

VERY HIGHLY TOXIC EFFECTS OF ENDOSULFAN ACROSS NINE SPECIES OF  
TADPOLES: LAG EFFECTS AND FAMILY-LEVEL SENSITIVITY

DEVIN K. JONES, JOHN I. HAMMOND, and RICK A. RELYEA\*

Department of Biological Sciences, University of Pittsburgh, Pittsburgh, Pennsylvania 15260, USA

(Received 23 January 2009; Accepted 2 April 2009)

**Abstract**—Pesticides are commonly used for health and economic benefits worldwide, but increased use has led to increased contamination of aquatic habitats. To understand potential impacts on nontarget organisms in these habitats, toxicologists generally use short-term (4-d) toxicity tests on model organisms. For most pesticides, few amphibian tests have been conducted, but there is growing concern about the potential impact of pesticides to amphibian populations. For the insecticide endosulfan, previous studies have found that low concentrations can be very highly toxic to amphibians and have suggested that this mortality may exhibit important lag effects. To estimate the lethal concentration of endosulfan that would cause 50% mortality after 4 d (LC50<sub>4-d</sub>) across a diversity of amphibians and the presence of lag effects, LC50<sub>4-d</sub> experiments were conducted on nine species of tadpoles from three families (Bufonidae: *Bufo americanus*, *B. boreas*; Hylidae: *Pseudacris crucifer*, *P. regilla*, *Hyla versicolor*; and Ranidae: *Rana pipiens*, *R. clamitans*, *R. cascadae*, *R. catesbeiana*) and then held the animals for an additional 4 d in clean water. The LC50<sub>4-d</sub> values for endosulfan ranged from 1.3 to 120 ppb, which classifies endosulfan as highly toxic to very highly toxic. Moreover, holding the animals for an additional 4 d in clean water revealed significant additional mortality in three of the nine species. Leopard frogs, for example, experienced no significant death during the initial 4-d exposure to 60 ppb but 97% death after an additional 4 d in clean water. A phylogenetic pattern also appears to exist among families, with Bufonidae being least susceptible, Hylidae being moderately susceptible, and Ranidae being most susceptible. Results from the present study provide valuable data to assess the impact of endosulfan on a globally declining group of vertebrates.

**Keywords**—Ecotoxicology    Amphibian decline    Nontarget    Anuran    Contaminant

## INTRODUCTION

Pesticides are widely used to control pest species and increase crop yields (e.g., Roundup-Ready® corn and soybeans, Monsanto, St. Louis, MO, USA) for economic gain. However, they are also commonly found in aquatic habitats, including streams, rivers, and ponds, at varying concentrations because of direct overspray, drift, atmospheric transport, agricultural and residential runoff, individual misuse, and improper disposal [1–5], [http://pubs.usgs.gov/circ/2005/1291/pdf/circ1291\\_front.pdf](http://pubs.usgs.gov/circ/2005/1291/pdf/circ1291_front.pdf). For example, surveys performed by the U.S. Geological Survey across 51 major river basins and groundwater [5] found at least one pesticide at every sample site, including water surrounding agricultural fields and urban areas. The challenge is to understand whether nontarget taxa might be affected by such contamination [1,6–11].

Amphibians are a diverse group of nontarget organisms that have traditionally received little pesticide testing. However, amphibians are now receiving increased attention from toxicologists because of recently discovered correlations between pesticide use and declining amphibian populations in the western United States [12–15]. A number of studies have documented a variety of mechanisms by which pesticides can directly and indirectly affect amphibians [6,7,11,16–18], but the traditional approach has been to assess the direct toxic effects in highly controlled, short-term (i.e., 1- to 4-d) laboratory experiments. Using such experiments, one can estimate the LC50 value of a pesticide (the concentration expected to kill 50% of a population). Unfortunately, amphibians are not tested as part of the registration process for the vast majority

of pesticides, so we have few LC50 data for amphibians despite their being a particularly sensitive group [19]. Hence, there is a substantial need to conduct LC50 tests on amphibians, particularly for those pesticides that are widely applied or for which there is some evidence of potentially high toxicity.

One of the assumptions of LC50 experiments is that the magnitude of toxicity observed over a short time period can be reasonably extrapolated over longer periods. Although additional mortality is always expected to occur with additional days of pesticide exposure, we generally do not expect lag effects that cause substantial death after the pesticide exposure has ended. In contrast to this expectation, Berrill et al. [20] reported that the insecticide endosulfan had lag effects in three species of larval anuran (wood frogs, *Rana sylvatica*; American toads, *Bufo americanus*; and green frogs, *Rana clamitans*). After an initial 4-d exposure to endosulfan, which caused little mortality, the investigators reported increased mortality of the exposed animals after being transferred to clean water (although no LC50 estimates were made). In a recent study using a single application of endosulfan in outdoor mesocosms at a very low concentration (6 ppb), Relyea [11] observed 84% death in larval leopard frogs (*R. pipiens*). Interestingly, this death appeared to not occur during the first few days of the experiment (R.A. Relyea, personal observation), again suggesting a potentially important role of lag effects. Both studies point toward an important and potentially widespread delayed effect of endosulfan exposure on amphibian larvae. Equally interesting in the Relyea [11] study was the fact that gray tree frogs (*Hyla versicolor*) in the same mesocosms suffered no death from endosulfan, suggesting that amphibian sensitivity might differ among families of amphibians and therefore have a phylogenetic basis.

