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Short Communication

ACUTE TOXICITY OF HERBICIDE FORMULATIONS AND CHRONIC TOXICITY OF TECHNICAL-GRADE TRIFLURALIN TO LARVAL GREEN FROGS (*LITHOBATES CLAMITANS*)

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Abstract—Fewer toxicity studies have been performed on herbicides than on insecticides despite heavier use of herbicides and evidence of herbicide formulation toxicity to amphibians. We conducted acute and chronic toxicity tests with the herbicide trifluralin (2,6-dinitro-*N,N*-dipropyl-4-(trifluoromethyl)aniline) on tadpoles. Herbicide formulations had lower median lethal concentrations than an insecticide formulation and technical-grade trifluralin. Chronic trifluralin exposure resulted in significantly smaller tadpoles at low concentrations (20 μg/L) compared with controls and 200-μg/L treatments. Environ. Toxicol. Chem. 2012;31:2029–2034.
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INTRODUCTION

There is a general concern regarding the potential for deleterious effects of agrochemicals on amphibian populations [1]. Insecticides are used at much lower quantities than fertilizers and herbicides [2], but many more insecticides than herbicides have been the subject of extensive research in amphibian ecotoxicology [3]. As an example of the relative use of different classes of pesticides, statistics on agrochemical use on cotton in West Texas for 2007 indicated that 226 million kg of fertilizers were applied, 4.98 million kg of herbicides were applied, and just less than 2.04 million kg of insecticides were applied [4]. The lack of studies on herbicide toxicity to amphibians may be driven, in part, by the fact that herbicide active ingredients are not typically considered toxic to animals.

However, data indicate that some triazine herbicides, such as atrazine, cause negative sublethal effects in amphibians (e.g., Hayes et al. [5,6]), although others have not found effects at similarly low environmentally relevant concentrations (e.g., Carr et al [7]). In addition, herbicide formulations can be very toxic to amphibian larvae, resulting from what are considered inactive additives such as surfactants, solvents, and other chemicals (e.g., Roundup [8–11]). For example, there has been considerable research into the acute and sublethal toxicity of glyphosate-based herbicides. Glyphosate formulations containing the polyethoxylated tallowamine surfactant appear to be highly toxic to amphibians (e.g., Relyea and Jones [10], and Mann and Bidwell [11]), and it seems apparent that the surfactant contributes strongly to toxicity as the technical-grade active ingredient has much lower toxicity [11]. Therefore, despite mechanisms of action that may be specific to plants, herbicide formulations could be causing important toxic effects on amphibians either through active ingredients that cause unexpected sublethal toxic effects or because of unregulated solvents and surfactants used in herbicide formulations.

Although there is some research on the effects of herbicides on amphibians, much of the research has focused on only a few chemicals and considerable data gaps exist. Specifically, highly used herbicides such as pendimethalin and trifluralin (the sixth and eighth most widely used herbicides in the United States [2]) have not received much research attention compared with the most widely used and well-known herbicides (e.g., glyphosate and atrazine). Previous research has suggested that trifluralin is highly toxic to amphibian larvae [12]. Fowler's toad (*Bufo woodhousii fowleri*) tadpoles exposed to multiple ($n = 18$) pesticides (herbicides and insecticides, active ingredients only) for 96 h were most sensitive to trifluralin (median lethal concentration [LC50] = 180 μg/L). This single trifluralin LC50 appears to be environmentally relevant, as the active ingredient has been detected in playa wetland water samples (South High Plains, TX, USA) at mean concentrations of 7.3 μg/L with concentrations as high as 437 μg/L (T.A. Anderson et al., The Institute of Environmental and Human Health, Texas Tech University, unpublished data). These two studies combined suggest that trifluralin (and possibly the closely structured herbicide pendimethalin) could be posing serious risk to amphibians in areas receiving runoff from agricultural fields in which these herbicides have been applied.

The objectives of the present study were as follows: (1) to evaluate and compare the toxicity of two common herbicide formulations, Treflan 4D (active ingredient = trifluralin) and Prowl 400EC (active ingredient = pendimethalin), to green frog (*Lithobates clamitans*) tadpoles; (2) to compare herbicide formulation toxicity to technical-grade trifluralin and a common insecticide formulation (Malathion 55%); and (3) to evaluate the long-term toxicity of environmentally relevant concentrations of trifluralin (99% pure) to tadpoles in a laboratory setting.

