

INTERACTIVE EFFECTS OF PESTICIDE MIXTURES, PREDATORS, AND ENVIRONMENTAL REGIMES ON THE TOXICITY OF TWO PESTICIDES TO RED-EYED TREE FROG LARVAE

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Abstract: Global amphibian declines have many corroborative causes, and the use of pesticides in agriculture is a likely contributor. In places with high pesticide usage, such as Costa Rica, agrochemical pesticides may interact with other factors to contribute to rapid species losses. Classical ecotoxicological studies rarely address the effects of a pesticide in combination with other stressors. The present study investigated the synergistic roles of 2 pesticides (chlorothalonil and endosulfan), predator stress, and environmental regimes (controlled laboratory environments versus ambient conditions) on the survival of red-eyed tree frog larvae (*Agalychnis callidryas*). No synergistic effects of pesticide mixtures or predator stress were found on the toxicity of either chlorothalonil or endosulfan. Both pesticides, however, were considerably more toxic under realistic ambient temperature regimes than in a climate-controlled laboratory. Overall, endosulfan displayed the highest toxicity to tadpoles, although chlorothalonil was also highly toxic. The median lethal concentration estimated to kill 50% of a tested population (LC50) for endosulfan treatments under ambient temperatures was less than one-half of that for laboratory treatments (3.26 µg/L and 8.39 µg/L, respectively). Studies commonly performed in stable temperature-controlled laboratories may significantly underestimate toxicity compared with more realistic environmental regimes. Furthermore, global climatic changes are leading to warmer and more variable climates and may increase impacts of pesticides on amphibians. *Environ Toxicol Chem* 2013;32:2379–2386. © 2013 SETAC

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INTRODUCTION

Declines in amphibian populations around the world have come to the forefront of research and conservation efforts over the past decade [1–3]. Globally, nearly one-third of all amphibians are considered threatened, and it is believed that 159 extinctions occurred within the past 20 yr [3,4]. Widespread amphibian declines and extinctions are pervasive within the Neotropics, where amphibian diversity is among the highest on the planet [5]. The loss of many amphibian populations within the Neotropics can be attributed to land-use changes and conversions of native habitats to agricultural or urban landscapes. However, many so-called enigmatic amphibian declines are occurring within areas largely undisturbed by direct anthropogenic effects [3,6]. Several past studies identified potential factors involved in both enigmatic and conventional amphibian declines, such as global climate change, invasive species, emerging infectious diseases, and increased uses of pesticides and other toxic chemicals [7].

Pesticides are a ubiquitous reality of global agriculture and may persist or accumulate in the environment [8]. Pesticide residues are widespread in agricultural landscapes where these substances are directly applied. Furthermore, pesticides are capable of aerial transport, and residues can be found among the most remote or pristine environments on the planet [9–12]. Nontarget effects of these chemicals may impact amphibians by causing direct mortality [13], sublethally disrupting physiological processes [14,15], or increasing susceptibility to other natural

or anthropogenic stressors [16–18]. Moreover, pesticides may cause indirect effects by disrupting prey or predators of amphibians [19–21].

Direct and indirect impacts of pesticides on amphibians have received considerable attention in recent years, although the overwhelming majority of such studies were conducted on common, wide-ranging, and generally nonthreatened North American species [22]. Although both amphibian species diversity and proportion of threatened species are much higher in the Neotropics, very few studies have examined impacts of pesticides on amphibians in these regions [22–24]. Considerable differences exist in climate, ecology, and agricultural practices of temperate and tropical regions, and these differences limit the applicability of studies focused on temperate regions to understand impacts in the tropics. Pesticide application rates may be higher in tropical regions than in temperate regions because of greater pest density and diversity, and year-round growing seasons [25–29]. Furthermore, pesticides that are prohibited in some countries or never approved for use in developed nations may be commonly used in tropical regions [30].