\* To whom correspondence may be addressed (relyea@pitt.edu).  
Published on the Web 4/9/2009.

Table 1. The species of tadpoles used in the endosulfan experiments including family name, number of egg masses, initial mass (mean  $\pm$  1 SE), and developmental stage

Common name	Latin name	Family	Egg masses	Mass (mg)	Gosner stage
American toad	<i>Bufo americanus</i>	Bufoidae	9	19 $\pm$ 2	27
Western toad	<i>Bufo boreas</i>	Bufoidae	10	63 $\pm$ 3	26
Spring peeper	<i>Pseudacris crucifer</i>	Hylidae	28	27 $\pm$ 3	26
Pacific tree frog	<i>Pseudacris regilla</i>	Hylidae	10	83 $\pm$ 3	26
Gray tree frog	<i>Hyla versicolor</i>	Hylidae	30	59 $\pm$ 3	26
Leopard frog	<i>Rana pipiens</i>	Ranidae	8	30 $\pm$ 2	25
Green frog	<i>Rana clamitans</i>	Ranidae	15	44 $\pm$ 3	25
Cascades frog	<i>Rana cascadae</i>	Ranidae	3	63 $\pm$ 4	27
American bullfrog	<i>Rana catesbeiana</i>	Ranidae	15	40 $\pm$ 6	25

The goal of the present study was to conduct experiments with a widely applied pesticide, endosulfan, for which we know little about the sensitivity of amphibians and for which there is some evidence of important lag effects that need further examination. We compare the toxicity of endosulfan (by estimating LC10, LC50, LC90, and LOEC [lowest-observed-effect concentration]) across nine species of tadpoles within three families (Bufoidae, Hylidae, and Ranidae) to quantify LC50 estimates and to test for potential lag effects on survival. Based on previous work, three hypotheses were tested. First, tadpoles exposed to higher levels of endosulfan will suffer higher mortality. Second, LC50 values will differ among species and exhibit patterns of sensitivity across families. Third, endosulfan will have substantial lag effects on tadpole mortality.

The insecticide endosulfan is a common organochlorine pesticide that has an excitatory effect on the neuromuscular system, can damage gill tissues and cause eye abnormalities [16,18], and is considered a potential endocrine disruptor [21]. It is composed of two isomers,  $\alpha$ - and  $\beta$ -endosulfan, which degrade into endosulfan sulfate. All three residues are found in various concentrations in soils, farm ditches, and stream runoff found near agricultural fields [4]. Application rates for endosulfan on crops such as corn, wine grapes, and walnuts average approximately 1.9 kg/ha (CA pesticide records; [http://www.pesticideinfo.org/Detail\\_ChemUse.jsp?Rec\\_Id=PC35085](http://www.pesticideinfo.org/Detail_ChemUse.jsp?Rec_Id=PC35085)). Endosulfan can be present at 700 ppb in pond water 10 m away from targeted application sites and 4 ppb in pond water 200 m away from such sites (when sprayed from a nozzle 3–4 m above the ground) [22]. It has been reported at 0.5 ppb in ponds near apple orchards in Ontario, Canada [1], and 2.5 ppb in Australian aquatic environments [23], and the U.S. Environmental Protection Agency (U.S. EPA) [21] places the expected environmental concentration for surface drinking water at 0.5 to 23.9 ppb. Endosulfan has also been detected in surveys of amphibians and fish tissues and has been reported to be highly toxic to fish, some amphibians, and crustaceans that inhabit natural ponds and streams [4,11,13,20,21,24].

## MATERIALS AND METHODS

### Experimental design

To examine the impact of endosulfan on tadpole survival, nine species were exposed to a range of concentrations over a 4-d period followed by an additional 4 d of exposure in clean water. All experiments were completed at the University of Pittsburgh's Pymatuning Laboratory of Ecology (PLE) under controlled laboratory conditions. The tadpoles represented a diverse group of species that allowed us to examine patterns of susceptibility among species, genera, and families (Table 1). All animals were collected as egg masses from ponds in

Pennsylvania and Oregon, USA, and the Oregon eggs were shipped overnight to PLE in an ice chest containing cold packs. Egg masses were hatched in covered outdoor culture pools containing well water, and the hatchlings were fed daily with rabbit chow (ad libitum). In toxicology tests, tadpoles can be exposed at similar sizes or similar developmental stage (and both approaches are defensible); we chose to expose each species at similar Gosner [25] developmental stages (Table 1).

For each species, except American toads and leopard frogs, we employed a randomized block design that contained two blocks (laboratory shelf heights) and two replicates of each treatment per block. For the first two species examined (American toads and leopard frogs), our experimental design used a single block with four replicates and included a negative control (water), a vehicle control (ethanol), and five nominal concentrations of endosulfan (1, 5, 10, 50, and 100 ppb). Ethanol was used as a vehicle because of endosulfan's insolubility in water; the amount of ethanol added for the vehicle control was the same as the amount in the highest endosulfan concentration treatment (concentration = 0.1%). Given that the 4-d mortality rates in leopard frogs and American toads were too low to obtain LC50 estimates, we added a sixth nominal endosulfan concentration of 500 ppb for the remaining seven species, moved to a two-block experimental design, and adjusted the corresponding vehicle control concentration of ethanol (concentration = 0.5%).

