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Effects of four types of pesticides on survival, time and size to metamorphosis of two species of tadpoles (*Rhinella marina* and *Physalaemus centralis*) from the southern Amazon, Brazil

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Pesticides have been implicated as one of the main factors responsible for amphibian population declines. Although Brazil is one of the countries that harbours the largest diversity of amphibians on the planet and is a leader in the use of pesticides, few studies have addressed the effects of these substances on amphibians in Brazil. We evaluated the effect of four herbicides (glyphosate, 2,4-D, picloram and a picloram and 2,4-D mixture) commonly used in the southern Amazon on tadpoles of *Rhinella marina* and *Physalaemus centralis*. To address the acute toxicity of each pesticide, we calculated $LC50_{96}$ values and compared them with values reported for several fish species provide by manufacturers, which are often used to infer toxicity of pesticides in Brazil. To address the chronic effects of each pesticide, we maintained tadpoles from Gosner stage 25 until stage 42 or metmorphosis and tested how fractions of $LC50_{96}$ (25%, 50%, and 75% of $LC50_{96}$) affected survival, time to metamorphosis and size of metamorphs of the tadpoles. Picloram and the mixture of picloram and 2,4-D showed the highest acute toxicity ($LC50_{96}$) among the pesticides tested, with a much higher value than those reported for fish. Survival was affected by different concentrations depending on the type of pesticide, without a standard for chronic toxicity. The time to metamorphosis was reduced only in *P. centralis*, with 2,4-D at 25 and 50% of the $LC50_{96}$ concentration. Therefore, with the other pesticides, the tadpoles were not able to accelerate their metamorphosis. The size of the metamorphs was increased or reduced depending on the concentration of the pesticide and the species, and in some cases, it was intermediate concentrations that had the greatest effect. These results indicate the need to reassess the current methods of estimating environmental risk because the effects on amphibian fauna are drastic and there is great expansion of agriculture areas in the Amazon.

Key words: Amazon, amphibian, ecotoxicology, pesticides, tadpoles

INTRODUCTION

Amphibian population decline is more severe than that of other animal groups (Blaustein et al., 2003; Stuart et al., 2004) and has been attributed to habitat destruction, increased ultraviolet radiation, pathogens, natural population fluctuations, and environmental contaminants and their synergistic effects (Halliday, 2008; Mann et al., 2009; Allentoft & O'Brien, 2010; Wake, 2012). Among the many environmental contaminants, pesticides are likely responsible for a large part of the loss of biodiversity (Lajmanovich et al., 2003; Davidson, 2004; Relyea, 2004; Mann et al., 2009) due to their intense application in agricultural areas, where frequent use and misuse contaminate groundwater and surface water, affecting aquatic species (Tomita & Beyruth, 2002; Spadotto, 2006).

Pesticide effects on amphibian larval development can be complex and cause immunosuppression, morphological and physiological changes, and endocrine disruption (Hayes, 2006; Mann et al., 2009). Firstly, immunosuppression caused by pesticides can make them more vulnerable to infection by pathogens (Carey & Bryant, 1995). Secondly, they can act as environmental stressors, reducing the time to metamorphosis and reducing the final size of the metamorphs (Denver & Crespi, 2006; Mann et al., 2009). Thirdly, the combination of pesticides and predators may affect the interactions between prey and predators and the morphology and physiology of tadpoles (Relyea, 2012). Finally, these changes during the larval phases can compromise the fitness and survival of the adult individual (Semlitsch et al., 2000; Chelgren et al., 2006; Mann et al., 2009).

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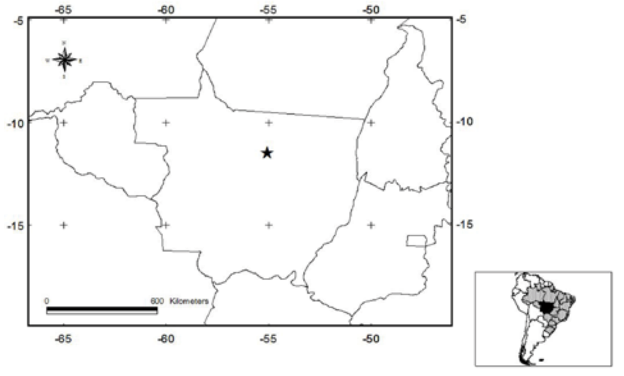


Fig. 1. Location of the study area (dark star) in the north of the state of Mato Grosso, Brazil.