MATERIALS AND METHODS

Study organisms

Green frog eggs were acquired from a commercial supplier (Charles D. Sullivan) and shipped on June 22, 2011 to The Institute of Environmental and Human Health, Texas Tech University. Eggs were evenly divided and placed into two

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40-L aquaria for hatching and maintenance of tadpoles prior to testing. Eggs took approximately 13 d to hatch and reach Gosner stage 25 [13]. For hatching, maintenance, and all experiments, moderately hard reconstituted fresh water was used (3 g $CaSO_4$, 3 g $MgSO_4$, 0.2 g KCl, and 4.9 g $NaHCO_3$ dissolved in 50 L of deionized water), hereafter called lab water. Tadpoles were fed ground rabbit chow and fish flakes ad libitum following hatching. All tadpoles used in experiments started in Gosner stage 25 [13]. Tadpoles were chosen in a haphazard manner and randomly distributed to treatments during experimental setup. All procedures involving tadpoles were approved by the Texas Tech University Institutional Animal Care and Use Committee (approval no. 10011-04).

Pesticide information

We purchased herbicide formulations that are used commonly by pesticide applicators. Treflan 4D (Dintec Agrichemicals; active ingredient = trifluralin, 478 g/L) is a dinitroaniline herbicide commonly used on cotton in the South High Plains. Prowl 400EC (BASF; active ingredient = pendimethalin, 400 g/L) is also a dinitroanaline herbicide used on cotton in the South High Plains. Trifluralin was applied at three times the amount of pendimethalin used on cotton in Texas in 2007 (1,056,870 kg trifluralin compared with 335,204 kg of pendimethalin, [4]) and was therefore the focal pesticide of interest in the present study. We also acquired a commercially available formulation of malathion (Malathion 55%, Hi-Yield Chemical) to compare with the toxicity of the herbicide formulations. Finally, technical-grade trifluralin with a guaranteed purity >99% was purchased from Chem Service.

Acute toxicity tests

Tadpoles were exposed to pesticides in glass jars filled with 400 ml of lab water. Four tadpoles were used in each jar, with five jars per concentration for each treatment. A range-finding test was first conducted to determine concentrations used in the definitive tests. Tests were conducted for 96 h; tadpoles were not fed during the test. Tests were conducted in an incubator (Low temp biochemical oxygen demand incubator; VWR) set at 25°C with a 14:10 light:dark cycle. Every 24 h, mortality at each concentration was recorded. In addition, tadpoles were held for an additional 4 d (with food) to determine any possible lag effects from pesticide exposure. Water samples were collected for concentration verification after spiking as well as at the end of the 4-d exposure period. Treflan 4D and Prowl 400EC acute toxicity tests were started on July 6, 2011, and technical-grade trifluralin and Malathion 55% toxicity tests began on July 11, 2011. Nominal exposure concentrations of both Treflan 4D and Prowl 400EC were 0, 0.5, 1, 2, 4, and 8 mg a.i. per liter of lab water. For Treflan 4D and Prowl 400 EC, 1 mg/ml stock solutions were created by adding 0.313 and 0.375 ml, respectively, of formulation to 150 ml of lab water. As an example, 3.2 ml of the 1 mg/ml solution was spiked into 400-ml jars to achieve concentrations of 8 mg/L. Exposure concentrations for Malathion 55% were 0, 3, 4, 5, 6, and 7 mg a.i. per liter of lab water. A stock solution of 0.400 mg/ml of Malathion 55% was created by adding 113.9 μl of formulation to 150 ml of lab water. The 7-mg/L treatments were then spiked with 7 ml of the stock solution to achieve the nominal concentration in 400-ml jars. Technical-grade trifluralin exposure concentrations were 0, 2, 3, 4.5, 6.75, and 10.125 mg/L. A 10-mg/ml stock solution of technical-grade trifluralin was created by dissolving 10 mg of trifluralin in 10 ml of acetone. To achieve a concentration of 10.125 mg/L, 0.405 ml of the stock solution was added to the 400 ml of lab water. Controls received the greatest volume of acetone (technical-grade trifluralin) or lab water (Treflan 4D, Prowl 400 EC, and Malathion 55%) used in the acute toxicity experiments.