For each species, the tadpoles were tested in groups of 10 animals held in 1-L plastic containers filled with 500 ml of carbon-filtered, ultraviolet-irradiated well water. The water was changed and the pesticide reapplied daily. For efficiency, large volumes of each treatment were prepared and then distributed to the appropriate experimental units. Experimental water was prepared daily by adding different amounts of a concentrated stock solution (0.1 mg active ingredient/ml; technical-grade endosulfan [99% purity; Chem Services]) to containers filled with 2.75 L of water. To achieve nominal concentrations of 1, 5, 10, 50, 100, and 500 ppb, we added 27.5, 137.5, 275, 1,375, 2,750, and 13,750  $\mu$ l of the stock solution to the 2.75 L of water. At 24-h intervals, we quantified survival, removed any dead individuals, and changed the water. Prior to the 48-h water change, we measured temperature and pH (in one species, these measurements were taken prior to the 24-h water change). Temperature across all species ranged from 17.9 to 20.8°C. Within each species, temperature variation was narrow, with the maximum difference between experimental units ranging from 0.4 to 1.4°C. The pH across all species ranged from 8.0 to 8.4.

At each water change, 125 ml were sampled from each newly mixed, large volume of treatment water and placed in a

precleaned glass amber jar for testing (a total of 500 ml of sampled water over the 4 d of exposure). To determine the actual concentrations, we tested a set of samples (one from each concentration) from each of two amphibian species plus a set of samples (one from each concentration) that was pooled from experiments conducted across nine species. The three sets of sampled water were shipped to the Mississippi State Chemical Laboratory for independent analysis of the actual endosulfan concentrations (excluding the ethanol vehicle controls). A weighted average was then used to estimate the actual mean concentrations based on the three samples, while a standard error was calculated from the nonweighted values. The actual mean concentrations ( $\pm 1$  SE) of endosulfan were  $1.7 \pm 0.3$ ,  $5.5 \pm 0.8$ ,  $6.5 \pm 0.8$ ,  $34.9 \pm 0.8$ ,  $59.5 \pm 12.6$ , and  $295.5 \pm 53.2$  ppb (henceforth referred to as 2, 6, 7, 35, 60, and 296 ppb, respectively). The three control samples had either no detectable endosulfan concentrations (first single-species sample) or very low endosulfan concentrations (second single-species sample = 0.017, pooled sample = 0.19 ppb). These data generated a weighted average concentration of  $0.16 \pm 0.06$  ppb.

After the initial 4 d of exposure to different endosulfan concentrations, we transferred animals to clean water for another 4 d. During the postexposure period, we changed the water every 24 h and fed the tadpoles a ration of ground Tetra fish flakes (Tetra) based on a per capita ration that was 2% of the mean tadpole mass. Daily mortality checks continued every 24 h. After 8 d, we counted all survivors and then euthanized them in 2% MS-222 (tricaine). Animal bodies and experimental water were disposed of in accordance with university protocols for environmental health and safety.

#### Statistical analysis

To determine whether the endosulfan treatments affected tadpole survival, we analyzed survival using the proportion of individuals surviving in an experimental unit as our response variable. The analysis of variance determined that there were never any block effects on tadpole survival ( $p$  values always  $> 0.4$ ), so we dropped this term from the analyses and pooled the degrees of freedom in the error term. Because of low variance in the highest and lowest concentration treatments (i.e., either all or no individuals survived across all replicates), we rank transformed the data and used these ranks as the response variable in a repeated-measures analysis of variance (rm-ANOVA) comparing survival from day 1 through day 8. The rm-ANOVA analysis, followed by planned contrasts between the controls and the range of endosulfan concentration treatments, tested three specific questions. First, does endosulfan concentration affect survival? Second, does the lethality of endosulfan change over time? Third, is there a lag effect on mortality (i.e.,  $LC_{50,4-d} < LC_{50,8-d}$ , given 4 d exposure to endosulfan + 4 d in clean water)?

To estimate the LC10, LC50, and LC90 values at 4- and 8-d intervals, we used probit analyses to fit a sigmoid-shaped curve to the data. Since low but detectable levels of endosulfan were present in the control samples, we cannot separate death due to the experimental conditions from death due to endosulfan. Thus, we included the controls in the analysis with the averaged observed endosulfan concentration. Based on these LC50 estimates, we tested whether the 4- and 8-d values were significantly different by comparing the overlap between the 84% confidence intervals. Simulation tests have shown this method approximates an  $\alpha = 0.05$  [26].

## RESULTS

For all species, the rm-ANOVAs indicated significant effects of endosulfan concentration, time, and their interaction ( $p \leq 0.03$  for all tests). Endosulfan significantly reduced survival in all species, and its effect was dose dependent. In five of the nine species, holding the tadpoles for an additional 4 d in clean water produced significant differences in the 4- and 8-d LC50 estimates (Table 2). Next, we detail the impacts of endosulfan for each species.

Leopard frog survival in the water and vehicle controls was high (100 and 98% after 4 d and 98 and 95% after 8 d, respectively). After 4 d, the 7-ppb concentration was the only treatment to experience significantly more death than the control (Fig. 1), and the amount of death in this treatment was low (10%). As a result, an LC50 estimate could not be calculated. After 8 d, all endosulfan concentrations were significantly different from the control, and the resulting LC50 estimate was 4.6 ppb (Table 2). Although 35 and 60 ppb of endosulfan caused no significant death after 4 d of exposure, survival decreased by 97% following an additional 4 d in clean water. Hence, endosulfan had a substantial time lag effect on leopard frog tadpoles.

