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Induction of Mortality and Malformation in *Scinax nasicus* Tadpoles Exposed to Glyphosate Formulations

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On the global scale several factors have been suggested as possible causes of amphibians declines, including ultraviolet radiation related to ozone layer depletion, climate change, and virulent pathogens (Wake 1991; Blaustein et al. 1994; Pounds et al. 1997). Similarly, there is an extensive literature on the toxic effects on larval amphibians of metals and chemicals used in insecticides and herbicides (Power et al. 1989). On a regional scale there have been cases of frog mortality and cessation of frog choruses following the application of glyphosate based herbicides (Tyler and Willams 1996). *Scinax nasicus* was selected to carry out the present study. This hylid has an extensive distribution on Neotropical region. Moreover it is frequently found in agricultural lands and urban territories.

Glyphosate (N-(phosphonomethyl)glycine) (GLY) is a broad spectrum, non-selective systemic herbicide that will kill most plants. For the agricultural practice, the application of GLY are 0.3-5.8 kg a.i./ha and are dependent on the type of use (WHO 1994). Approximately up to 3 % of applied GLY may recuperate in aquatic environments (Urban and Cook 1986). In a field experiment in a temperate coastal rainforest in British Columbia, Canada, the highest concentration of GLY in water was 162 µg/L (Feng et al. 1990). The presence of GLY in surface water is most likely to occur as a result of heavy rainfall after recent application, with subsequent rapid dissipation into stream sediment (WHO 1994). Some of the surfactants used in agricultural formulations have been found to be significantly more toxic to fish, amphibians and aquatic invertebrates than the herbicide itself (Mitchell et al. 1987; Servizi et al. 1987; Wan et al. 1989; Mann and Bidwell 1999). The objective of this study was to investigate, under laboratory conditions, the acute toxicity of commercial glyphosate formulations (GLY-F) in *S. nasicus* tadpoles, through their survival and larvae malformation.

MATERIALS AND METHODS

S. nasicus tadpoles (250 individuals, stage 18-24: Gosner 1960) were collected from a temporary pond in the Floodplain Paraná River (31° 42´S; 60° 34´O, Paraná, Argentina) and maintained under laboratory conditions. The tadpoles were acclimatized to a 12 h-12 h light-dark cycles in glass tanks with artificial

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pond water (APW) of pH 6.8, conductivity 149 μmhos/cm^{-1}, dissolved oxygen concentration 5.5 ± 1 mg/L, hardness 66.6 mg/L of CO_3Ca at 22 ± 2 °C for 7 days.

The 96-h acute toxicity test was conducted according to USEPA (1975; 1989) standard methods, with prometamorphic larvae (from stages 25-26) (Gosner 1960). The glass tanks (35 cm diameter and 60 cm high) with 4 L of APW and 10 tadpoles (average weight: 0.01 ± 0.001 g) per tank were used in the experiments. The assayed product was the herbicide GLYFOS ®, commercial formulation containing 48 % GLY as isopropylamine salt and inert ingredients POEA (polyoxethylamine) (ESP 2001). In the acute toxicity survival test, the concentrations used were: 3.07, 3.84, 4.8, 6 and 7.5 mg of GLY-F/L. Tests were conducted at 22 ± 2 °C and 12:12 light:dark. Both control and test solutions were in triplicate. Solutions were renewed daily. Mortality was recorded every 24 h. The LC50 with confidence limits ($p \leq 0.05$) were estimated by using an analysis program based on Finney (1971). Data from control and experimental groups were analyzed by one-way analysis of variance in conjunction with LSD test.

Control and treated tadpoles that survived acute tests at 24, 48, 72 and 96 h, were fixed in 10 % formalin solution. Following fixation, the tadpole's external morphology was examined with binocular microscopy. Tadpoles were stained with Alcian blue for cartilage visualization and cleared according to Wassersug (1976). Their branchial skeletons were then examined with a binocular Olympus SZX9 microscope equipped with a Olympus SC 35 camera.