Chronic toxicity tests of trifluralin

Tadpoles at Gosner stage 25 were randomly assigned to 20-L glass aquaria filled with 10 L of lab water. Five tadpoles were used in each 20-L aquarium and there were six replicate aquarium for each concentration treatment. Tests were conducted in a temperature-controlled animal facility (22 ± 3°C) with a 14:10 light:dark cycle. Dissolved oxygen was maintained with air pumps and polyethylene tubing. Tadpoles were given 7 d to acclimate to tanks prior to spiking with trifluralin. The first spiking occurred on July 20, 2011. Trifluralin concentrations represented an environmentally realistic concentration of 20 μg/L, a relatively worst-case scenario of chronic exposure to a high concentration of 200 μg/L, and a control treatment of 0 μg/L. Water was changed every 4 d, and trifluralin (99% pure) was spiked after every water change. Trifluralin stock solutions were created fresh for each water change by dissolving technical-grade trifluralin (~15 mg, range: 13.9–16.8 mg) in 10 ml of acetone. Spike volumes were adjusted to maintain nominal concentrations. Control tanks received an acetone-only spike equal to the volume delivered to the 200-μg/L tanks. Tadpoles were given ground rabbit chow and fish flakes ad libitum during tests, and fresh food was provided after each water change. Tadpole mass was measured at each water change starting at day 26 until the termination of the experiment at day 62 using a Mettler–Toledo PR 2002 balance (accurate to 0.01 g). To measure tadpole mass, all five tadpoles from a given replicate were combined and placed into a previously tared, glass jar containing lab water. Tadpoles were captured using a brine shrimp net that was placed on a paper towel to remove excess water prior to measuring mass. Attempting to remove excess water reduced the addition of water from the treatments that would bias measurements. At the end of the experiment, tadpoles were observed under a dissecting scope to record Gosner stage [13] to determine any effects of treatment on development.

Water samples were taken once at the beginning of the experiment (experimental day 1) to verify concentrations and once in the middle of the experiment (experimental day 20). Water samples were taken immediately before a water change and then after the water change and trifluralin spike. Water quality (pH, dissolved oxygen, conductivity, and temperature) was monitored by collecting a random sample from each treatment before and after water changes. Water quality never deviated outside of acceptable ranges: pH ranged from 6.8 to 8.0, dissolved oxygen ranged from 5.04 to 7.09 mg/L, conductivity ranged from 279.2 to 451 μS/cm, and temperatures ranged from 20.9 to 24.9°C. In almost all cases, water quality improved following water changes (i.e., dissolved oxygen increased, conductivity decreased, etc.).

Chemical analysis

Concentrations of trifluralin, pendimethalin, and malathion were verified using a Hewlett-Packard 6890 gas chromatograph equipped with an electron capture detector. All water concentrations (percentage of nominal concentration) reported here were corrected for extraction efficiencies. For Treflan 4D, Prowl 400EC, and technical trifluralin acute studies, 10 ml water samples were randomly collected from two replicates for each treatment. Water samples were extracted with 10 ml of hexane,

shaken for 30 min using a VMR Orbital Shaker, and centrifuged for 10 min at 1,712 *g* (Beckman Allegra6R Centrifuge). The measured concentrations ranged from 105 to 137% of the nominal concentrations for technical-grade trifluralin, 100 to 117% of the nominal concentrations for Treflan 4D, and 68 to 106% for Prowl 400EC. For malathion, 5-ml water samples were collected randomly from two replicates for each treatment, and diluted to 10 ml with lab water. Diluted samples were extracted with 10 ml hexane, shaken for 20 min, and centrifuged for 10 min. Water concentrations ranged from 88 to 101% of the nominal concentrations (after correcting for extraction efficiency). For the trifluralin chronic study, 20-ml water samples were randomly collected from two replicates for each treatment, extracted with 5 ml hexane, shaken for 30 min, and centrifuged for 10 min at 1,712 *g*. Measured technical-grade trifluralin concentrations ranged from 75 to 100% of the nominal concentrations.

Statistical analysis

All statistical analyses were performed using R (Ver 2.13.1 [14]). We used two methods for estimating LC50 values including a logistic model and a log-logit model. Two methods were used as no single estimation method provided robust estimates of all LC50s because of differences in the data among chemicals: data either did not contain partial mortality (Treflan 4D and Prowl 400 EC) or data did not contain multiple treatments with mortality greater than 50% (Malathion 55% and technical-grade trifluralin). For the two herbicide formulations, LC50 values were estimated by fitting a logistic model using the "drm" function within the "drc" package in R [15]. For Malathion 55% and technical-grade trifluralin, a log-logit model was used to estimate LC50s using the "glm" function of the "MASS" package in R [16]. Significant differences between LC50 values were determined by comparing confidence intervals calculated for each LC50.