Chlorothalonil, a broad-spectrum, non-systemic foliar fungicide, is favored in banana agriculture throughout Central America [31]. Chlorothalonil is the third most commonly used pesticide in Costa Rica [32], where applications in banana plantations may exceed 45 treatments per year [31]. Although chlorothalonil is widely used around the globe, little attention has been paid to the impacts of chlorothalonil and other fungicides on amphibians in tropical regions. Endosulfan is a persistent organochlorine insecticide that was recently banned for use in the United States by the US Environmental Protection Agency [33]. Endosulfan is widely used in tropical countries, and in recent years its use has steadily increased in Costa

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Rica [32]. While the use of these pesticides predominantly occurs in lowland agricultural areas, both appear capable of long-distance aerial transport to higher elevations [10]. Daly et al. [10] examined chlorothalonil and endosulfan residues in the soil and air of protected areas throughout Costa Rica and found the highest concentrations in midelevation sites, a pattern consistent with regions suffering from the most severe amphibian declines [5,34,35].

Classical studies generally employ short-duration single-stressor laboratory assays that aim to identify impacts of pesticides on amphibians. These studies isolate toxicity effects from effects of other stressors. However, in real environments amphibians are exposed to a variety of other stressors, including conspecific and interspecific competitors, predators, and variable or extreme temperatures. Agricultural regions commonly employ more than a single pesticide, and as a consequence, amphibian-inhabited environments may be exposed to combinations of pesticides. While some studies have shown stressor interactions to be important [21,36–39], other studies failed to find evidence for synergistic effects of interactions among stressors [19,40].

In the present study, we performed subchronic static-renewal toxicity tests on a common, nonthreatened species of tropical phyllomedusine tree frog, the red-eyed tree frog (*Agalychnis callidryas*). We examined the responses from exposures to 2 commonly used agrochemical pesticides of Costa Rica: endosulfan and chlorothalonil. Our objective was to assess impacts on survival and cumulative tadpole biomass of these 2 pesticides, either alone or in conjunction with 3 other types of natural or anthropogenic stressors: pesticide combinations, predators, and varying ambient temperatures.

METHODS

Study site and system

We conducted the present study at La Selva Biological Station, a 1600-hectare protected reserve located in the northeastern lowlands of Costa Rica (10°26'N, 83°59'W, 30–151 m above sea level). La Selva is home to 52 species of amphibians [41]; S.M. Whitfield, unpublished data). Between 1970 and 2005, amphibian populations at La Selva experienced gradual population declines with a reduction in density of 75% [42]. Reptile populations also suffered from severe declines during this time frame. While the La Selva reserve itself is predominantly old-growth rainforest, the surrounding region has been converted from relatively undisturbed forests to mixed-use agricultural pastures, banana, pineapple, and palm heart plantations, and residual forest fragments [43,44].

We collected our study animals as newly laid egg masses from a variety of seasonal ponds within the La Selva reserve. We chose red-eyed tree frogs as our study species because they are a common, nonthreatened species that breeds continuously throughout the wet seasons of approximately 8 mo [45]. We transferred egg masses to a laboratory and reared them under ambient conditions until hatching. Following hatching, we randomly distributed the tadpoles at Gosner stage 25 to 10-L tubs

containing 8 L of ultraviolet (UV)-treated and charcoal-filtered well water for holding and acclimatization in both indoor and outdoor (ambient) laboratories. Tadpoles were acclimatized for 2 d to environmental conditions before initiating trials.

General study design

We conducted 3 separate experiments to 1) compare toxic effects of 2 pesticides between laboratory and ambient environmental regimes, 2) test for interactive effects of pesticide combinations, and 3) test for interactive effects of pesticides and predator stress. For each of the 3 experiments, we conducted 12-d static-renewal toxicity tests. Our basic experimental unit was 10 randomly chosen tadpoles in a 10-L polypropylene tub containing 8 L of UV-treated and charcoal-filtered well water. Every 2 d we fed tadpoles organic alfalfa leaf powder (StarWest Botanicals) at a ratio of 20% of initial body mass. After 6 d, we doubled the food ratio to account for mass increase. These methods were modeled on prior studies to facilitate direct comparison with an existing body of literature (e.g., Relyea [37] and Relyea [38]).