In American toads, survival also was high in the water and vehicle controls (92 and 100% after 4 d and 92 and 98% after 8 d, respectively). After 4 d, none of the concentrations significantly differed from the control (survival  $\geq 95\%$ ; Fig. 1). As a result, an LC50 estimate could not be generated. After 8 d, only the highest concentration (60 ppb) differed from the control (50% survival; Fig. 1), but we were still unable to estimate an LC50 value because of a lack of higher concentrations that would cause greater death (Table 2). Because American toads exposed to 60 ppb of endosulfan exhibited no significant death after 4 d but 50% death if they were held in clean water for an additional 4 d, we conclude that endosulfan had a significant time lag effect on American toad tadpoles.

For spring peepers, survival in the water and vehicle controls was initially very good but slightly declined over time (95 and 92% after 4 d and 90 and 82% after 8 d, respectively). After 4 d, only the 296-ppb concentration exhibited significantly greater death than the control (Fig. 1). After 8 d, the 7-, 35-, 60-, and 296-ppb concentrations all exhibited significantly greater mortality than the control. Based on these results, the LC50 estimates were 120 ppb after 4 d but 26.6 ppb after an additional 4 d in clean water (Table 2). Based on the 84% confidence intervals for the LC50 estimates, endosulfan had a significant (and large) lag effect on mortality of spring peepers.

In gray tree frogs, survival remained high in the water and vehicle controls (98 and 100% after 4 d and 95 and 95% after 8 d, respectively). After 4 and 8 d, the 7-, 35-, 60-, and 296-ppb concentrations had significantly greater mortality than the control (Fig. 1). The LC50 estimates were 9.0 ppb after 4 d and 6.0 ppb after an additional 4 d in clean water (Table 2). Based on the 84% confidence intervals, the two estimates are significantly different, but the effect was quite small and may be attributable to a small decline in survival over time that we observed in the control treatments.

Pacific tree frogs also experienced high survival in the water and vehicle controls (98 and 100% after 4 d and 95 and 95% after 8 d, respectively). After 4 and 8 d, the 7-, 35-, 60-, and 296-ppb concentrations had significantly more death than the control (Fig. 1). The LC50 estimate was 21.4 ppb after 4 d and 13.9 ppb after an additional 4 d in clean water (Table 2). Based on the 84% confidence intervals, the difference in LC50

Table 2. Lethal concentration (LC) values LC10, LC50, and LC90 (and 84% confidence intervals) and lowest-observed-effect concentrations (LOEC) for nine species of tadpoles after 4 d of exposure and 4 d of clean water to the insecticide endosulfan. All units are in ppb. Values denoted by (—) were not estimable from the data because of low death rates. Species in italics have significantly different LC50 estimates between 4 d of endosulfan exposure and 4 d in clean water. Values for LOEC with (\*) represent discontinuous trends within the results

Species	4-d LC10	4-d LC50	4-d LC90	4-d LOEC	8-d LC10	8-d LC50	8-d LC90	8-d LOEC
<i>Leopard frog</i>	—	—	—	7*	1.2 (0.8, 1.6)	4.6 (3.9, 5.5)	17.7 (13.7, 25.6)	2
<i>American toad</i>	—	—	—	—	0.9 (0.4, 1.6)	26.6 (19.4, 37.5)	768 (399, 1,918)	60
<i>Spring peeper</i>	18.7 (12.4, 25.4)	120 (93.5, 161)	769 (486, 1,483)	296	1.1 (0.7, 1.5)	6.0 (4.9, 7.3)	33.5 (24.7, 50.5)	35
<i>Gray tree frog</i>	2.3 (1.7, 3.0)	9.0 (7.6, 10.7)	34.4 (26.3, 49.4)	7	1.7 (1.1, 2.4)	13.9 (11.1, 17.5)	114 (79.0, 187)	7
<i>Pacific tree frog</i>	2.7 (1.7, 3.7)	21.4 (17.0, 27.0)	172 (117, 283)	7	4.9 (3.1, 7.0)	46.8 (36.5, 61.5)	446 (282, 832)	7*
<i>Western toad</i>	5.0 (2.9, 7.6)	76.1 (56.3, 109)	1,147 (623, 2,726)	7*	1.5 (1.2, 1.8)	3.0 (2.6, 3.4)	5.8 (5.0, 7.0)	6
<i>Green frog</i>	1.9 (1.5, 2.2)	3.2 (2.8, 3.6)	5.4 (4.8, 6.3)	6	6.0 (5.5, 6.1)	6.7 (6.5, 7.1)	7.6 (7.1, 9.0)	7
<i>Cascades frog</i>	4.8 (3.6, 5.9)	15.0 (12.7, 17.9)	47.4 (37.4, 64.0)	7	0.2 (0.1, 0.3)	0.9 (0.7, 1.2)	3.8 (3.0, 5.3)	2
<i>Bullfrog</i>	0.4 (0.2, 0.5)	1.3 (1.0, 1.6)	4.7 (3.7, 6.3)	6	—	—	—	—

estimates was not significant. Hence, Pacific tree frog tadpoles experienced no lag effect of endosulfan.

For western toads, survival in the water and vehicle controls was 100% after both 4 and 8 d. After 4 and 8 d, tadpoles exposed to 7, 60, and 296 ppb of endosulfan had significantly greater death than the control (Fig. 1). Interestingly, this species exhibited a discontinuous pattern of mortality with increasing endosulfan concentrations, and the reasons for the pattern are unclear. Using these data, the LC50 estimates were 76.1 ppb after 4 d and 46.8 ppb after an additional 4 d in clean water (Table 2). Based on the 84% confidence intervals, western toad tadpoles experienced no lag effect of endosulfan exposure.