Effects of trifluralin on mass and growth rate during the chronic trifluralin study were assessed using repeated measures analysis of variance. For all statistical analyses, alpha was 0.05. All data met assumptions of analysis of variance, and no transformations were needed. For both chronic and acute toxicity, aquaria or jars were the unit of replication, not individual tadpoles.

RESULTS

Acute toxicity tests

Both Treflan 4D (LC50 = 2.81 mg/L) and Prowl 400EC (LC50 = 2.47 mg/L) were more toxic to tadpoles than Malathion 55% (LC50 = 6.47 mg/L) or technical trifluralin (LC50 = 9.76 mg/L) (Table 1). In the case of both Treflan 4D and Prowl 400EC, toxicity curves were very sharp over our concentration range, and all mortality occurred within 24 h of the start of exposures. In contrast, technical-grade trifluralin showed a more gradual toxicity, which increased over time. No latent effects were found in our studies, with 8-d mortality matching 96-h mortality exactly for all acute tests (data not shown).

Chronic toxicity tests

Mortality was very low throughout the entire chronic exposure, with only 1 tadpole mortality in the 20-μg trifluralin/L treatment. After 62 d, only one tadpole had forelimb emergence. Treatment ($F_{2,15} = 4.75$, $p = 0.025$), time ($F_{9,135} = 318.9$, $p < 0.001$), and the time × treatment interaction ($F_{18,135} = 1.85$, $p = 0.025$) had significant effects on tadpole mass (Fig. 1). Interestingly, the lower, more environmentally relevant, exposure concentration of 20 μg/L resulted in considerably smaller tadpoles compared with controls and 200-μg/L treatments. No significant difference was found between control and 200-μg/L treatments. Tadpole growth rate was significantly affected only by time ($F_{8,120} = 3.02$, $p = 0.004$). Treatment and the interaction

Table 1. Acute toxicity of two herbicide formulations, Treflan 4D (active ingredient = trifluralin) and Prowl 400 EC (active ingredient = pendimethalin), compared with an insecticide formulation (Malathion 55%) and technical-grade trifluralin (>99% purity)[a]

Chemical	Concentration (mg/L)	96-h mortality (%)	LC50 (mg/L)	SE	Lower CI	Upper CI
Treflan 4D	0	0	2.81A	0.028	2.76	2.86
	0.5	0				
	1	0				
	2	0				
	4	100				
	8	100				
Prowl 400EC	0	0	2.47A	0.803	0.90	4.04
	0.5	0				
	1	0				
	2	5				
	4	100				
	8	100				
Trifluralin	0	0	9.76B	0.843	8.11	11.41
	2	0				
	3	0				
	4.5	10				
	6.25	35				
	10.125	45				
Malathion 55%	0	0	6.47C	0.280	5.92	7.02
	3	5				
	4	5				
	5	20				
	6	20				
	7	75				

[a] 96-h mortality represents the proportion of tadpoles that had died by 96 h for each concentration. Standard errors (SE) are provided for each median lethal concentration (LC50) along with associated 95% confidence intervals (CI). Lethal concentrations with different letters are significantly different.

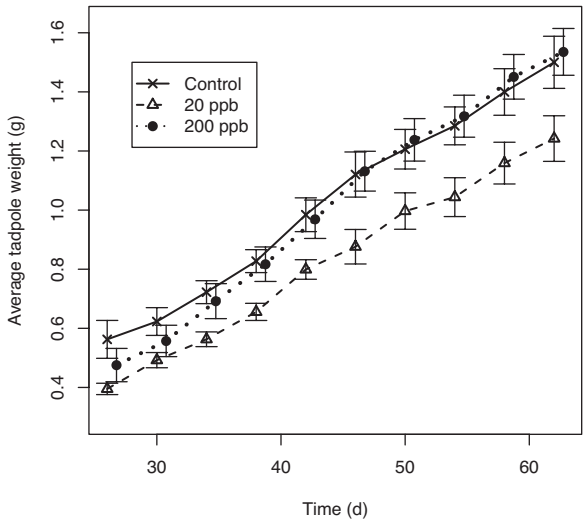

Fig. 1. Green frog (*Lithobates clamitans*) tadpole growth during the chronic experiment with exposure to 0, 20, or 200 μg/L technical-grade trifluralin. Tadpole mass is the average tadpole mass (the total mass of tadpoles/five tadpoles). Time, treatment, and their interaction are significantly affecting growth. Treatment differences are due to smaller tadpoles in the 20-μg/L treatment. The 200-μg/L data are offset by 0.5 d to ease viewing, but all data were collected on the same day. Error bars represent ± 1 standard error.