We tested 2 pesticide formulations: Daconil 50SC, which contained 50% of the active ingredient chlorothalonil, and Thionex, which contained 35% of endosulfan; each of these formulations also contained inert ingredients. Each of the 3 experiments was replicated 3 times on different dates (temporal blocks) and utilized tadpoles from different egg masses to broaden genetic diversity in our experiment. In the first block, we tested 5 concentrations each of chlorothalonil (50 µg/L, 25 µg/L, 12.5 µg/L, 6.25 µg/L, and 0.625 µg/L) and endosulfan (100 µg/L, 50 µg/L, 25 µg/L, 12.5 µg/L, and 1.25 µg/L), as well as a control containing no pesticide. However, we experienced very high mortality at these initial concentrations of endosulfan, and therefore adjusted concentrations in the second and third blocks of the experiment to 25 µg/L, 12.5 µg/L, 6.25 µg/L, 3.125 µg/L, and 0.3125 µg/L. We used a total of 1400 tadpoles in 140 experimental tanks for the 3 experiments in the present study. Because we used the same methodology and pesticide applications for 3 different experiments, some of the experimental replicates overlapped between experiments; this allowed us to reduce the number of total animals used in the present experiment and prevent unnecessary duplication.

Each day, we assessed the number of surviving tadpoles and removed any that died. Tadpoles were determined to be dead if they did not move in response to gentle probing. For all experimental tanks, the water was changed every fourth day and pesticide treatments were immediately reapplied. At the end of the experiment, we measured pH, dissolved oxygen, and conductivity within each experimental tank (Table 1). We also measured the cumulative masses of surviving tadpoles in each treatment group to estimate average tadpole biomass.

Experiment 1: Artificial and natural environmental regimes

To test for effects of environmental regimes on the toxicity of 2 pesticides, we conducted trials in 2 locations with different environmental properties: a climate-controlled laboratory typical for short-term toxicity assays, and an outdoor shade house to

Table 1. Water properties from laboratory and ambient environments^a

Experiment setting	pH	Dissolved oxygen (mg/L)	Conductivity (mS/cm)
Laboratory	6.34 (6.13–6.54)	3.85 (1.67–6.03)	0.08 (0.06–0.10)
Ambient	6.64 (6.18–7.11)	3.40 (1.39–5.40)	0.10 (0.02–0.14)

^aData are expressed as means, with 95% confidence intervals in parenthesis.

determine if controlled laboratory conditions underestimate stress induced by more natural ambient conditions (e.g., higher temperatures, day to night temperature fluctuations, ambient UVB radiation). The shade house was designed to approximate environmental conditions of interior forest habitats in the rainforest at La Selva. We used the concentrations described in the *General study design* section for trials in both laboratory and ambient conditions. To track water temperatures during the experiment, we recorded water temperature in tubs every 30 min using iButtons (Embedded Data Systems; Figure 1). Both the laboratory and the shade house had the same light/dark cycles regulated by natural lighting conditions, which provided similar photoperiods to our test organisms. For the present experiment, we analyzed 740 tadpoles among 74 tanks that received identical pesticide treatments under both artificial laboratory and natural outdoor environmental regimes.

Experiment 2: Pesticide combinations

To determine if very low concentrations of a secondary pesticide increased lethal effects of a primary pesticide, we conducted trials using a range of concentrations (described in *General study design*) for a primary pesticide in the presence or absence of a low, ecologically relevant concentration of a secondary pesticide. For both endosulfan and chlorothalonil, we tested the range of concentrations described previously in the presence or absence of a secondary pesticide (0.18 $\mu\text{g/L}$ of chlorothalonil as a secondary pesticide in trials for which endosulfan was the primary pesticide, and 0.02 $\mu\text{g/L}$ of endosulfan as a secondary pesticide in trials for which chlorothalonil was the primary pesticide). Our concentrations

for secondary pesticides represent the Canadian Water Quality Guidelines for the Protection of Aquatic Life (hereafter called ALQs) [46]. These relatively low-concentration ALQs are generally thought to have no effect on aquatic organisms. The individual and combined chemical treatments allowed us to determine whether multiple chemicals interact and whether the current ALQ levels accomplish the prevention of impacts from pesticide cross-exposures. For the present experiment, which was conducted entirely within the climate-controlled laboratory, we analyzed 700 tadpoles among 70 tanks that received treatments of pesticide combinations.