Green frog survival was initially excellent in the water and vehicle controls but declined slightly over time (98 and 95% after 4 d and 95 and 88% after 8 d, respectively). After 4 and 8 d, the 6-, 7-, 35-, 60-, and 296-ppb concentrations caused significantly greater mortality than the control (Fig. 1). Based on these data, the LC50 estimates were 3.2 ppb after 4 d of endosulfan exposure and 3.0 ppb after an additional 4 d in clean water (Table 2). Thus, the green frog tadpoles experienced no lag effect in response to endosulfan exposure.

For Cascades frogs, survival in the water and vehicle controls was 100% after both 4 and 8 d. After 4 and 8 d, the 7-, 35-, 60-, and 296-ppb concentrations caused significantly more death than the control (Fig. 1). The LC50 estimate was 15.0 ppb after 4 d but 6.7 ppb after an additional 4 d in clean water, which is significantly lower based on the 84% confidence intervals (Table 2). Thus, Cascades frog mortality exhibited a small lag effect following exposure to endosulfan.

Bullfrog survival in the water and vehicle controls was high over the first 4 d (93 and 100%, respectively) but declined slightly over the following 4 d (90 and 100%). After the first 4 d, all concentrations except 2 ppb caused significantly more death than the control (Fig. 1). After 8 d, all concentrations were significantly different from the control. The LC50 estimates were 1.3 ppb after 4 d and 0.9 ppb after 8 d (Table 2). Based on the 84% confidence intervals, no significant lag effect occurred.

## DISCUSSION

We found that the insecticide endosulfan can be very highly toxic to amphibian larvae at low concentrations, that species differ in sensitivity to endosulfan, and, for several species, that the mortality after 4 d of exposure to endosulfan may substantially underestimate lethality of the pesticide. Using categories defined by the U.S. EPA (<http://www.epa.gov/espp/litstatus/effects/redleg-frog>) and LC50<sub>4-d</sub> estimates from the present study (Table 2), endosulfan would be classified as very highly toxic (i.e., LC50 < 100 ppb) to six species (western toads, Pacific tree frogs, gray tree frogs, Cascades frogs, green frogs, and bullfrogs) and highly toxic (i.e., 100 < LC50 < 1,000 ppb) to one species (spring peepers). For the remaining two species (American toads and leopard frogs), the LC50<sub>4-d</sub> value could not be estimated because the value is higher than our highest concentration of 60 ppb. However, based on our LC50<sub>8-d</sub> estimates and assuming that the LC50 for American toads is close to 60 ppb (Fig. 1), endosulfan would be classified as very highly toxic to all nine species (Table 2).

If one considers the LC50<sub>8-d</sub> estimates among species with regard to family affiliations, an interesting phylogenetic pattern emerges. Generally, it appears that members of the toad family (Bufonidae) are the least susceptible, members of