between treatment and time did not significantly affect growth rates ($p > 0.19$; Fig. 2). At the end of the experiment, Gosner developmental stages did not differ between treatments ($F_{2,15} = 0.735$, $p = 0.50$; data not shown).

DISCUSSION

We found that herbicide formulations were more acutely toxic to amphibians at concentrations lower than an insecticide formulation and technical-grade trifluralin. We found no evidence of lag effects in our acute experiments, as there was no additional mortality in the 4 d in clean lab water (with food provided) following pesticide exposures. Environmentally

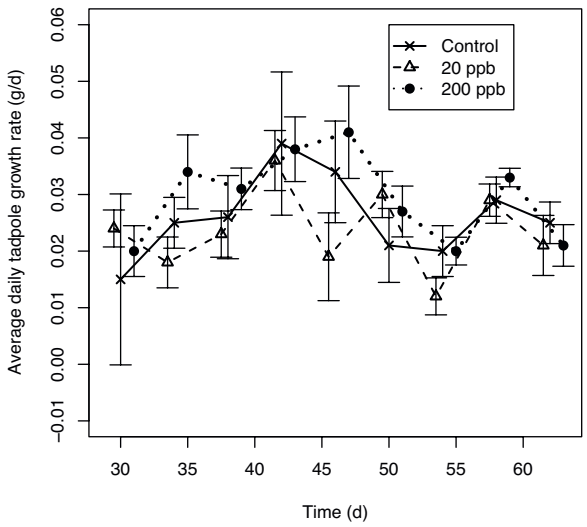

Fig. 2. Green frog (*Lithobates clamitans*) tadpole growth rates during the chronic experiment with exposure to 0, 20, or 200 μg/L technical-grade trifluralin. Growth rate was calculated as $(X_{i+1}–X_i)/4$ d, in which the average tadpole mass from one period (X_i) was subtracted from the next period (X_{i+1}) and divided by 4 d. Only time is significantly affecting growth rate. Both 200- and 20-μg/L data are offset to ease viewing, but all data were collected on the same day. Error bars represent ± 1 SE.

relevant concentrations of technical-grade trifluralin resulted in smaller tadpoles than controls throughout the chronic exposure experiment.

The concentrations that caused acute toxicity to amphibians are unlikely to be environmentally relevant. Following label specifications for Treflan 4D, a direct accidental overspray would result in a concentration of 102 μg/L in a 1-ha pond that is 1 m deep. Many playa wetlands are less than 1 m deep, but it would take a 1-ha lake of only 10 cm depth to result in a concentration near toxicity for amphibians. Label specifications are similar for Prowl 400EC, and following label specifications a direct application to a pond would result in 168 μg/L of pendimethalin in a 1-ha pond that is 1 m deep. Interestingly, a previous study found maximum trifluralin concentrations of 437 μg/L in playa wetlands in West Texas (T.A. Anderson et al., The Institute of Environmental and Human Health, Texas Tech University, unpublished data). Consequently, very large applications of Treflan 4D to small shallow wetlands could result in concentrations high enough to cause acute toxicity to amphibians, but would likely be very rare.

Our results suggest that the formulated herbicide was more toxic than the technical-grade active ingredient. Mann and Bidwell [11] similarly reported that Roundup formulations of the herbicide glyphosate are much more toxic than the technical-grade glyphosate acid or the isopropylamine salt of glyphosate alone. However, formulations are not always more toxic than the active ingredient. Puglis and Boone [17] found that formulations of seven pesticides were significantly more toxic than technical-grade active ingredients in only four cases (malathion, imidacloprid, bifenthrin, and glyphosate) and less toxic for three insecticides (carbaryl, permethrin, and β-cyfluthrin). These results were not consistent across pesticide groups (organophosphates, pyrethroids), which further highlights the contribution of so-called inert ingredients to the toxicity of pesticides to nontarget organisms. Additional research is needed to better understand the relative toxicity of formulated and technical-grade pesticides and to determine whether any patterns emerge in relative toxicity.