Experiment 3: Predator stress

We conducted assays with our range of pesticide concentrations (described in *General study design*) applied in the presence and absence of a caged predator to test if predation stress increased susceptibility to chlorothalonil and endosulfan. We constructed small (6.25 cm diameter \times 3 cm height) cages enclosed with mesh, which were placed in each experimental tank. We collected odonate larvae, which were all morphologically similar aeshnids, from nearby ponds and placed them in the cages within the experimental tanks. We placed empty cages in additional experimental units as our controls. Odonate larvae were fed 1 tadpole every other day and were replaced within 1 d if they died. For the present experiment, which was conducted entirely within the ambient shade house, we analyzed 690 tadpoles among 69 tanks that received identical pesticide treatments with and without the additions of predators.

Statistical analysis

We analyzed the number of tadpoles surviving at the end of the experiment (Supplemental Data, Appendix 1) with a generalized linear model (R MASS package) with binomial errors and used the following predictor variables: concentration of chlorothalonil, concentration of endosulfan, predator stress (present or absent), and environmental regime (laboratory versus ambient). We based the number of residuals for the analysis on the number of tubs used in each experiment. We analyzed the dose-response relationships for each treatment and estimated median lethal concentration (LC50) values using the US Environmental Protection Agency's Benchmark Dose Software, a tool widely used in policy and conservation to obtain reference values for assessing health risks and setting regulatory levels [47] (Supplemental Data, Appendix 2). We analyzed the total biomass of surviving tadpoles at the end of the experiment with a generalized linear model with Gaussian error distributions and used the following predictor variables: concentration of chlorothalonil, concentration of endosulfan, predator stress (present or absent), and environmental regime (laboratory versus ambient).

RESULTS

Lethal effects

Our data indicate strong dose-response effects of chlorothalonil and endosulfan on the survival of tadpoles (Figure 2). Control tadpoles experienced very high survival (99.231%), regardless of temperature regime or predatory presence. Endosulfan caused 96.25% mortality at 12.5 $\mu\text{g/L}$ when all treatments were pooled and caused 100% mortality at all higher concentrations. Our highest concentration of chlorothalonil (50 $\mu\text{g/L}$) produced 81.67% tadpole mortality when all treatments were pooled. Endosulfan was lethal at lower concentrations than chlorothalonil. The final (day 12) LC50 estimates (95%

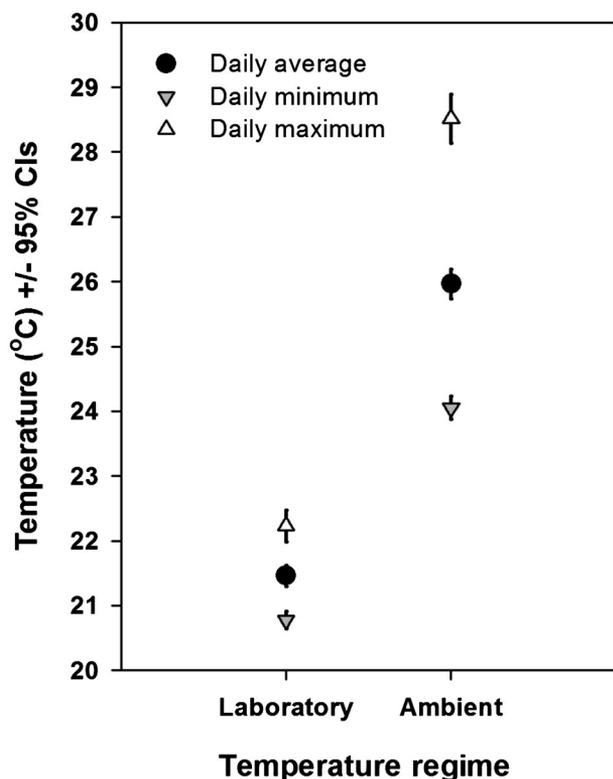


Figure 1. Characteristics of the temperature regimes used in the present study, derived from data from temperature dataloggers measuring water temperature every 30 min in laboratory and ambient conditions. Water temperature was both warmer and more variable in ambient conditions than in the laboratory. CI = confidence interval.

