (0.83) B	0.250 (0.034) B	12.45 (0.25)
<b>Endosulfan</b>						
0	0.079 (0.025) A	7.74 (0.65) A	0.197 (0.087)	11.19 (1.50)	0.515 (0.133) A	14.60 (1.72)
0.8	0.03 (0.013) B	6.78 (0.52) AB	0.132 (0.019)	10.11 (0.63)	0.298 (0.057) B	12.57 (0.91)
3.12	0.079 (0.011) AC	6.34 (0.66) B				
12.5	0.032 (0) BC	6.42 (0) AB				
200	0.062 ABC	7.13 AB				

<sup>a</sup> Values are presented as the mean (standard deviation). Values with the same uppercase letter for a chemical and column cannot be distinguished at  $p < 0.05$ .

Table 4. Time to climax, body mass, and snout–vent length (SVL) at metamorphosis for *Pseudacris regilla* and *Rana boyleii* exposed to chlorpyrifos and endosulfan<sup>a</sup>

Dose ( $\mu\text{g/L}$ )	<i>Pseudacris regilla</i>			<i>Rana boyleii</i>		
	Body mass (g)	SVL (mm)	Days to climax	Body mass (g)	SVL (mm)	Days to climax
Chlorpyrifos						
Control	0.35 (0.08)	14.1 (1.3)	42 (5) A	0.96 (0.27) A	17.5 (1.6) A	86 (13) A
0.8	0.43 (0.11)	14.9 (1.4)	40 (5) A	0.91 (0.20) A	17.6 (1.5) A	90 (18) A
3.12	0.36 (0.11)	14.6 (1.7)	44 (5) AB	0.92 (0.14) A	17.6 (1.1) A	92 (15) AB
12.5	0.37 (0.12)	13.9 (1.8)	43 (6) A	0.92 (0.19) A	17.2 (1.6) AB	95 (20) AB
50	0.31 (0.11)	13.8 (1.6)	43 (6) AB	0.76 (0.18) AB	16.8 (1.9) AB	101 (17) AB
200	0.35 (0.10)	14.5 (1.4)	50 (8) B	0.50 (0.14) B	15.0 (1.7) B	120 (95) B
Endosulfan						
Control	0.35 (0.08)	14.1 (1.3)	41 (4)	0.96 (0.27)	17.5 (1.6)	87 (12)
0.8	0.34 (0.09)	13.6 (1.1)	41 (5)	1.09 (0.39)	17.4 (1.4)	96 (1)
3.12	0.33 (0.08)	12.9 (2.6)	44 (10)			
12.5	0.26 (0.09)	11.4 (1.2)	46 (9)			

<sup>a</sup> Values are presented as the mean (standard deviation). Values with the same uppercase letter for a chemical and column cannot be distinguished at  $p < 0.05$ .

close linear relationship to water concentrations (each  $r^2$  value  $\geq 0.997$ ,  $p < 0.0001$ ). At 0.8 and 3.12  $\mu\text{g/L}$  of endosulfan, sulfate dominated, but as the concentration of endosulfan in water increased, the relative percentage of endosulfan I increased and that of endosulfan II decreased.

## DISCUSSION

The present study demonstrates that both chlorpyrifos and endosulfan are highly toxic to *P. regilla* and *R. boyleii* and that *R. boyleii* is more sensitive than *P. regilla* to these insecticides. For chlorpyrifos, the LC50s were in the few hundred parts-per-billion range, and the estimated LC50 for *P. regilla* was approximately fivefold greater than that for *R. boyleii*. Endosulfan, however, was considerably more toxic than chlorpyrifos in both species, with the estimated LC50s in the few or sub parts-per-billion range. Endosulfan was 21-fold more toxic than chlorpyrifos in *P. regilla* and nearly 121-fold more toxic in *R. boyleii*. This makes endosulfan very highly toxic to this species. In an acute toxicity test on *R. boyleii*, however, 100% of tested animals died within 24 h at 5 to 40  $\mu\text{g/L}$  of chlorpyrifos oxon, the common degradate of chlorpyrifos. The 24-h LC50 for the parent compound was approximately 3 mg/L [25], however, so this insecticide, when converted by bacteria or other factors, also may pose a hazard to *R. boyleii* in subacute or chronic exposures. This difference in sensitivity is important, because *P. regilla* populations are still compar-