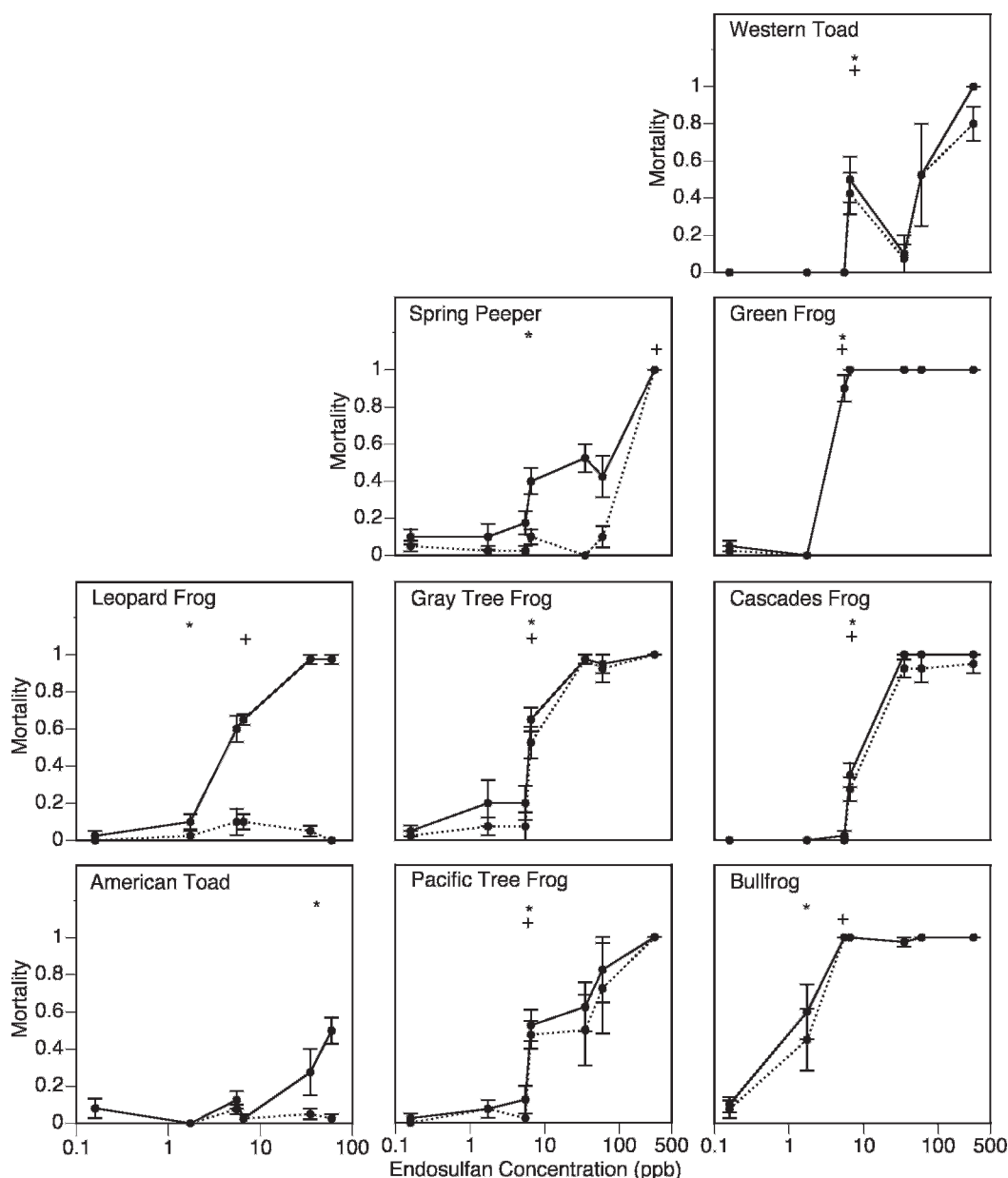


Fig. 1. Mortality (means  $\pm$  1 SE) of tadpoles when exposed to a range of endosulfan concentrations. Dotted lines indicate mortality after 4 d, while solid lines indicate mortality after 8 d. The lowest-observed-effect concentration (compared to the control) is marked with a plus sign (+) for mortality after 4 d and an asterisk (\*) for mortality after 8 d. Note that the first two experiments (*Bufo americanus* and *Rana pipiens*) were tested with up to only 60 ppb of endosulfan. All other species were tested with up to 296 ppb of endosulfan.

the tree frog family (Hylidae) are intermediate in susceptibility, and members of the true frog family (Ranidae) are the most susceptible. However, to rigorously determine if this apparent pattern really exists, we need to test many more species from each family and conduct a phylogenetic analysis on the species' susceptibilities. If the pattern holds, it then offers helpful predictive ability regarding the relative impacts on larval anurans when exposed to endosulfan contamination in nature.

An important comparison to make when examining lethal concentration values is where the estimates fall relative to environmentally expected values. Estimates from wind-blown overspray indicate that endosulfan concentrations can reach 700 ppb when sprayed 10 m from a pond and 4 ppb when sprayed 200 m from a pond [23]. Actual endosulfan concentrations were 0.53 ppb in ponds near apple orchards in Ontario, Canada [1], and 2.5 ppb in the Gwydir River basin

in Australia [24]. A recent U.S. EPA model [21] estimates the environmentally expected values of surface drinking water for endosulfan at 4.5 to 23.9 ppb for acute exposures and 0.5 to 1.5 ppb for chronic exposures. Combining the U.S. EPA chronic estimates with our 8-d lethal concentration values (Table 2), larval populations of leopard frogs, spring peepers, gray tree frogs, Pacific tree frogs, and green frogs could experience an approximate 10% decrease in survival, whereas bullfrog tadpoles could experience an approximate 50% decrease in survival. Combining U.S. EPA acute estimates with 8-d values from the present study, larval populations of western toads could experience an approximate 10% decrease in survival, all the hylids (spring peepers, gray tree frogs, and Pacific tree frogs) could experience an approximate 50% decrease in survival, and all the ranids (leopard frogs, green frogs, Cascades frogs, and bullfrogs) could experience an

approximate 90% decrease in survival. Of course, the true effect of endosulfan exposure on amphibian populations in nature is difficult to estimate because of other potentially additive and synergistic interactions with other pesticides, biotic factors, and abiotic conditions.

While few toxicity experiments using endosulfan have been conducted on amphibians, our LC50<sub>4-d</sub> values are similar to or less than those previously reported. For example, our experiments found that LC50<sub>4-d</sub> values across seven species range from 1.3 to 120 ppb (LC50<sub>4-d</sub> values could not be estimated for two of the nine species). Among the few tests that have been conducted, LC50<sub>4-d</sub> values range from 1.8 to >4,700 ppb across a variety of *Bufo* and *Rana* [18,27–29]. Some of this variation is likely due to differences in testing protocols. For example, Harris et al. [29] used static renewals in which endosulfan concentrations were renewed every 24 h, whereas Bernabò et al. [18], Gopal et al. [27], and Vardia et al. [28] used static tests in which replicates were dosed with endosulfan at the start of the experiment but the concentration was never renewed. Moreover, the earlier tests did not extend beyond 4 d, preventing one from observing the highly lethal effects in some species. Values in the literature do not show an apparent pattern of susceptibility among anuran families, but this is perhaps not surprising given differences in testing protocols and the fact that the phylogenetic pattern in our data appeared only when we examined the LC50<sub>8-d</sub> values.

Other aquatic organisms have also been tested with endosulfan with similar levels of reported toxicity. Fish such as striped bass (*Morone saxatilis*), rainbow trout (*Oncorhynchus mykiss*), and bluegill sunfish (*Lepomis macrochirus*) have static LC50<sub>4-d</sub> estimates that range from 0.1 to 1.7 ppb [4,21]. Endosulfan LC50 studies also have been completed on crustaceans, including amphipods and crayfish. Static LC50<sub>4-d</sub> values for the amphipods *Gammarus palustris* [24] and *Hyalella azteca* [4] are 0.43 and 5.7 ppb, respectively, whereas the static LC50<sub>4-d</sub> value for the red swamp crayfish (*Procambarus clarkii*) is 120 ppb, [24], matching the present study's highest LC50<sub>4-d</sub> value among the nine anurans. Collectively, these results suggest that larval anurans can have sensitivities that overlap with the sensitivities of fish and crustaceans.