On the other hand, detoxification of the organism (Maltby, 1999; Costa et al., 2008) may reduce the availability of energy (Orlofske & Hopkins, 2009; Kooijman, 2009) necessary to complete larval development, which would increase the time tadpoles are exposed to contaminants and predators in the aquatic environment (Relyea, 2004) and affect their survival.

Despite the fact that Brazil hosts one of the greatest diversities of amphibians on the planet (Mittermeier et al., 1992), and is a world leader in the use of pesticides (IBGE, 2012), amphibians are poorly represented in ecotoxicological studies (Silvano & Segalla, 2005; Kopp et al., 2007). In contrast, there is a significant increase in the number of toxicological studies on amphibians around the world (e.g., Hopkins, 2007), a global trend that has arisen due to evidence of amphibian population declines (Mann et al., 2009). Few protocols of environmental risk assessment used by government regulatory agencies (Costa et al., 2008) use amphibians as target organisms (Kopp et al., 2007) and the LC50 is unknown for many pesticides and for amphibian species. Thus, when considering amphibian species richness, the extensive list of registered pesticides and environmental heterogeneity existing in Brazil, risk estimates based on environmental toxicity tests using algae, microcrustaceans and fish (Tomita & Beyruth, 2002; Spadotto, 2006) may not be appropriate for estimating the risk of pesticides on amphibians. The commonly used ecotoxicological tests (LC50, EC50, NOEC, and LOEC) have received various criticisms (Chapman et al., 1996; Chapman & Caldwell, 1996; Kooijman, 2009; Jager & Zimmer, 2012). Furthermore, pesticide manufacturers do not provide all the necessary information, such as confidence intervals, test protocols, and fish species tested. It reinforces the need to adopt new models for estimating the environmental risk of pesticides, and to assess the effects of low-concentration pesticides on amphibians and what decisions we can make to conserve their diversity.

Considering the absence of studies on the effects of pesticides on Brazilian amphibians, this study addressed the acute and chronic effects of four types of pesticides on tadpoles of two species of anurans from southern Amazonia. The following questions were tested and discussed: is the estimated acute toxicity for fish suitable for evaluating the harmful effects of pesticides on amphibians? Considering that the toxicity for different species was standardised by the acute toxicity test, do the chronic effects of concentrations below the $LC50_{96}$ differ between species? Do pesticides at concentrations below the $LC50_{96}$ cause negative effects on survival, time to metamorphosis or size of metamorphs?

MATERIALS AND METHODS

Collection and housing of animals

Eggs of *Rhinella marina* (Linnaeus, 1758) and *Physalaemus centralis* (Bokermann, 1962) were collected in temporary ponds in southern Amazonia in the state of Mato Grosso (Fig. 1), Brazil, in the municipalities of Claudia (11°30’54’’ S, 54°53’27’’ W) and Sinop (11°52’21’’ S, 55°32’07’’ W). Temporary ponds form during the rainy season (December to February), which accounts for more than 70% of annual rainfall. Tadpoles of these species were obtained by hatching eggs in the laboratory from spawn nests collected between January and April 2009 in temporary ponds within the study area. To reduce parental effects, at least six distinct egg masses per species were collected from different locations, all of which were separated by at least 1 km. Eggs were hatched in a container (30 cm wide x 20 cm high x 50 cm long; a total volume of 30 L) containing rainwater and tadpoles were fed with rabbit food *ad libitum* until they reached stage 25 of Gosner (1960), at which point they were used in the experiments (Relyea, 2012). The pH of the water

Table 1. Concentration values (mg/L) used to find the $LC50_{96}$ in acute toxicity experiments for the two frog species and four types of pesticides. This information was based on the label of products provided by manufacturers.

Species	Concentration	Glyphosate	2,4-D	Picloram	Picloram and 2,4-D
R. marina	C1	8	50	0.02	7.5
	C2	16	100	0.04	15
	C3	32	200	0.08	30
	C4	64	400	0.16	60
	C5	128	800	0.32	120
P. centralis	C1	8	50	0.06	7.5
	C2	16	100	0.12	15
	C3	32	200	0.24	30
	C4	64	400	0.48	60
	C5	128	800	0.96	120

was adjusted to 7 and the temperature was maintained between 28°C and 32°C during the experiments. Theses values are similar to those found in the ponds from which the eggs were collected. Initial exploratory tests showed that the dissolved oxygen remained constant (2 mg/L) in the experiments and, therefore, this variable was not monitored.