atively stable in California, even in montane areas, whereas *R. boyleii* is one of the species that has declined in recent years [8]. Pesticide sampling of snow and standing water in the Sierras has been limited. Water samples from the Sierras of California have contained endosulfan concentrations within the range of lethality for *R. boyleii*, however, and some have exceeded the LC50 for this species [13]. In that study, endosulfan sulfate was not quantified, so the total concentrations of endosulfan would have been even higher. Fellers et al. [16] found water concentrations of endosulfan ranging from 0.94 to 4.08 ng/L among four sites in *Rana muscosa* habitat. Chlorpyrifos concentrations, not including the oxon form, ranged from 0.17 to 12 ng/L, and sites with declining or extirpated concentrations had higher concentrations of both pesticides than found at sites with extant populations of *R. muscosa*. In a survey of 103 wetland sites sampled over 2002 and 2003, 43 sites were positive for endosulfan in sediment, and 24 were positive for chlorpyrifos (Sparling et al., unpublished data). Endosulfan concentrations ranged from the limit of detection (0.77 mg/kg) to 48.7 mg/kg, and concentrations of chlorpyrifos or chlorpyrifos oxon ranged from 0.77 to 161 mg/kg. Sediment concentrations do not directly reflect water concentrations, but tadpoles often inhabit surface layers of sediment and the higher

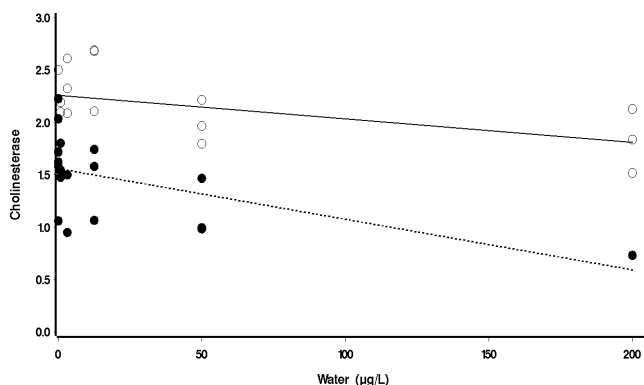


Fig. 1. Cholinesterase (ChE) activity ( $\mu\text{mol/g}$  tissue/s) in *Pseudacris regilla* (solid line and circles) and *Rana boyleii* (dashed line and dots) tadpoles (Gosner stages 35–41) exposed to chlorpyrifos.

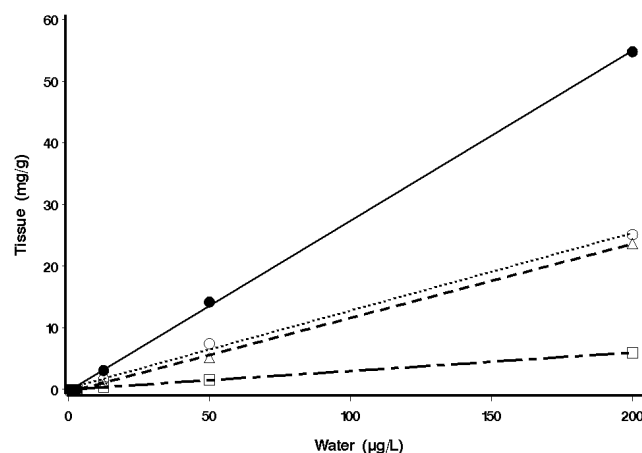


Fig. 2. Endosulfan residues in *Pseudacris regilla* metamorphs versus aqueous concentration of endosulfan. Total endosulfan (solid line), endosulfan I (large dashes), endosulfan II (dotted), endosulfan sulfate (small dashes) are shown.

sediment concentrations at least suggest that. Coupled with its more terrestrial lifestyle, the lower sensitivity of *P. regilla* to these contaminants seems to place it at a lower risk than *R. boylei*.