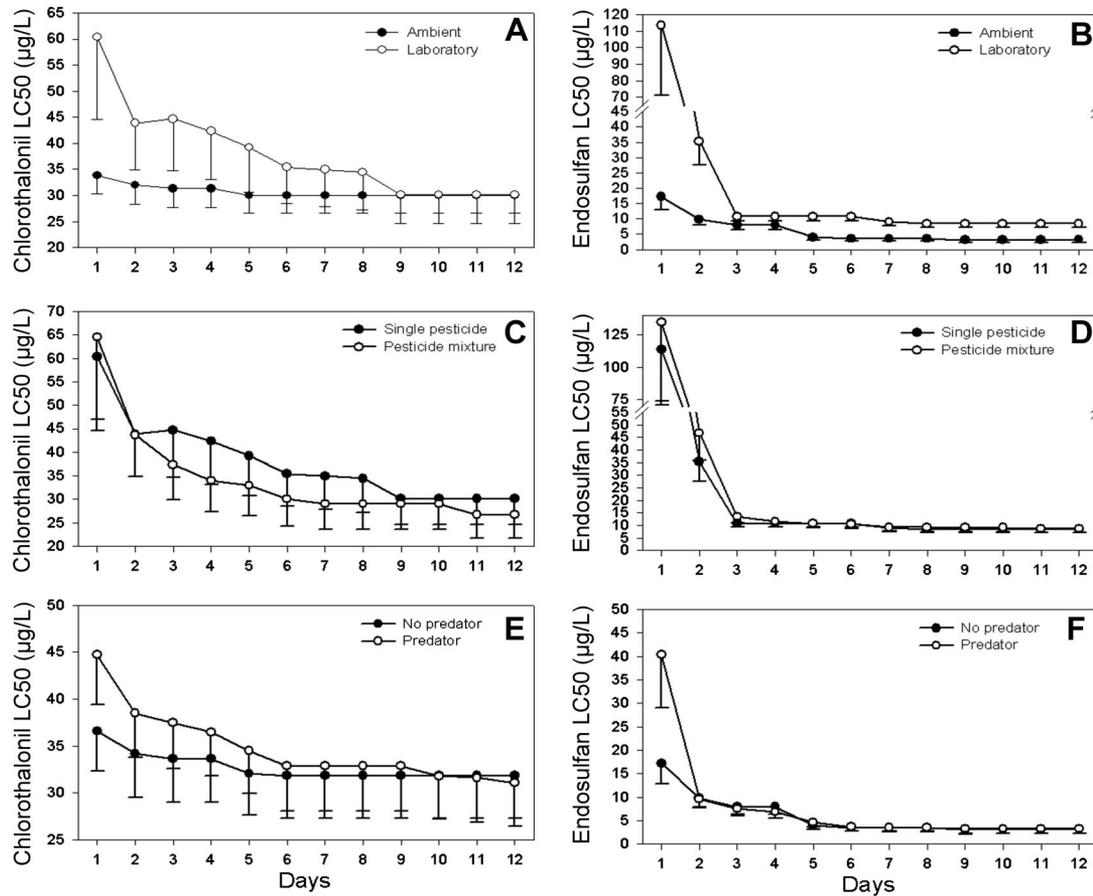


Figure 2. Median lethal concentration (LC50) estimation for chlorothalonil and endosulfan. Error bars indicate 95% lower confidence interval. The LC50 values were estimated with the US Environmental Protection Agency Benchmark Dose Software and analyzed with R.

confidence interval [CI] for endosulfan-containing treatments ranged from 3.26 µg/L to 8.67 µg/L among treatments, while LC50s for chlorothalonil-containing treatments ranged from 26.72 µg/L to 30.16 µg/L among treatments (Figure 2).

Both chlorothalonil and endosulfan were considerably more lethal under ambient environmental conditions than in the climate-controlled laboratory (Figure 2A and B; Table 2). We observed no significant additional lethal effects of a secondary pesticide relative to a single pesticide in laboratory treatments (Figure 2C and D). Our LC50 estimates for chlorothalonil and endosulfan were similar among individual and pesticide combination treatments (Figure 2C and D). We detected no main effect of predators, and no interaction between predators

with either chlorothalonil or endosulfan (Table 2; Figure 2E and 2F). Again, LC50 estimates were similar in the presence or absence of a predator.