Pesticide background information

The pesticides used in the experiments were obtained from commercial formulas and selected among the most widely used herbicides in the region (IBAMA, 2010), except for picloram, chosen to be applied in a mixture with 2,4-D and has high persistence in the environment. The herbicides tested were: i) glyphosate 480 (Roundup), Agripec® (concentration: 480 g/L), a non-selective herbicide whose mode of action is inhibition of amino acid synthesis. The active ingredient is glyphosate, and its $LC50_{96}$ for fish (species name not provided by the manufacturer) is 7.5 mg/L; ii) U46 D-FLUID 2,4-D, Nufarm® (concentration: 806 g/L), a herbicide used for broadleaf weed control in agricultural and nonagricultural settings. Its mode of action is as an auxin mimic; the active ingredient is 2,4-dichlorophenoxyacetic acid, and its $LC50_{96}$ for fish (Rainbow Trout) is 250 mg/L; iii) Padron, Dow Agroscience® (picloram; concentration: 240 g/L), which kills or damages annual and perennial broadleaf herbs and woody plants. It acts as an auxin mimic or synthetic growth hormone and causes uncontrolled and disorganised growth in susceptible plants. The active ingredient is 4-amino-3,5,6 tricloropicolinic acid and its $LC50_{96}$ for fish (species name not provided by the manufacturer) is 11.9 mg/L; and iv) Tordon, Dow Agroscience® (picloram+2,4-D: 64 and 240 g/L, respectively), a selective herbicide that acts as an auxin mimic or synthetic auxin. The active ingredient is 4-amino-3,5,6-trichloro-2-pyridinecarboxylic acid, and the $LC50_{96}$ for fish (species name not provided by the manufacturer) is 131.99 mg/L. These data were provided by the respective manufacturers.

Acute toxicity experiments

To determine the concentrations to be evaluated in the chronic toxicity test, we determined the $LC50_{96}$ (acute toxicity) of pesticides for each species by exposing them to different concentrations (Table 1) for 96 hours. Given the unavailability of information for native species, we used data for exotic species in the literature and the data for fish provided by the manufacturers to carry out pilot experiments. The experiments were performed in circular transparent containers (capacity 1 L) with 500 ml of the test solutions for treatments with pesticides and 500 ml of clean water (rainwater) for the control experiments. For each concentration, four replicates of five individuals each were carried out. The tadpoles were fed *ad libitum* for 6 hours before the experiments. Containers were reviewed twice daily for removal and registration of dead individuals.

The $LC50_{96}$ values were determined using the nonparametric statistics Trimmed Spearman-Karber method (Hamilton, 1977; Costa et al., 2008). This test, with or without trimming of data from the distribution tails, is appropriate and the most commonly applied technique to generate $LC50_{96}$ estimates and the associated 95% confidence limits for living organisms (Mann et al., 2009; Knillmann et al., 2012). The $LC50_{96}$ statistical analysis was performed using the program TSK (TSK, 2009).

Chronic toxicity experiments

To determine the effect of chronic toxicity, we evaluated the effects of chronic exposure through experiments with fractions of 0% (control), 25%, 50% and 75% of the $LC50_{96}$ value estimated in the acute toxicity experiment. We did not use the values of concentrations in the natural environment or the other studies, because this information does not exist for water bodies of the region or in Brazil and, mainly, due to the differences in formula among the pesticides studied. However, we used, preliminarily, an index of environmental risk obtained by the quotient method (Solomon, 1996; Spadotto, 2006), calculated from the LC50, and we estimated the environmental concentration according to Generic Estimated Exposure concentration (GENEEC) protocols (Parker et al., 1995; EPA, 2012), which considers the concentration in a lake with a volume of 20 million litres of water, set in a crop of 10 hectares. The results from GENEEC protocols were similar to the fractions value found in the acute toxicity experiment (Ricardo L.T. Andrade, pers. comm.). Then, we used only the results of acute toxicity to create the fractions and for

Table 2. Values fractions (mg/L) used in chronic toxicity experiments to assess the effects of lower concentrations of pesticides, being 0% (control) and 25, 50 and 75 % of $LC50_{96}$ values found in the acute toxicity test.

