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Low concentrations of atrazine, glyphosate, 2,4-dichlorophenoxyacetic acid, and triadimefon exposures have diverse effects on *Xenopus laevis* organ morphogenesis

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Abstract

Many chemicals are released into the environment, and chemical contamination has been suggested as a contributing factor to amphibian declines. To add to a growing body of knowledge about the impact of individual chemicals on non-target organisms, we examined the specificity of deformities induced by exposure to four pesticides (atrazine, 2,4-dichloropheoxyacetic acid (2,4-D), triadimefon, and glyphosate) in the model amphibian species, *Xenopus laevis*. We focused on the period of organ morphogenesis, as it is frequently found to be particularly sensitive to chemical exposure yet also commonly overlooked. We found similar levels of intestine malformations and edemas, as well as disruption of skeletal muscle, in atrazine and triadimefon exposed tadpoles. The effects of 2,4-D were only apparent at the highest concentrations we examined; glyphosate did not induce dramatic malformations at the concentrations tested. While researchers have shown that it is important to understand how chemical mixtures affect non-target organisms, our results suggest that it is first crucial to determine how these chemicals act independently in order to be able to identify consequences of individual pesticide exposure.

Key words: amphibian; pesticide; organ morphogenesis; teratogenesis; toxicology; *Xenopus laevis*

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Many anthropogenic chemical mixtures can be detected in the environment, yet the exact combinations that animals encounter are difficult to predict. How exposure to combinations of agricultural chemicals can affect wildlife is highly dependent on the precise mixture, animal, and life stage studied (Fort et al., 2004; Hayes et al., 2006; Ortiz-Santaliestra et al., 2006). One of the ways in which humans negatively impact wildlife habitats is through the use of pesticides. For example, giant toads (*Bufo marinus*) are more likely to have ambiguous sexually dimorphic traits and reproductive tissue in areas of intense agricultural activity as compared to non-agricultural urban areas (McCoy et al., 2008).

While it is important to know how chemical mixtures interact *in vivo*, it is crucial also that we understand potential single-chemical effects. This is useful in determining which chemicals could interact to affect a common physiological process and should be examined in concert. Therefore, we compared four pesticides to determine specificity of effects caused by each chemical. Atrazine is a pre-emergent herbicide that inhibits photosynthesis in broadleaf plants (Giddings et al., 2005). 2,4-dichlorophenoxyacetic acid (2,4-D) is also an herbicide commonly used to control broadleaf plants and acts by mimicking auxin, a plant growth hormone (Stebbins-Boaz et al., 2004). Previous research has indicated negative effects of 2,4-D exposure on hormone dependent oocyte maturation in *Xenopus laevis* (Stebbins-Boaz et al., 2004). Glyphosate kills weeds by inhibiting a critical plant enzyme and is argued to be harmless to amphibians (Mann and Bidwell, 1999). The fourth pesticide we examined was the agricultural fungicide, triadimefon, which can disrupt craniofacial development in both frogs (Groppelli et al., 2005; Papis et al., 2006, 2007) and mice (Groppelli et al., 2005).

Our goal was to evaluate if these four pesticides affect *Xenopus laevis* tadpoles differently during organ morphogenesis. This period of development is particularly sensitive to exposure of various chemicals, including ammonium nitrate (Ortiz-Santaliestra et al., 2006), cypermethrin (Greulich and Pflugmacher, 2003), and carbaryl (Bridges, 2000). Our previous work also indicated that exposure to atrazine, but not to pure glyphosate, during organ morphogenesis induced extensive malformations in tadpoles (Lenkowski et al., 2008). We observed 100% mortality when tadpoles were exposed to 2,4-D doses up to 260 mg/L previously used in the literature (Morgan et al., 1996; Lenkowski et al., 2008). To extend these findings, we have examined the effects of exposure to lower concentrations of 2,4-D (20 to 70 mg/L), glyphosate in its commonly

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used formulation RoundUp®, and triadimefon on organ morphogenesis.