Tadpole biomass

Both chlorothalonil ($F_1 = 11.552, p < 0.0001$) and endosulfan ($F_1 = 64.467, p < 0.0001$) had strong negative effects on total tadpole biomass (Figure 3). Tadpole biomass was higher in ambient conditions than in the climate-controlled laboratory ($F_1 = 13.098, p = 0.0004$). There were no main effects of predators or pesticide combinations on tadpole biomass (Table 3).

Table 2. Analysis of deviance between treatment regimes on tadpole survival

Factor	df	Deviance	Residual df	Dev p (> Chil)	NULL
Chlorothalonil	1	37.92	137	1443.5	<0.0001
Endosulfan	1	860.99	136	582.5	<0.0001
Temperature	1	7.92	135	574.59	0.005
Predator	1	0.17	134	574.42	0.684
Block	1	0.1	133	574.32	0.750
Chlorothalonil:endosulfan	1	27.22	132	547.1	<0.0001
Chlorothalonil:predator	1	0.2	131	546.89	0.651
Endosulfan:predator	1	15.81	130	531.09	<0.0001
Chlorothalonil:temperature	1	0.1	129	530.98	0.748
Endosulfan:temperature	1	40.98	128	490.01	<0.0001
Residuals			138	1481.42	

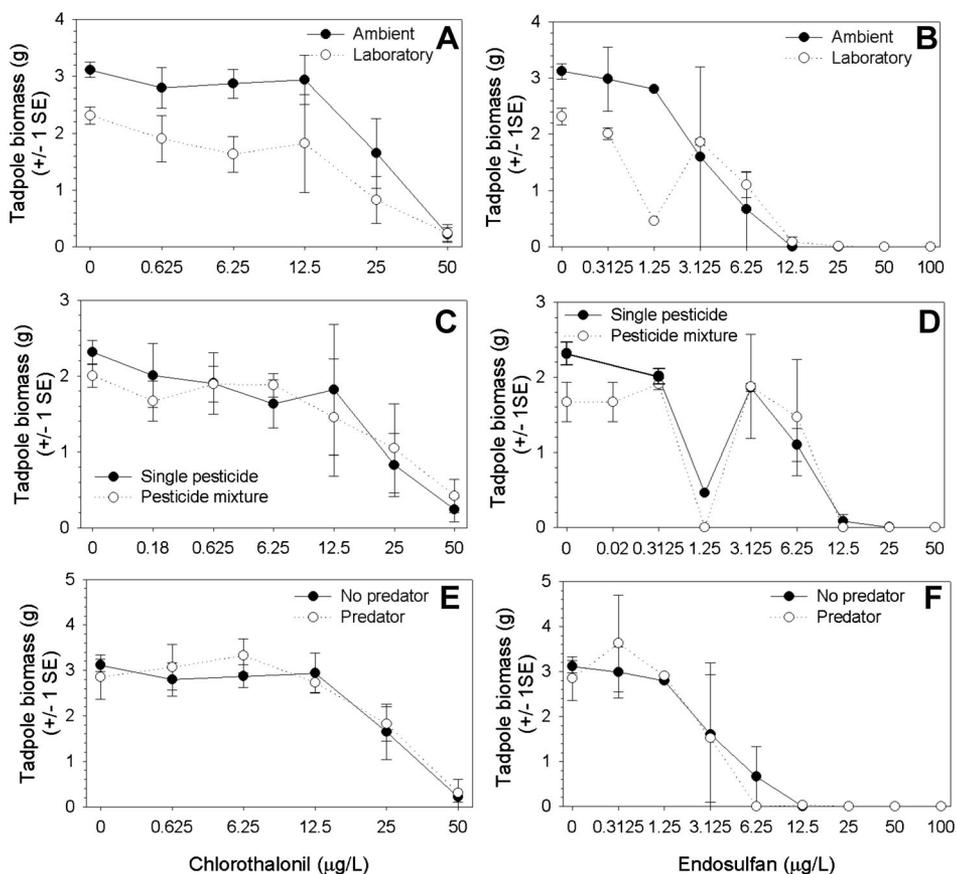


Figure 3. Experimental effects on tadpole biomass. SE = standard error.