Species	Pesticide	Sublethal concentration		
		25%	50%	75%
R. marina	Glyphosate	8.00	16.00	24.00
	2,4-D	70.71	141.42	212.13
	Picloram	0.075	0.15	0.225
	Picloram and 2,4-D	10.60	21.21	31.82
P. centralis	Glyphosate	4.92	9.85	14.77
	2,4-D	128.93	257.87	386.81
	Picloram	0.127	0.255	0.382
	Picloram and 2,4-D	6.99	13.99	20.99

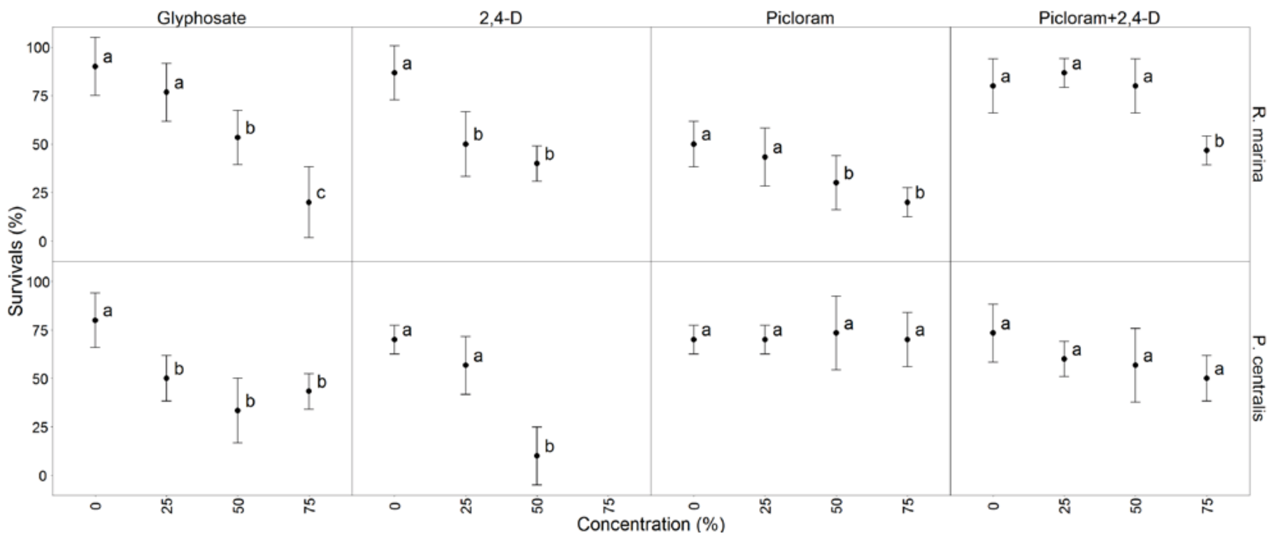


Fig. 2. Survival (mean±1 standard deviation) of individuals in the chronic toxicity experiment. Pesticide treatments are percentages of the $LC50_{96}$ obtained in the acute toxicity experiment. Different letters show treatment differences based on Scott-Knott cluster test (*p*<0.05). The analysis does not include the value of 75 % for 2,4-D, due to mortality of all tadpoles of both species tested.

testing chronic effects on tadpoles. It was performed because in Brazil, specifically the Mato Grosso state, the use of pesticides is 3.2 times greater than that in the world (Pignati & Machado, 2007). Ninety percent of the pesticides applied are lost to the environment and approximately 1% is carried by runoff to water bodies (Spadotto, 2006), exceeding the environmental safety standards established by regulatory agencies (Peltzer et al., 2008; Lajmanovich et al., 2010).

The concentrations were different for each type of pesticide and for each anuran species used. The concentrations of glyphosate applied to *R. marina* were 8, 16, and 24 mg/L, corresponding to 25, 50, and 75% of the value of acute toxicity ($LC50_{96}$). Different concentrations were observed for other pesticides and the other species (*P. centralis*; see Table 2). Furthermore, controls were established using clean water (rainwater). For each concentration mentioned above, there were five replicates, each with six tadpoles, using pots (diameter 139 mm x 91.5 mm height) with 500 ml of solution (Olsen & Daly, 2000). Individuals were fed every 48 hours with rabbit food (80 mg). The photoperiod was set at 12:12hs (light:dark). Every 3 days, containers were cleaned and test solutions were renewed. Every 12 hours, the containers were inspected for removal and registration of dead individuals. Metamorphs were preserved in 5% formalin and morphometric measurements were taken. The experiment was terminated by death or metamorphosis - considered as stage 42 of Gosner (1960) of the last individual. Development time was considered as the number of days from the beginning of the experiment until metamorphosis.