Of the nine species tested, two species had significant, small lag effects, and another three species (from all three families of anurans) had significant, large lag effects. For example, leopard frogs experienced no significant mortality after the initial 4 d of endosulfan exposure (at 60 ppb) but suffered 97% mortality during the subsequent 4 d while living in clean water. The lag effect is also revealed by changes in the LOEC between days 4 and 8 (Table 2). Collectively, this suggests that the toxicity estimates based on a 4-d exposure to endosulfan could dramatically underestimate the effects of endosulfan on many species of larval amphibians.

The decision to hold the tadpoles for an additional period of time to observe potential lag effects of endosulfan was influenced by two previous studies [11,20]. Berrill et al. [20] reported large magnitudes of postexposure death of wood frog, green frog, and American toad tadpoles. After 4 d of static (single dose) exposure, the three species of tadpoles were removed from contaminated water and held for an additional number of days in clean water (tadpoles were fed daily, and complete water changes were conducted every 3 d). For American toads, the initial 4-d exposure caused at least 10% mortality in all endosulfan concentrations. After an additional 5 d in clean water, mortality was 60 to 90% in the endosulfan treatments (41,

139, and 252 ppb). For wood frogs, the initial 4-d exposure caused approximately 10% mortality across all endosulfan concentrations. After an additional 7 d in clean water, mortality was approximately 80% in the two lower endosulfan concentrations (68 and 138 ppb) and 100% in the highest endosulfan concentration (364 ppb). For green frogs, the initial 4-d exposure caused approximately 10% mortality in the endosulfan concentrations (53, 130, and 345 ppb). After an additional 10 d in clean water, mortality was approximately 30% in two endosulfan treatments (53 and 345 ppb) and 80% in the middle concentration (130 ppb). Unfortunately, no LC50 values were estimated for the initial 4-d exposures or for the extended holding times. In a second study examining endosulfan's impact on tadpoles, Relyea [11] added endosulfan at a very low concentration (6 ppb) to mesocosm communities containing tadpoles. The single application of endosulfan killed 84% of leopard frog tadpoles, but this mortality did not appear to occur in the first few days of the experiment (R.A. Relyea, personal observation). This observation is consistent with the current study's discovery of substantial lag effects in leopard frogs, American toads, and spring peepers. Hence, combined with the findings of Berrill et al. [20] and Relyea [11], our data suggest that lag effects of endosulfan may be common in amphibians.

## CONCLUSIONS

The present study found that endosulfan can cause high levels of mortality in amphibian larvae at concentrations that are expected and found in nature, although the frequency of such contaminations is currently unknown. Although a lag effect from exposure to endosulfan was previously reported by Berrill et al. [20], this is the first study to estimate LC50 values after 4 d of endosulfan exposure followed by another 4 d in clean water. In doing so, we are able to quantify a strong lag effect of endosulfan exposure across several species of amphibians. This discovery means that predictions based on traditional 4-d tests may dramatically underestimate LC50 values. As with all LC50 tests, the effects of endosulfan under more natural conditions and within a community context still need further investigation. Future research should examine whether continuous exposure to endosulfan would cause the same amount of death as an initial exposure to endosulfan followed by an exposure to clean water to test whether the ultimate lethal impacts of endosulfan are determined early in exposure. The potential for lag effects in other common pesticides also should be investigated to determine if current observations are characteristic only of endosulfan. The variation among species in their LC50<sub>8-d</sub> estimates spanned more than an order of magnitude between the least and most susceptible species and appeared to show phylogenetic patterns. By continuing to conduct comparative studies that shed light on possible phylogenetic patterns in pesticide sensitivity, toxicologists may gain insights into those clades and traits that help determine species susceptibility.

*Acknowledgement*—We thank Steven Bagnall, Jenise Brown, Shane Hanlon, and Tim Schwartz for their help with the experiments. Our thanks to Andy Blaustein, Paul Bradley, and Julia Buck at Oregon State University for their help with egg collection. Rickey Cothran provided comments that improved the present paper, and the U.S. National Science Foundation funded this research.

## REFERENCES

1. Harris ML, Bishop CA, Struger J, Van Den Heuvel MR, Van Der Kraak GJ, Dixon DG, Ripley B, Bogart JP. 1998. The functional