DISCUSSION

We found significant dose–response effects of both chlorothalonil and endosulfan on the survival of red-eyed tree frog tadpoles. In general, we found no major effects of pesticide combinations or predators on either survival or resulting biomass. We observed higher mortality from both pesticides in ambient climate conditions than in cooler, more stable, and more controlled laboratory settings. However, while our data indicate that both pesticides were more toxic in ambient conditions than standard laboratory conditions, the specific responses of how environmental regimes affected toxicity varied among pesticides. Chlorothalonil produced more rapid mortality in our ambient environmental regime than in our laboratory environmental regime, but our final (day 12) LC50 estimates

were similar for laboratory and ambient environments for chlorothalonil (Figure 2A). Endosulfan also produced more rapid initial mortality in ambient environmental regimes than in laboratory conditions, and the day 12 LC50 for endosulfan indicated persistently higher mortality in ambient conditions (Figure 2B). The ambient LC50 for endosulfan at day 12 was 3.26 $\mu\text{g/L}$, less than half of the value from laboratory trials (8.39 $\mu\text{g/L}$).

A number of factors could contribute to the marked differences in toxicity between laboratory and ambient environmental conditions, including either temperature averages or temperature variability, or differences in levels of UVB radiation. Higher average temperatures may increase toxicity either because metabolic rates increase with temperature or

Table 3. Analysis of variance on surviving tadpole biomass

Factor	<i>df</i>	<i>SS</i>	<i>MS</i>	<i>F</i>	<i>p</i>
Chlorothalonil	1	8.920	8.920	8.0526	0.0053
Endosulfan	1	59.545	59.545	53.7551	<0.0001
Temperature	1	11.763	11.763	10.6194	0.0014
Predator	1	0.045	0.045	0.0408	0.8403
Block	1	0.142	0.142	0.1281	0.7210
Chlorothalonil:endosulfan	1	0.803	0.803	0.0025	0.3962
Chlorothalonil:predator	1	0.003	0.003	0.0025	0.9599
Endosulfan:predator	1	0.450	0.450	0.4061	0.5250
Chlorothalonil:temperature	1	0.401	0.401	0.3622	0.5484
Endosulfan:temperature	1	1.619	1.619	1.4614	0.2286
Residuals	127	140.679	1.108		

SS = sum of squares; *MS* = mean squares.

because higher temperature reduces dissolved oxygen, necessitating higher energy expenditure for gas exchange to maintain homeostasis. Higher variability in temperatures may also be important stressors, perhaps more important than temperature itself [48]. Furthermore, pesticide toxicities may increase with higher UVB exposure [49]. In any case, the differing values of our laboratory and ambient regimes are a great concern because most environmental standards are only supported through laboratory studies, which may underestimate risks to amphibians that generally live in variable environments. Although the ALQs for endosulfan and chlorothalonil fall below our measured benchmark dose levels, future research should begin to incorporate varying environmental conditions into their studies to better determine the roles of natural environments on pesticide toxicity.

While several studies found increased toxicities of pesticides in conjunction with predator-induced stress [21,36,38,39], not all studies have indicated such synergistic responses [19,40]. The results of these studies may suggest that some amphibian species show interactive effects of pesticides and predators, while other species do not follow similar trends when subjected to the same experimental treatments. Therefore it is possible that *A. callidryas* will not show interactive effects between predators and pesticides in many conditions. Alternatively, we only conducted our predator–pesticide interactions in ambient conditions. It is possible that the stress caused by high or fluctuating temperatures in both predator and predator-free treatments was stronger than any stress response from predators alone.