For each individual that reached metamorphosis, we measured total length, body length, body height, body width, tail height, tail muscle height, oral disc width and intraocular distance, according to Altig & McDiarmid (1999). Measurements were taken with a stereomicroscope and an ocular micrometer. We ordered the data in a Principal Component Analysis (PCA) and performed the Pearson correlation analysis between the first axes (captured 65% of the data variation) of the PCA and measure of the metamorphs. The Pearson correlation analysis showed that body length was correlated with all variables and with first axes (the lower value of *r* was 0.57 and the higher value of *p* was 0.002) that best represented the variability in the size of metamorphs.

The chronic effects of the pesticides on the survival and time to metamorphosis were tested by ANOVA with Scott-Knott grouping a posteriori (α=0.05). The Scott-Knott test was used as a post hoc, as it is more powerful and robust than Tukey, and it controls the type I error rates almost always in agreement with the nominal levels for all distributions and for being robust to the normality violation (Borges & Ferreira, 2003). To evaluate the effect of pesticides on the size of the metamorphs, an ANOVA was used, with Scott-Knott test a posteriori, to compare the control with other experiments, and with body length as the response variable. To remove the effect of time to metamorphosis on the size of individuals (Bridges, 2000), we used time as a covariate. However, the time was not statistically significant in any treatment and so it was removed from all analyses. In the treatment with 386.81 mg/L (0.75% of $LC50_{96}$) of 2,4-D, only one individual of *R. marina* and none of *P. centralis* reached metamorphosis. These treatments were excluded from the analyses. Statistical analyses were performed using the R Environment package (R Development Core Team, 2013).

RESULTS

Acute toxicity experiment

For glyphosate and 2,4-D, $LC50_{96}$ values for both species of tadpoles were lower than reported for fish by manufacturers. Picloram and the mixture of picloram and 2,4-D showed the highest acute toxicity among the pesticides tested, with a much higher value than those reported for fish (Table 3). The value concentrations used

Table 3. $LC50_{96}$ values in mg/L obtained for the two frog species for the three types of pesticides and the picloram and 2,4-D mixture. Confidence intervals (95%) are provided in parentheses. The $LC50_{96}$ for fish were obtained from the pesticide manufacturers. The fish name is provided below the LC50 value.

Species	Glyphosate	2,4-D	Picloram	Picloram and 2,4-D
Rhinella marina	32.00 (24.88–41.16)	282.84 (227.6–351.49)	0.30 (0.26–0.35)	42.43 (34.93–51.53)
Physalaemus centralis	19.70 (17.07–22.73)	515.75 (456.66–582.48)	0.51 (0.42–0.62)	27.99 (23.49–33.36)
Fish	7.50 Undefined fish species	250.00 Rainbow trout	11.59 Undefined fish species	131.99 Undefined fish species

to assess chronic toxicity were different among the types of pesticides (Table 2).