X. laevis tadpoles were cultured in 0.1× Marc's Modified Ringer's solution (MMR; 0.01 mol/L NaCl, 0.2 mmol/L KCl, 0.1 mmol/L $MgCl_2$, 0.2 mmol/L $CaCl_2$, 0.5 mmol/L HEPES, pH 7.5), staged according to Nieuwkoop and Faber (NF) (1994), euthanized with MS-222, and fixed 1 hr at room temperature in MEMFA (0.1 mol/L MOPS, pH 7.5, 2 mmol/L EGTA, 1 mmol/L MgSO4, 3.7% formaldehyde). Animals were treated humanely according to Tufts IACUC approved protocol #M2008-01.

Pesticides used in this study were atrazine, 2,4-D, triadimefon, and glyphosate in RoundUp® formulation. Working dilutions and corresponding vehicle controls when necessary were made in 0.1× MMR (Table 1). Because many pesticides have short environmental half-lives, we performed brief 48 hr exposures with static renewal at 24 hr. Exposures began at NF stage 41, were performed in triplicates, and maintained at 18°C. Tadpole density per exposure was 13–18 tadpoles in 10 mL of MMR. There was no mortality in atrazine, 2,4-D, and glyphosate exposures. Mortality was 0% in 7.29 and 21.88 mg/L triadimefon exposures, but significantly increased in 36.47 and 51.05 mg/L exposures (8.5%, $p < 0.005$; 25.4%, $p < 0.0001$; respectively). Mortality occurred between 24 and 48 hr, and these tadpoles were still included in our data (Fig. 1). Replicates were aggregated for analysis. Frequency data were analyzed using χ^2 contingency tables or Fisher's exact test when expected frequencies were less than 5.

Fixed tadpoles were washed in PBTr (1X PBS, 2 mg/mL bovine serum albumin, 0.1% Triton X-100) at room temperature (RT) and incubated overnight at 4°C with a primary antibody known to identify *X. laevis* muscle

Table 1 Pesticides, stocks, and working concentrations used in experiments

Pesticide	Vehicle	Stock (mg/mL)	Working concentration (mg/L)
Atrazine	DMSO[a]	10	0.35, 3.5, 10, 35 (0.1% and 0.35% DMSO controls)
Triadimefon	Ethanol	58.75	7.29, 21.88, 36.47, 51.05
2,4-D	0.1X MMR[b]	0.2	20, 40, 60, 70
Glyphosate (RoundUp®)	0.1X MMR	180	0.25, 0.5, 1, 5

[a] DMSO: dimethyl sulfoxide; [b] MMR: Marc's Modified Ringer's solution.