- integrity of northern leopard frog (*Rana pipiens*) and green frog (*Rana clamitans*) populations in orchard wetlands. I. Genetics, physiology, and biochemistry of breeding adults and young-of-the-year. *Environ Toxicol Chem* 17:1338–1350.
2. McConnell LL, LeNoir JS, Datta S, Seiber JN. 1998. Wet deposition of current-use pesticides in the Sierra Nevada mountain range, California, USA. *Environ Toxicol Chem* 17:1908–1916.
  3. LeNoir JS, McConnell LL, Fellers GM, Cahill TM, Seiber JN. 1999. Summertime transport of current-use pesticides from California's central valley to the Sierra Nevada mountain range, USA. *Environ Toxicol Chem* 18:2715–2722.
  4. Wan MT, Kuo J, Buday C, Schroeder G, Van Aggelen G, Pasternak J. 2005. Toxicity of  $\alpha$ -,  $\beta$ -, ( $\alpha + \beta$ )-endosulfan and their formulated and degradation products to *Daphnia magna*, *Hyalella azteca*, *Oncorhynchus mykiss*, *Oncorhynchus kisutch*, and biological implications in streams. *Environ Toxicol Chem* 24:1146–1154.
  5. Gilliom RJ, Hamilton PA. 2006. Pesticides in the nation's streams and ground water, 1992–2001—A summary. Fact Sheet. U.S. Geological Survey, Reston, VA.
  6. Rohr JR, Crumrine PW. 2005. Effects of an herbicide and an insecticide on pond community structure and processes. *Ecol Appl* 15:1135–1147.
  7. Boone MD, Semlitsch RD, Little EE, Doyle MC. 2007. Multiple stressors in amphibian communities: Effects of chemical contamination, bullfrogs, and fish. *Ecol Appl* 17:291–301.
  8. Relyea RA, Diecks N. 2008. An unforeseen chain of events: Lethal effects of pesticides at sublethal concentrations. *Ecol Appl* 18:1728–1742.
  9. Relyea RA, Hoverman JT. 2008. Interactive effects of predators and a pesticide on aquatic communities. *Oikos* 117:1647–1658.
  10. Rohr JR, Schotthoefer AM, Raffel TR, Carrick HJ, Halstead N, Hoverman JT, Johnson CM, Johnson LB, Lieske C, Piwoni MD, Schoff PK, Beasley VR. 2008. Agrochemicals increase trematode infections in a declining amphibian species. *Nature* 455:1235–1239.
  11. Relyea RA. 2009. A cocktail of contaminants: How mixtures of pesticides at low concentrations affect aquatic communities. *Oecologia* 159:363–376.
  12. Davidson C, Shaffer HB, Jennings MR. 2001. Declines of the California red-legged frog: Climate, UV-B, habitat, and pesticides hypotheses. *Ecol Appl* 11:464–479.
  13. Sparling DW, Fellers GM, McConnell LL. 2001. Pesticides and amphibian population declines in California, USA. *Environ Toxicol Chem* 20:1591–1595.
  14. Davidson C, Shaffer HB, Jennings MR. 2002. Spatial tests of the pesticide drift, habitat destruction, UV-B, and climate-change hypothesis for California amphibian declines. *Conserv Biol* 16:1588–1601.
  15. Davidson C. 2004. Declining downwind: Amphibian population declines in California and historical pesticide use. *Ecol Appl* 14:1892–1902.
  16. Harris ML, Chora L, Bishop CA, Bogart JP. 2000. Species- and age-related differences in susceptibility to pesticide exposure for two amphibians, *Rana pipiens*, and *Bufo americanus*. *Bull Environ Contam Toxicol* 64:263–270.
  17. Relyea RA. 2005. The impact of insecticides and herbicides on the biodiversity and productivity of aquatic communities. *Ecol Appl* 15:618–627.
  18. Bernabò I, Brunelli E, Berg C, Bonacci A, Tripepi S. 2008. Endosulfan acute toxicity in *Bufo bufo* gills: Ultrastructural changes and nitric oxide synthase localization. *Aqua Toxicol* 86:447–456.
  19. Hopkins WA. 2007. Amphibians as models for studying environmental change. *ILAR Journal* 48:270–277.
  20. Berrill M, Coulson D, McGillivray L, Pauli B. 1998. Toxicity of endosulfan to aquatic stages of anuran amphibians. *Environ Toxicol Chem* 17:1738–1744.
  21. Rossi LA. 2002. Reregistration eligibility decision for endosulfan. EPA 738-R-02-013. Reregistration Report. U.S. Environmental Protection Agency, Washington, DC.
  22. Ernst WR, Jonah P, Doe K, Julien G, Hennigar P. 1991. Toxicity to aquatic organisms of off-target deposition of endosulfan applied by aircraft. *Environ Toxicol Chem* 10:103–114.
  23. Muschal M. 2001. Central and north west region's water quality program. CNR2000.067. CNWRWQP Pesticide Report. Centre for Natural Resources, NSW Department of Land and Water Conservation, NSW, Australia.
  24. Leight AK, Van Dolah RF. 1999. Acute toxicity of the insecticides endosulfan, chlorpyrifos, and malathion to the epibenthic estuarine amphipod *Gammarus palustris* (Bousfield). *Environ Toxicol Chem* 18:958–964.
  25. Gosner KL. 1960. A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologica* 16:183–190.
  26. Payton ME, Greenstone MH, Schenker N. 2003. Overlapping confidence intervals or standard error intervals: What do they mean in terms of statistical significance? *J Insect Sci* 34:1–6.
  27. Gopal K, Khanna RN, Anand M, Gupta GSD. 1981. The acute toxicity of endosulfan to fresh-water organisms. *Toxicol Lett* 7:453–456.
  28. Vardia HK, Rao PS, Durve VS. 1984. Sensitivity of toad larvae to 2,4-D and endosulfan pesticides. *Arch Hydrobiol* 100:395–400.
  29. Harris ML, Bishop CA, Struger J, Ripley B, Bogart JP. 1998. The functional integrity of northern leopard frog (*Rana pipiens*) and green frog (*Rana clamitans*) populations in orchard wetlands. II. Effects of pesticides and eutrophic conditions on early life stage development. *Environ Toxicol Chem* 17:1351–1363.