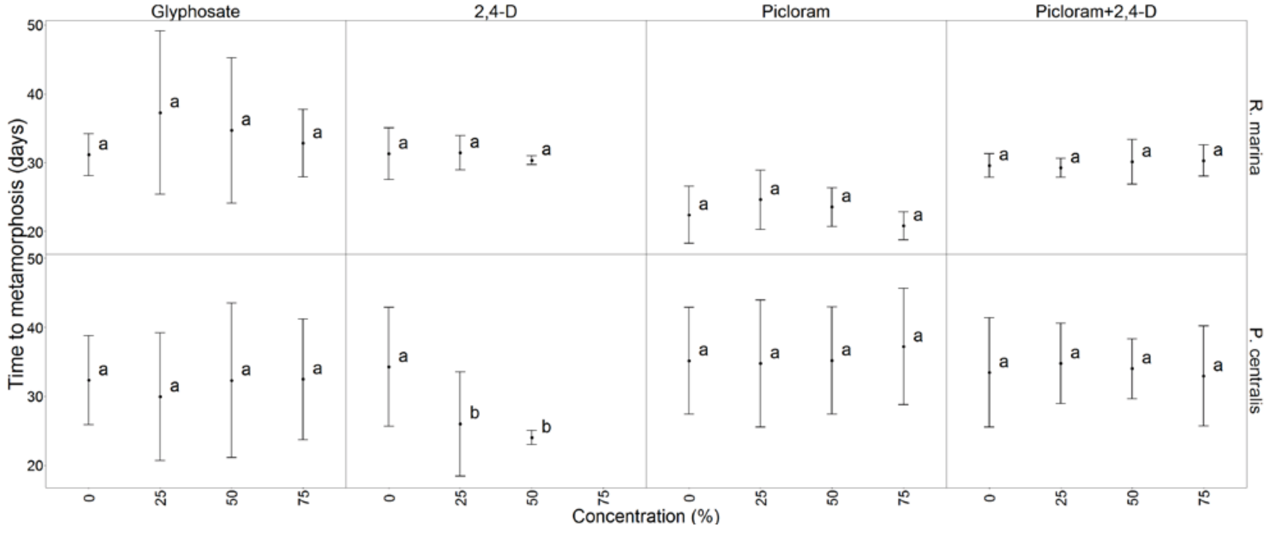
Survival was affected by different concentrations depending on the type of pesticide, without a standard for chronic toxicity. The survival of *R. marina* was affected by glyphosate ($F_{3,16}$=19.35; p<0.0001), 2,4-D ($F_{3,16}$=38.42; p<0.0001), picloram ($F_{3,16}$=5.90; p<0.01), and picloram and 2,4-D ($F_{3,16}$=13.04; p<0.0001). The differences among the concentrations tested can be seen in Fig. 2. *Physalaemus centralis* was affected by glyphosate ($F_{3,16}$=11.63; p<0.001) and 2,4-D ($F_{3,16}$=47.22; p<0.0001), but not by picloram or the mixture of picloram plus 2,4-D (p=0.96 and p=0.10, respectively). The effect of treatment with 2,4-D at 257.87 mg/L (75% of sublethal concentration, p=0.001) on *P. centralis* should be viewed with caution due to the low number of survivors. Despite fractions of $LC50_{96}$ being specific for each species, the effects of chronic exposure were often different between *R. marina* and *P. centralis* (Fig. 2).

The time to metamorphosis was not affected by any concentration of any pesticide used in the chronic toxicity test with *R. marina* (p>0.05). At concentrations of 8.00 mg/L (25% of $LC50_{96}$) and -16.00 mg/L (50% of $LC50_{96}$) of glyphosate, *R. marina* showed a longer time to metamorphosis but, due to large variations between individuals, this was not statistically significant when compared with control (Fig. 3). Only *P. centralis* showed accelerated time to metamorphosis for treatment with 2,4-D ($F_{2,35}$=5.65; p=0.007) at 128.09 mg/L (25% of $LC50_{96}$; p=0.003) and 257.87 mg/L (50% of $LC50_{96}$; p=0.04). However, at a concentration of 257.87 mg/L, few tadpoles survived.

For *R. marina*, glyphosate (p=0.8) and 2,4-D (p=0.06) did not affect the size of the metamorphs. Picloram ($F_{3,37}$=6.40; p=0.001), at concentrations of 0.075 mg/L (25% of $LC50_{96}$; p=0.002) and 0.15 mg/L (50% of $LC50_{96}$; p=0.003), caused a reduction in the size of individuals and the mixture of picloram plus 2,4-D ($F_{3,89}$=5.24; p=0.002), at concentrations of 10.60 mg/L (25% of $LC50_{96}$; p=0.004), 21.22 mg/L (50% of $LC50_{96}$; p<0.001), and 31.80 mg/L (75% of $LC50_{96}$; p=0.003), increased the size of tadpoles (Fig. 4).

For *P. centralis*, the size of metamorphs increased by glyphosate ($F_{3,89}$=2.95; p=0.04) at a concentration of 4.93 mg/L (25% $LC50_{96}$; p=0.038). 2,4-D ($F_{2,39}$=8.31; p<0.001) induced an increase in the size of metamorphs, mainly at a concentration of 257.88 mg/L (50% of $LC50_{96}$; p<0.001), and picloram ($F_{3,70}$=3.44; p=0.02) reduced the size at the concentration of 0.130 mg/L (25% of $LC50_{96}$). The mixture of picloram and 2,4-D also increased the size of

Fig. 3. Time to metamorphosis in days (mean±1 standard deviation) of individuals in the chronic toxicity experiment. Pesticide treatments are percentages of the $LC50_{96}$ obtained in the acute toxicity experiment. Different letters show treatment differences based on Scott-Knott cluster test (p<0.05).