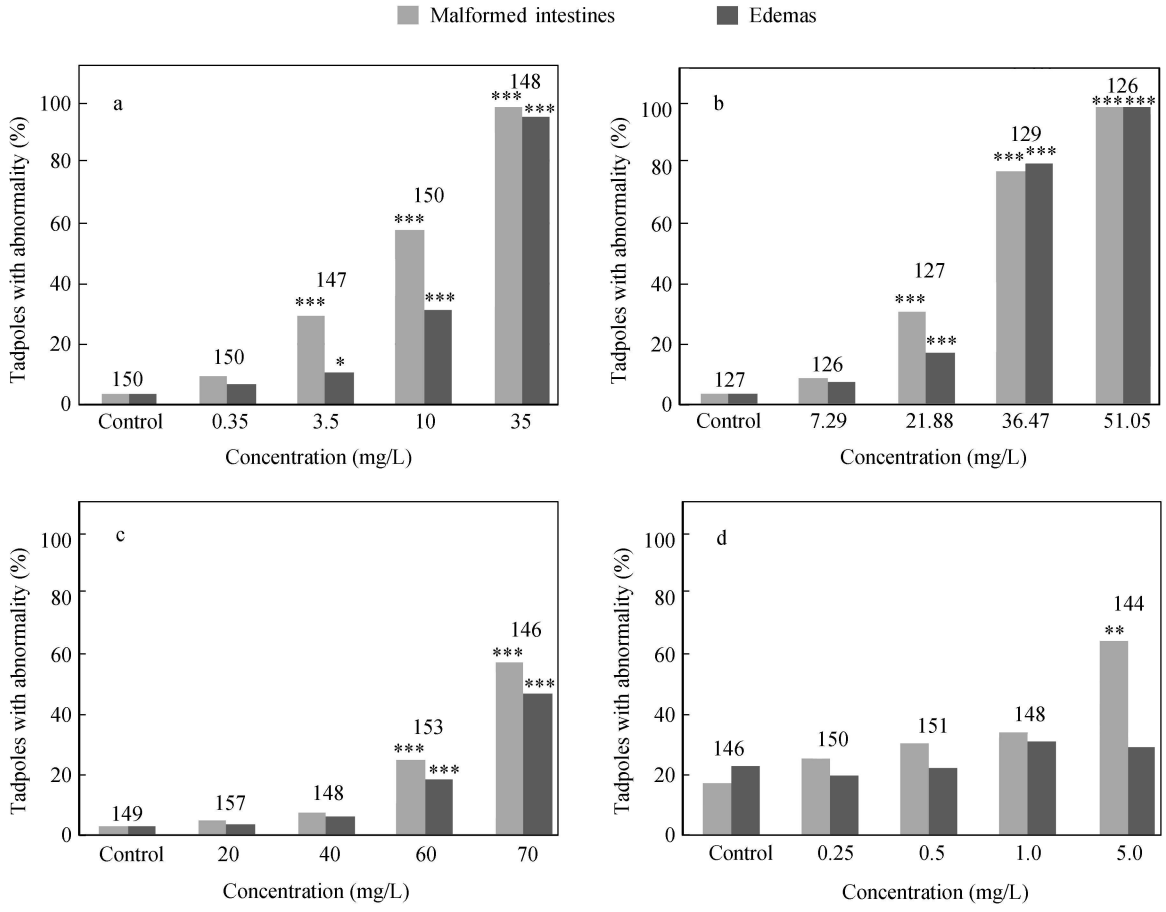

Fig. 1 Incidence of intestine malformations and edemas in tadpoles exposed to atrazine (a), triadimefon (b), 2,4-D (c), and glyphosate (d) starting at NF stage 41 for 48 hr. Sample size above bars. DMSO control values for atrazine exposures were not significantly different from non-treated controls; atrazine treatments that were significantly different from non-treated controls were also different from DMSO controls, determined by χ^2 contingency tables or Fisher's exact probability test as appropriate. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0001$.

sarcoplasmic reticulum (12/101; Developmental Studies Hybridoma Bank). Tadpoles were washed several times with PBTr at RT and incubated overnight at 4°C with alkaline phosphatase-conjugated antibody. Antibody localization was visualized with a chromogenic reaction using 5-bromo-4-chloro-3-indolyl phosphate salt and nitro-blue tetrazolium chloride at RT.

While many researchers describe how individual chemicals affect various signaling pathways (Papis et al., 2007; Stebbins-Boaz et al., 2004), still others strategically test chemical-stressor mixtures to define interactions (Boone et al., 2005; Chen et al., 2004; Hayes et al., 2006). Ecosystems are constantly bombarded with many pesticides including the widely used pesticides atrazine, triadimefon, 2,4-D, and glyphosate. In the same way atrazine has been shown to hinder normal amphibian development, any of these pesticides (as well as others) has the potential to disrupt development in species which are not targeted by a particular pesticide. Here, we describe how these four individual agricultural pesticides affect organ morphogenesis in the model amphibian species *Xenopus laevis*.