A few studies have examined lethal effects of chlorpyrifos or endosulfan in other amphibian larvae. Two estimates of 96-h LC50s for chlorpyrifos were 1 µg/L for American toads (*Bufo americanus*) and 3,000 µg/L for northern leopard frogs (*Rana pipiens*) [26]. In several species of amphibians, acute (96-h) exposures of endosulfan decreased escape and feeding behavior, reduced growth, and increased mortality at concentrations of 45 µg/L and higher [27]. Endosulfan at 0.8 µg/L interacted with water temperatures to result in increased mortality compared to controls in Australian Blue Mountains treefrog (*Litoria citropa*) tadpoles during a 96-h exposure [28]. Over a 30-d period, 10 µg/L of an endosulfan I and II mixture resulted in decreased feeding and growth and, ultimately, a 50% mortality rate in the streamside salamander (*Ambystoma barbouri*) [29]. Along with these studies, our data show that concentrations necessary to induce mortality in amphibians generally decrease with duration of exposure and that *R. boylei* are exceptionally susceptible to endosulfan. Increased toxicity with longer exposure has been commonly observed [30] for gray treefrogs (*Hyla versicolor*) exposed to carbaryl.

Because of the relatively rapid degradation of insecticides, especially chlorpyrifos, the exposure regimen in the present study is best described as episodic. In an ancillary study on endosulfan using the same methods and aquaria, J.A. Hunt (unpublished data) determined that 25 to 80% of the total endosulfan could dissipate within the 4 d between treatments, depending on the initial concentrations. Similarly, Mazanti et al. [31] reported that the half-life for chlorpyrifos in conditions similar to ours was approximately 0.2 to 0.3 d. Populations of amphibians inhabiting Sierra Nevada meadows, however, likely also would be exposed episodically because of repeated thawing and freezing of snow packs. Risk from insecticides can be compounded by episodic bursts of endosulfan and chlorpyrifos into ponds and streams from snowmelt as a result of the dynamics of water solubility and freezing/thawing cycles [32,33].

Overl mortality is the most extreme endpoint of toxicity; however, sublethal effects can occur at concentrations far lower than those that result in acute lethality [18]. Other endpoints include growth, development, time to metamorphosis, and for chlorpyrifos and other organophosphorous pesticides, ChE depression. These factors are important in that they can affect behavior and increase the vulnerability of anuran larvae to predators and hydrological events.

In the present study, chlorpyrifos up to 200 µg/L did not affect growth or development in *P. regilla*. Exposure to chlorpyrifos did increase the time to metamorphosis in this species. Although size at metamorphosis was greater in *P. regilla* at the higher concentrations, the ANCOVA demonstrated that this difference was the result of delayed maturation, not a direct result of the insecticide. In *R. boylei*, chlorpyrifos exposure eventually led to smaller body sizes and delayed maturation. In this species, the time to metamorphosis was not related to changes in body size. These sublethal effects from chlorpyrifos support the concept of a heightened sensitivity in *R. boylei* compared to *P. regilla*. Endosulfan negatively affected body size, developmental rates, and time to metamorphosis in both species, thus supporting the higher toxicity of this insecticide compared to chlorpyrifos. Reduced rates of growth can make

tadpoles subject to a greater range of predators for extended periods [29]. Similarly, delayed development and metamorphosis can be problematic if ponds dry before metamorphosis is complete.

Cholinesterase activity declined with the concentration of chlorpyrifos, and the rate of decline was steeper for *R. boylei* than for *P. regilla*. Endosulfan is not an inhibitor of ChE, so tadpoles exposed to it were not tested. Reduced ChE has been related to impaired behavior and increased mortality in *Rana sphenoccephala* tadpoles [34]. Sparling et al. [14] found that 60 to 100% of the *P. regilla* collected in Sequoia National Park (CA, USA) had ChE values that were 50% or less of those found in the reference population in Lassen Volcanoes National Park (CA, USA). *Pseudacris regilla* collected from sites where *Rana* sp. populations were rated as poor had significantly lower ChE activities compared with those collected from sites where populations were rated as good. In the present study, *P. regilla* did not experience a 50% reduction in ChE activity, even at 200 µg/L, and the regression line estimated that *R. boylei* experienced a 50% decline around 100 µg/L of chlorpyrifos. Extrapolation of laboratory data suggests that the tadpoles in the field had been exposed to considerable concentrations of organophosphorous pesticides.