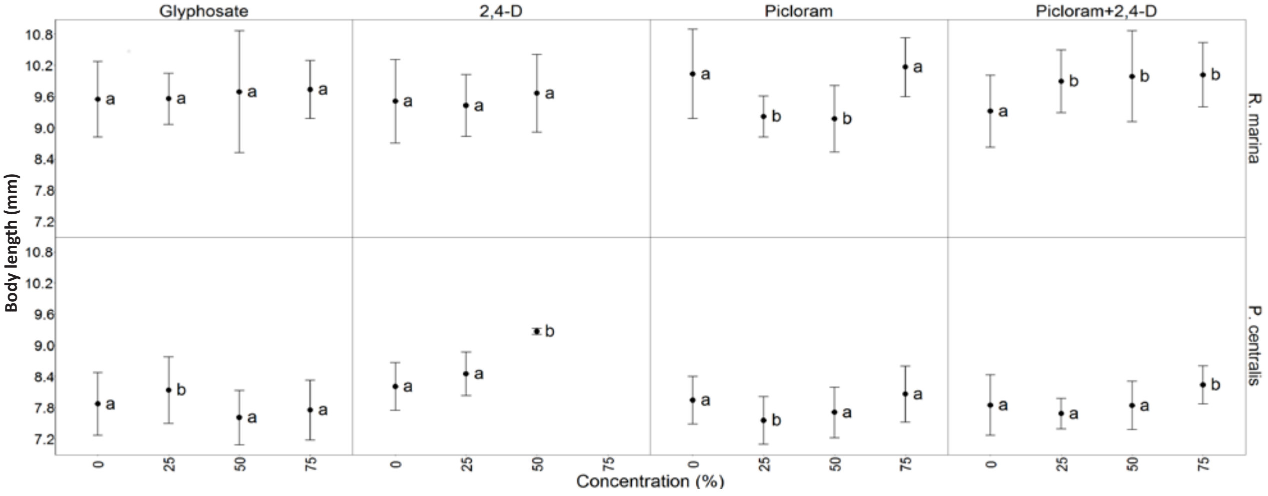


Fig. 4. Effect of different concentrations of pesticides on the size of the metamorphs (mean±1 standard deviation). Different letters show treatment differences based on Scott-Knott cluster test (p<0.05).

metamorphs ($F_{3,67}$=4.63; p=0.005) at a concentration of 21 mg/L (75% $LC50_{96}$; p=0.012, Fig. 4).

DISCUSSION

The acute toxicity test showed great variation between the tested species and type and formula of pesticide used. This variation found by us and in the literature is harmful to species conservation, due to the susceptibility of different organisms to identical chemical agents (e.g., Mayer & Ellersieck, 1986). For example, in this study, the lower values obtained for $LC50_{96}$ using glyphosate 480 to *R. marina* and *P. centralis* were 24.88 and 17.70 mg/L, respectively. Mann and Bidwell (1999) reported for glyphosate Roundup® that the $LC50_{48}$ for tadpoles of four Australian frog species ranged from 8.1 to 32.3 mg/L. Reyes et al. (2003) found effects of glyphosate Glifosan® on tadpoles of *Osteopilus septentrionalis* at a concentration of 20.81 mg/L ($LC50_{48}$). Lower values ($LC50_{96}$ at 2.64 mg/L) were found by Lajmanovich et al. (2003) for *Scinax nasicus* with glyphosate Glyfos®. For 2,4-D, in other organisms, the $LC50_{96}$ values were 45 mg/L for fish (*Salvelinus namaycush*) to 389 mg/L for planktonic crustaceans (*Daphnia magna*) (Sarikaya & Yılmaz, 2003; Verschueren, 1983; USDI, 1980). The values obtained show that extrapolations based on tests of acute toxicity with specific organisms are dangerous and inappropriate to be generalised by regulatory agencies, due the variations in LC50 values among taxonomic groups and types of pesticides.