RESULTS AND DISCUSSION

At 3.07 mg GL-F/L, tadpole mortality at 48-h was lower than controls but not significantly (Fig 1). Within the range of 3.84 to 7.5 m GLY-F/L survival was lower at 48 h. An elevated mortality rate was detected at all concentration at 96-h. The 96-h LC50 was 1.8 times lower than the 24-h LC50, indicating an increase of GLY-F toxicity when exposure time was prolonged (Table 1). In accordance with Pauli and Berril (1996) the low concentration of GLY-F that causes toxicity in the tadpoles to mimic concentrations that might occur in water following typical agricultural applications.

The 48 h-LC50 for tadpoles treated in this study with GLY-F/L was 3.62 mg GLY/L. Mann and Bidwell (1999) found a concentration higher than the values for tadpoles of Australian frogs (*Crinia insignifera*, *Heleioporus eyrei*, *Limnodynastes dorsalis*, and *Litoria moorei*) (48-LC50: 8.1-32.2 mg GLY/L). Several authors indicated their significant variation among and within amphibian species with respect to pesticide tolerance (Bridges and Semlitsch 2000).

Larval maldevelopment (craniofacial and mouth deformities, eye abnormalities and bent curved tails) (Fig 2) occurred in all tests and increased with time and GLY-F concentration. These effects were combined into all percent external malformation and tabulated in Table 2. Malformations were minimal at 3.07 mg/L

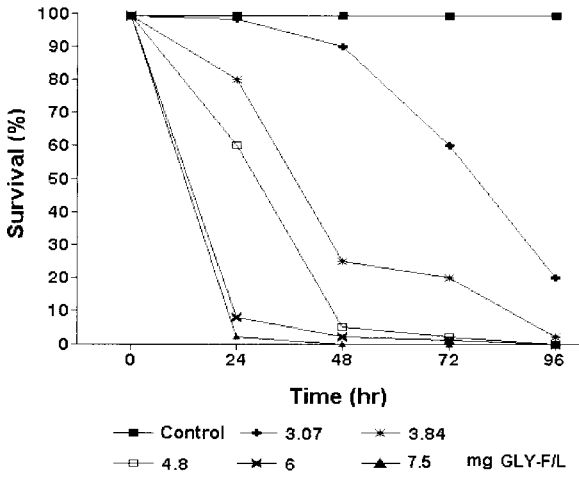

Figure 1. Survival curves for *Scinax nasicus* tadpoles.

Table 1. Acute toxicity response (LC50) of *Scinax nasicus* tadpoles exposed by glyphosate formulation.

Time	LC50	Confidence Limits 95 %	
(h)	(mg GLY/L)	Lower	Upper
24	4.78	4.23	5.35
48	3.62	3.28	5.02
72	3.23	3.07	3.36
96	2.64	2.19	2.84

n = 30

exposed for one day, whereas greater that 90 % were malformed at a GLY-F level of 7.5 mg/L. The current test confirmed the malformation effects of GLY-F on tadpoles.

Schultz et al. (1985) and Riggin and Schultz (1986) hypothesized that various pesticides may alters the synthesis of collagen in amphibian. Lajmanovich et al. (1998) found that the gills of *S. nasicus* tadpoles were very sensitive to different herbicides. The hyobranchial skeletons of *S. nasicus* tadpoles exposed to GLY-F show alterations in their cartilage structure consistent with disruption of collagen formation. The dispersant of GLY may be the culprit. This agent reduces surface tension on the leaves, allowing spray droplets to completely cover the surface. Such detergents interfere with the ability of frogs to breathe through their skin and tadpoles to breathe through their gills (Tyler 1997). This reduction in branchial cartilage is more marked in the individuals exposed to concentrations of 4.8 and 6 mg GLY-F/L - 48 h (Fig 3). In the extreme the ceratobranchials cartilages appeared as thin sheets stained. In the exposed individuals to a concentration of 6

mg/L, besides the ceratobranchials I to IV, the ceratohyals and the hypohyals were also reduced. Higher dose of GLY-F cause partial destruction of branchial arches and notable reduction of the structure of the branchial apparatus.

Table 2. Percent mortality and external morphology alterations in *S. nasicus* exposed to glyphosate formulation in static-renewal larval tests.