In the present study, uptake of chlorpyrifos and endosulfan (in *P. regilla*) was highly correlated to exposure concentration, and the highest mean whole-body concentrations were 588 and 9,899 ng/g for *P. regilla* and *R. boylei*, respectively. In one *P. regilla* exposed to 200 µg/L of endosulfan, we found the total endosulfan concentration to be 54,820 ng/g dry weight (~5,500 ng/g wet wt). Sparling et al. [14] found that more than 50% of *P. regilla* adults and tadpoles collected from Sequoia and Yosemite National Parks had detectable concentrations of chlorpyrifos or diazinon in their bodies; however, mean concentrations of chlorpyrifos were only 13 and 8 ng/g wet weight in the two parks, respectively. Endosulfan in one form or another was detected in 60 to 86% of the *P. regilla* collected from areas in the central and southern Sierra Nevada Mountains of California, with the highest detected total endosulfan concentration being 21.9 ng/g wet weight. In a field study of 20 mountain yellow-legged frogs (*R. muscosa*) [16] collected from Sequoia National Park, the highest detected concentration of endosulfan I was 1.4 ng/g; neither endosulfan II or endosulfan sulfate was quantified.

The form of endosulfan can have a significant effect on toxicity. The present study started with a 50:50 mixture of endosulfan I and II, and these products degraded over time into endosulfan sulfate. Endosulfan I tends to be more toxic than endosulfan II or endosulfan sulfate, but the combination of both endosulfan I and II is more toxic than endosulfan I by itself [35]. Because all three forms are toxic, studies that report concentrations of only one or two of the compounds are overly conservative in risk assessment, and the amount of toxic endosulfan in an environment may be greater than is typically reported.

Daly and Wania [5] argued that mountainous areas are especially suited for chemical transport. Aerial transport brings pesticides and other organic contaminants into the upper elevations, where they are spread by diurnal winds and then precipitate onto land and water through the facilitation of cold temperatures. A likely scenario for amphibian exposure to toxic concentrations of pesticides in the mid (~2,000 m) or higher elevations of the Sierra Nevada Mountains in California is through snowmelt. These mountain areas receive the bulk of

their precipitation as winter snow, of which several meters can accumulate [12]. Dry deposition, however, can occur during the spring and summer, when pesticides are being applied at their greatest rates [13]. Adult amphibians begin to breed as soon as snow melts and open water appears. Organic compounds can be released from melting snow in much higher concentrations than from either wet or dry precipitation [32,33].

Low temperatures increase the half-life of insecticides and, thus, lengthen exposure periods. In years with unusually high snowfall, tadpoles can be exposed to endosulfan for the entire duration of development and to chlorpyrifos for periods exceeding the mean time to death as found in the present study.

A few factors associated with the present study need to be considered when assessing hazards to natural populations of California amphibians. First, the measured concentrations of insecticides in water were less than the nominal values used to calculate dose–response relationships. The difference between nominal and measured concentrations may have been caused by breakdown of the parent compound before extraction of samples, intake by the tadpoles (which would still contribute to the toxicity), or adsorption of the insecticides to glass surfaces of the tanks (where it would become biologically unavailable), among other factors. Depending on the amount of pesticide actually absorbed, realized LC50s could be up to 40% lower than those estimated in the present study. Second, in the field, amphibians are exposed to several contaminants simultaneously, including dichlorodiphenyldichloroethylene, DDT, *trans*-nonachlor, chlordane, lindane, diazinon, malathion, trifluralin, and chlorothalonil [12,13,15]. Many of these chemicals could have additive or synergistic effects that alter overall toxicity. Third, tadpoles raised in glass aquaria are protected from many of the stressors that can augment the effects of pesticides, including reduced food availability, inter- and intraspecific competition [36], disease [37], predation [30,38], and ultraviolet radiation [7]. Exposure to pesticides at concentrations deemed to be sublethal in the laboratory can be substantially more serious under field conditions [39].

Environmentally realistic concentrations of insecticides in the Sierra Nevada Mountains of California may have the ability to inflict serious damage on native amphibians. In comparison to those of several other species, *P. regilla* populations seem to be stable or declining at a slower rate. A possible cause of their relative success is their reduced dependence on standing water. *Pseudacris regilla* adults lay their eggs in water and move to upland habitat shortly afterward. Hatching is rapid compared with some of the other species; and time to metamorphosis is less than that of *R. boylei*. The congeneric *R. muscosa*, which is listed by the U.S. government as an endangered species in the southern end of its distribution, can be exposed to waterborne contaminants for two to three summers as tadpoles before metamorphosing [40]. Thus, exposure to chlorpyrifos and endosulfan poses serious risk to amphibians in the Sierra Nevada Mountains.

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