We first determined if acute exposure to concentrations of triadimefon that have previously been examined (Groppelli et al., 2005) only during organ morphogenesis would result in malformations similar to those in atrazine-exposed tadpoles. Atrazine exposure significantly increased intestinal malformations and edemas at 3.5, 10, and 35 mg/L as previously shown (Fig. 1a; Lenkowski et al., 2008). Similarly, triadimefon exposure caused a significant increase in intestinal malformations and edemas at 21.88, 36.47, and 51.05 mg/L (Fig. 1b). Parallel malformations are further illustrated by the disruption of differentiated skeletal muscle in exposed animals (Fig. 2). Cranial muscles in atrazine and triadimefon exposed tadpoles were reduced as compared to non-treated controls (arrowheads, Fig. 2). Although Gropelli et al. (2005) exposed *X. laevis* embryos during neurulation in their studies, the reduction of cranial muscles we observed following triadimefon exposure during organ morphogenesis (Fig. 2) is similar to the reduction of craniofacial muscles they reported. Additionally, the net-like structure of the hypaxial muscle normally encases the intestine and was disrupted in exposed tadpoles (arrows, Fig. 2a,e and 2b-c, g, i, respectively). These anomalies varied in severity depending on the degree of malformation in an individual tadpole. Therefore, higher concentrations of chemical exposure exhibited more dramatic disruption of these muscles. Disruptions in hypaxial muscle could be a secondary effect of pesticide-induced edemas or the direct result of molecular changes induced by pesticide exposure, however, this was not addressed by our study here. The estimated expected environmental concentration (EEC) for triadimefon is 41 μg/L (US EPA, 2006), several fold less than the concentrations at which we observed overt malformations. As indicated by previous studies, however, triadimefon belongs to a family of triazoles, some of which have been shown to cause similar effects (Groppelli et al., 2005). Therefore, although the concentrations of triadimefon we used are higher than the EEC, further studies should determine if triadimefon is able to synergize with other triazole fungicides at lower concentrations to disrupt organ morphogenesis.

The similarities of malformations caused by atrazine and triadimefon might be due to the commonalities found in their chemical structures. Muscle degradation following atrazine or triadimefon exposure might be a direct effect of altered cell signaling that would breakdown the muscle tissue, an indirect effect due to the presence of edemas, or a toxic response. Interestingly, triadimefon exposure during gastrulation can disrupt retinoic acid signaling and alter craniofacial morphogenesis later in development (Papis et al., 2007). It is possible that exposure during organ morphogenesis also disrupts this pathway, although that was not tested in this study. Furthermore, the way in which deformities manifest in atrazine and triadimefon are slightly different. The edemas located in the anterior