A quick comparison revealed that tadpoles showed a higher sensitivity to picloram and picloram and 2,4-D mixture, while several species of fish with values of acute toxicity provided by pesticide manufacturers showed higher sensitivity to the pesticides glyphosate and 2,4-D (see Table 3). A similar result was found in preliminary tests with tadpoles of *Elachistocleis* sp. that are found in the studied region, with $LC50_{96}$ values of 0.14 and 5.66 mg/L to picloram and the mixture of picloram and 2,4-D, respectively (Figueiredo, 2010). Amazingly, the $LC50_{96}$ of picloram for both species of tadpole exceeded more than two dozen times the acute toxicity of $LC50_{96}$ reported for fish. The lack of studies addressing the effects of picloram on amphibians is harmful to species conservation, mainly in areas of plantation in which there is intense picloram use, as in the south of the Amazon. However, our tests were based on only two species of tadpoles and the information for fish provided by the manufacturers is quite obscure, with confidence intervals, testing protocols and, sometimes, even the fish species used in the tests not being reported.

When conducting the chronic toxicity test after the standardisation of toxicity using the $LC50_{96}$ for each species, we discovered differences in survival between species when exposed to relatively low concentrations of pesticides for an extended period. Other studies have shown a wide variation in effects between different species, pesticides and even commercial formulations (Relyea & Jones, 2009; Mann et al., 2009). However, significant direct effects on the survival of *Rana pipiens* and *Hyla versicolor* tadpoles was found by Relyea (2005) in the evaluation of glyphosate (at 3.8 mg/L, the concentration recommended by the manufacturer). Adverse effects were also found in *Bufo americanus* and *Pseudacris triseriata* at lower concentrations of two commercial formulations of Roundup®, up to 0.7 mg/L (Williams & Semlitsch, 2009). Nevertheless, when exposed long-term to pesticides, the organism attempts detoxification and tissue repair, which increase energy expenditure (Costa et al., 2008). This should affect the time to or the size at metamorphosis (Orlofske & Hopkins, 2009; Kooijman, 2009). In fact, 2,4-D accelerated metamorphosis in *P. centralis*, permitting survival (see Table 2), possibly as an escape response to physiological stress (Loman & Claesson, 2003; Márquez-García et al., 2009). A reduction in the time to metamorphosis was also observed for other species of tadpoles in experiments with glyphosate (Williams & Semlitsch, 2009) and cypermethrin (Greulich & Pflugmacher, 2003). However, this effect was only restricted to this treatment and despite the early metamorphosis, the metamporphs showed no reduction in size. That said, at a concentration of 257.87 mg/L (50% fraction), there was an increase in size, but the small number of metamorphs restricts broad generalisations. As for *R. marina*, for the mixture of picloram and 2,4-D, there was an increase in the size of the metamorphs in all treatments in relation to control. This is probably a hormesis effect; it has been observed in some cases and may be due to overcompensation by homeostatic mechanisms to contaminants, with a reallocation of the energy assimilated from nutrients (Jager & Zimmer, 2012).

On the other hand, exposure to lower concentrations of picloram reduced the size of the metamorphs of both tested species whereas, at higher concentrations, this effect was not observed. Hayes et al. (2006) observed similar results using pesticides that cause immunosuppression and endocrine disruption in *Xenopus laevis* and revealed that these adverse effects may be due to an increase in plasma levels of the stress hormone corticosterone, but there are no reports suggesting that picloram affects the hormonal system of vertebrates. However, the immunosuppression can be caused by pesticides and affect the immune system, growth and development in tadpoles (Hayes et al., 2006). Possibly, this effect can be occurring with the tested species, because individuals used in the tests are from populations that live in the contaminated areas and can exhibit genotype selection due to resistance of their parents to pesticides, which may make a difference in acute and chronic toxicity tests (Semlitsch et al., 2000; Bridges & Semlitsch, 2001; Allentoft & O'Brien, 2010).

In conclusion, our study showed that the $LC50_{96}$ value for acute toxicity was higher in fish with picloram, and picloram and 2,4-D, and that these data are inadequate for estimating the environmental risk of these pesticides to amphibians, because toxicological effects are more evident when individuals are exposed for longer periods and at lower concentrations than the $LC50_{96}$. The chronic toxicity effects differed between species and types of pesticides. Concentrations below the $LC50_{96}$ affected the survival of *R. marina* and *P. centralis* with glyphosate and 2,4-D; the acceleration of metamorphosis, possibly due to physiological stress, was only found *P. centralis*, with 2,4-D; and the size of metamorphs was affected only in *R. marina*, with picloram, and picloram and 2,4-D. More complex are the effects on size that were dependent on the species and pesticide concentration, the implication of which remains to be clarified. Furthermore, the survival of some individuals, even at higher concentrations, suggests that future studies should address the loss of genetic variability due to selection of genotypes resistant to the pesticides commonly used in the study area.

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