Days Exposure	GLY-F, mg/L	% mortality	% malformations [a]
1	3.07	2	5
	3.84	20	55
	4.8	40	70
	6	92	70
	7.5	98	70
2	3.07	10	55
	3.84	75	70
	4.8	95	70
	6	98	70
	7.5	100	90
3	3.07	40	60
	3.84	80	75
	4.8	98	75
	6	99	75
	7.5	100	-
4	3.07	80	75
	3.84	98	90
	4.8	100	90
	6	100	90
	7.5	100	-

[a] Includes both dead and alive

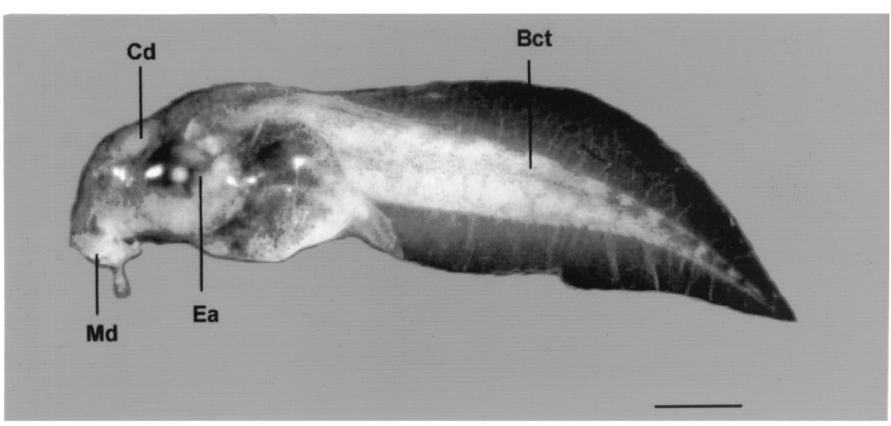

Figure 2. External malformation of *Scinax nasicus* tadpoles after 3.84 mg GLY-F/L - 24 h. Cd: Cranial deformities, Md: Mouth deformities, Ea: eye abnormalities and Bct: Bent curved tails. (X 12) (Bar, 1.5 mm).

Mitchell et al. (1987), McComb et al. (1990) and Giesy et al. (2000) considered that under normal usage, GLY herbicide did not present hazards for aquatic environment and aquatic fauna, because both the GLY and surfactant would be diluted in the water body. However, Mann and Bidwell (1999) found that in lentic, or ephemeral water bodies, at normal application rates, the concentration of

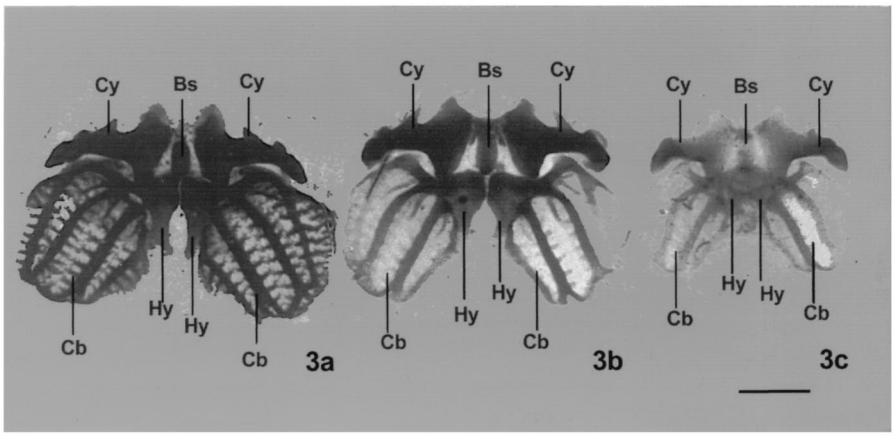

Figure 3. Hyobranchial skeletons alterations of *Scinax nasicus* tadpoles. (a) Control, observe the normal branchial arches. Cb: Ceratobranchials, Cy: Ceratohyals, Hy: Hypohyals and Bs: Basibranchial. (b) 4.8 mg GLY-F/L - 48 h. Note the size loss of that cartilaginous arc. (c) 6 mg GLY-F/L - 48 h. (X 30) (Bar, 0.75 mm).

surfactant may reach toxic levels. Considering that most amphibians are dependent on seasonal bodies of waters for their life cycles, GLY and surfactants may indeed reach harmful levels (Berger 1989). Given the effects of GLY-F on tadpole morphology we suggest that its use in the proximity of temporary pond tadpole habitats should be regulated.

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