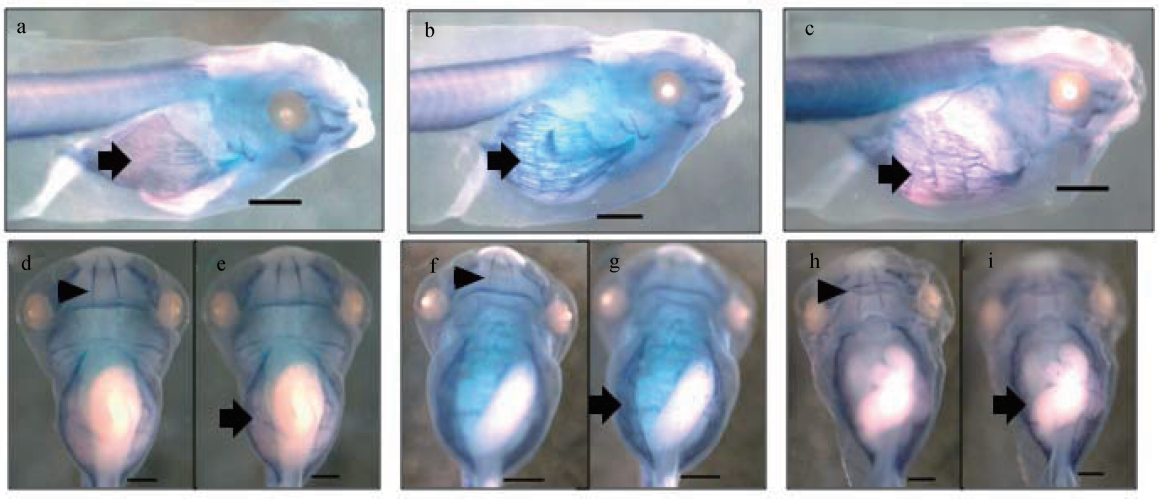

Fig. 2 Degradation of skeletal muscle in atrazine and triadimefon exposed tadpoles. Skeletal muscle visualized using the 12/101 antibody in tadpoles exposed to 35 mg/L atrazine (b, f-g), 36.47 mg/L triadimefon (c, h-i), and non-treated controls (a, d-e). Lateral (a-c) and ventral views (d-i) are the same tadpole for each treatment. Ventral views are different focal planes to better show the jaw muscle (d, f, h; black arrowhead) and hypaxial muscle (e, g, i; arrow) that are disrupted in atrazine and triadimefon-exposed tadpoles. Scale bars: 0.5 mm.

region of the tadpoles are more severe in triadimefon than in atrazine groups (Fig. 2c versus 2b). Also, axis malformations are common in atrazine-exposed tadpoles (Lenkowski et al., 2008), an abnormality not observed in triadimefon-exposed tadpoles (data not shown). Because of these differences, it is possible that the molecular response to these pesticides is slightly different and should be explored further.

The malformations observed in tadpoles exposed to 2,4-D and glyphosate help demonstrate the specificity of pesticide-induced malformations. 2,4-D can be very toxic, as our preliminary studies showed 100% mortality above 70 mg/L within hours (data not shown), but no specific deformities were observed. Lower concentrations of 2,4-D (60 and 70 mg/L) exposure caused a significant increase in intestinal malformations and edemas (Figure 1c). Most edemas in 2,4-D exposed tadpoles were mild (example from other treatments, Fig. 2b versus 2c), whereas in atrazine and triadimefon-exposed tadpoles there were severe edemas at higher concentrations. These results strongly contrast with previous studies showing that malformations occur after exposure to concentrations between 240 and 250 mg/L (Morgan et al., 1996), over three times the concentrations we used in our study. Glyphosate (RoundUp®) caused a significant increase in intestinal malformations only at 5 mg/L active ingredient (Fig. 1), dramatically lower than in other treatments (15.2% compared to up to 100% in other pesticide treatments). Sublethal and lethal effects of glyphosate in its pure form and commercial formulations have been previously examined (Mann and Bidwell, 1999; Perkins et al., 2000). Similar to these studies, we found that glyphosate in its formulation RoundUp® does not cause overt malformations at 5 mg/L, higher than the aquatic EEC of glyphosate formulations (1840 μg/L; Carey et al., 2008). Glyphosate does not directly affect tadpoles in our study, but this does not exclude the possibility of negative indirect effects on feeding tadpoles due to reduced algal communities exposed to glyphosate in the wild. Although there were significant increases in the frequency of malformations in 2,4-D and glyphosate treatments, these malformations were mild and tadpoles did not exhibit muscle degradation seen in atrazine and triadimefon exposure (data not shown).

As a conclusion, because the effects of a chemical contaminant on developmental processes can be very specific to the chemical, dose, species, and developmental stage, it is important to continue identifying how individual pesticides can impact development in different animals. Our results show that during organ morphogenesis in *X. laevis*, atrazine and triadimefon exposure both cause malformations of skeletal muscle, while the effects of 2,4-D and glyphosate (RoundUp®) appear less severe. *X. laevis* is a model system that is widely used in developmental studies to identify common vertebrate developmental processes and signaling pathways that may be disrupted by chemical exposure. Our results suggest that further investigation should identify if similar tissue malformations, such as those induced by atrazine or triadimefon exposure, have a common underlying molecular mechanism.

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