We found much lower toxicity for technical-grade trifluralin than Sanders [12]. In Sanders's [12] study, the technical-grade trifluralin LC50 for Fowler's toads (0.180 mg/L) was approximately 1/50 of the LC50 found in the present study (9.76 mg/L). However, toads were exposed to trifluralin at 15°C in Sanders's study [12], compared with 25°C in the present study. Lower temperatures may increase toxicity of other pesticides (e.g., endosulfan [18]), although the generality of this relationship remains uncertain and is likely related to the toxic mode of action. It is also important to consider interspecies differences. Toxicity estimates of 50-fold are not uncommon between species of amphibians and have been reported previously (e.g., cadmium and chloroform [19]). The combination of interspecies differences and temperature may contribute to the discrepancy between the two studies. However, our LC50 is closer to the 120-h LC50 of 11.8 mg/L derived for the red-bellied toad (*Bombina bombina* [20]). Sayim [20] performed experiments at 22°C, suggesting that the discrepancy between Sanders [12] and recent reports of toxicity may be related to the temperature of the experiments. We are not aware of other studies of pendimethalin on tadpoles, so our results appear to be the first record of toxicity of this herbicide to amphibians.

The effect of chronic exposure to trifluralin on tadpole growth could potentially have significant ecological effects. Amphibians that emerge smaller from the aquatic phase may

have lower survival as adults [21,22]. However, smaller froglets may be able to "catch up" in the terrestrial phase within a year of emerging from the larval stage [23]. Tadpole survival in ponds and wetlands is generally low [24,25], so it is unlikely that acute toxicity would result in large population-level effects. Weaker, more susceptible tadpoles would more likely succumb to predation or food shortage than the stronger, healthier 1 to 5% that survive the larval stage. However, sublethal effects (such as lower growth) that affect all tadpoles in a particular pond could potentially have larger effects on the population than short-term acute toxicity, which results in a small increase in mortality (e.g., 5–20%).

Finally, the nonmonotonic response found in the present chronic study was unexpected and interesting in that it contrasts with the dose–response paradigm. However, previous research has found nonmonotonic responses of chlorothalonil [26] and atrazine [27] in amphibians. There are several possible explanations for our results. One relates to potential discrepancies in food availability among treatments. However, we made every effort to provide tadpoles with adequate food to allow ad libitum feeding after each water change, and uneaten food was observed at each water change. In addition, the smaller body size in 20-μg/L treatments was consistent through time (Fig. 1), suggesting that smaller growth was consistent across the entire experiment. Metabolic enzyme physiology may also explain the difference between treatments. Regardless of the mechanism for the effect, 200 μg/L may cause an increase in metabolic enzymes (e.g., CYP450 enzymes) to break down trifluralin, but these enzymes may not be stimulated at 20 μg/L. Another possible explanation involves low-concentration endocrine disruption by trifluralin. Nonmonotonic responses are common for endocrine-disrupting chemicals [28]. However, determining the mechanism of this apparent nonmonotonic response was beyond the scope of the present study. More studies are needed to determine the mechanism behind this effect, with thyroid hormone disruption a logical starting point.

CONCLUSIONS

Formulations of two common herbicides (trifluralin and pendimethalin) were more toxic than a formulation of a common insecticide and the technical-grade active ingredient trifluralin. Acute toxicity was found at concentrations that are not likely to be ecologically relevant except in extreme cases (e.g., direct overspray of a small shallow wetland). However, chronic exposure to environmentally relevant concentrations (20 μg/L) of trifluralin resulted in smaller tadpoles, which could manifest as significant ecological effects in later stages. Chronic exposure to higher concentrations of trifluralin (200 μg/L) did not result in smaller tadpoles. This apparent "nonmonotonic" response found in our research suggests an endocrine-disrupting mechanism for trifluralin, but more research is needed to confirm this hypothesis. Our results highlight the importance of testing toxicity of herbicides in addition to insecticides, especially regarding formulations. Furthermore, research involving pesticide toxicity to amphibians should expand beyond common pesticides that have been intensively studied in the amphibian ecotoxicological literature (e.g., glyphosate, atrazine, carbaryl, malathion) to include emerging and common pesticides that have not yet been investigated.

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