Both chlorothalonil and endosulfan were lethal at relatively low concentrations. To our knowledge, only 3 published toxicity studies on amphibians have produced LC50 estimates, or estimates killing 50% of the tested population, for chlorothalonil [50–52], yet our LC50 estimates are lower than most of these. Some variation in LC50 estimates may be attributable to the formulations used, water chemistry, variation among species, or our methodology. The concentrations of chlorothalonil and endosulfan used in the present study were nominal concentrations, and if the chlorothalonil or endosulfan experienced significant degradation or adsorption to container materials, our estimates of LC50 values may be higher than true values. Furthermore, if the pesticide formulations used in the present study contained toxic inactive ingredients, then our LC50 values may overestimate toxicity of chlorothalonil and endosulfan. Still, the LC50 estimates for chlorothalonil used in the present study are similar to LC50 estimates for other Costa Rican species (S.M. Whitfield, unpublished data), and 2 other recent studies have highlighted the highly toxic effects of chlorothalonil to amphibians and other aquatic organisms [52,53]. In any case, the present study confirms the need for additional attention to chlorothalonil in particular and also fungicides, which are a much more significant portion of pesticide use in tropical regions than temperate regions.

The lethal effects of endosulfan were relatively mild during the first 4 d of the present study at concentrations that produced over 95% mortality over longer intervals (12.5 $\mu\text{g/L}$ and greater). Our LC50 values at 48 h for endosulfan were more than 4 times (laboratory) and 3 times (ambient) higher than our LC50 estimates for day 12 (Figure 2), although LC50 estimates appeared to stabilize after day 5. A considerable number of studies have examined impacts of endosulfan on amphibians, and LC50 estimates for endosulfan with amphibians are extremely variable because of delayed chemical effects [54]. Again, this raises concern for regulatory practices because many pesticide standards and regulations are set through 48-h toxicity

testing and may be dramatically underestimating the lethality of many pesticides.

While we have no data on pesticide concentrations from aquatic habitats at La Selva, where our test species might be exposed, the soil concentrations of endosulfan and chlorothalonil were 49 pg/g and 138 pg/g , respectively [11]. These soil concentrations are several orders of magnitude below our lowest LC50 estimate. Considering these levels of exposure and the LC50 values produced by the present study, it appears unlikely that either endosulfan or chlorothalonil are likely to cause direct mortality to a broad range of amphibian species at this site. However, it is unclear whether other pesticides, for which we have no data in the reserve (or in other national parks in tropical countries), could be capable of such impacts. Furthermore, we should also consider the environmental fates of these pesticides to help understand exposure risks. Compound-specific properties and environmental conditions determine the rates of chemical degradation [8] and influence solubilities or lipophilicities of many substances. Degradation products may persist longer than their original compound, accumulate [55], or be more toxic than the parent compound [56]. It is also unclear if pesticide residues have sublethal effects at concentrations far below the LC50 values. Of particular interest to amphibian declines is whether sublethal concentrations of pesticides may reduce amphibian immune capacity to resist emerging infectious diseases [13,57].

Although the concentrations of our test pesticides in protected areas are considerably lower than our LC50 estimates, we expect that amphibians in agricultural areas of Costa Rica may be exposed to concentrations above our lethal estimates. Studies of pesticide residues in Central American aquatic systems are not as common as those in temperate North America [58,59]. Because degradation rates are often greater at high temperatures, it may be more difficult to detect maximum concentrations in warmer climates. Chlorothalonil and endosulfan are likely to pose significant risks to amphibian populations in or near agricultural areas, yet concentrations in protected reserves are several orders of magnitude lower than our LC50 estimates.

The present study represents one of the first efforts to assess impacts of pesticides on amphibians in the Neotropics, a region that hosts the greatest amphibian diversity on the planet. While research conducted in temperate North America has explored impacts of pesticides on amphibians, we support the need for more work with tropical species in tropical environments. A greater proportion of amphibian species is threatened in tropical regions than in temperate regions, and conservation plans for many species require immediate attention. Studies have shown phylogenetic trends in susceptibility to pesticides [54], but tropical regions host broad clades that are not found or are poorly represented in temperate regions (here, phylomedusine hylids). There are pronounced underlying differences between pesticide usages in temperate and tropical agriculture, and relatively weak regulatory structures in tropical nations may expose environments to pesticides not commonly used in North America. We argue that more studies focused on species and pesticides commonly used in tropical regions will be necessary to realistically assess threats of pesticides to the majority of the planet's amphibian biodiversity.

SUPPLEMENTAL DATA

Appendices 1 and 2.

Tables S1 and S2.

Figures S1 and S2. (2.8 MB DOC).

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