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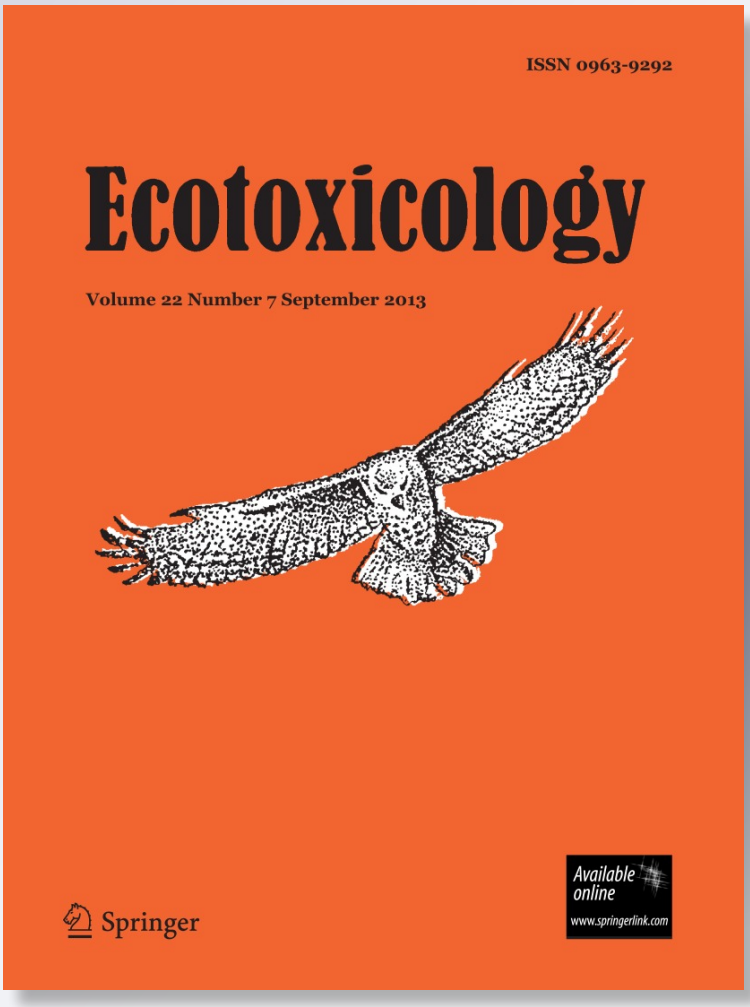

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Cholinesterase activities and behavioral changes in *Hypsiboas pulchellus* (Anura: Hylidae) tadpoles exposed to glufosinate ammonium herbicide

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Abstract In this study, amphibian tadpoles of *Hypsiboas pulchellus* were exposed to herbicide Liberty®, which contains glufosinate ammonium (GLA), for 48 h to the following concentrations: 0 (control), 3.55, 4.74, 6.32, 8.43, 11.25, 15, 20, 26.6, and 35.5 mg GLA L^{-1}. Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) activities, as well as swimming capabilities (swimming speed and mean distance) were measured in tadpoles whose concentrations displayed survival rates >85 %. Our results reveal that sublethal concentrations of GLA significantly inhibited both AChE and BChE activities in tadpoles with respect to the control, showing a concentration-dependent inhibitory effect. The highest inhibition percentages of AChE (50.86 %) and BChE (53.02 %) were registered in tadpoles exposed to 15 mg GLA L^{-1}. At this concentration, a significant increase of the swimming speed and mean distance were found in exposed tadpoles with respect to the control, as well as a negative and significant correlation between swimming speed and BChE activity, thus suggesting that this enzyme inhibition is related to an increase in swimming speed. Therefore, exposure of tadpoles to GLA in the wild at concentrations similar to those tested here may have adverse consequences at population level because neurotransmission and swimming performance are essential for tadpole performance and survival.

Keywords Cholinesterases · Swimming activity · Herbicide · Tadpoles

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Introduction

The modification of cholinesterases ChEs activity can have adverse effects on motor activity, as observed in several invertebrate and vertebrate species (Thompson et al. 1991; Sturm et al. 1999; Barata et al. 2004; Sánchez-Hernández 2006). Indeed, when ChEs are inhibited, excess of the neurotransmitter acetylcholine (ACH) builds up, which initially results in hyperactivity and then leads to uncontrolled muscular spasms, decreased activity and paralysis, thus causing respiratory failure and finally death (Walker et al. 2001). Studies on behavioral effects of pesticides on amphibian tadpoles suggest that some pesticides, particularly organophosphates (OPs), can reduce motor activity even at low concentrations (e.g. Britson and Threlkeld 1998; Widder and Bidwell 2008; Robles-Mendoza et al. 2011).

The study of behaviour provides multiple approaches to quantifying responses of contaminated organisms (Broomhall 2005; Denoël et al. 2010; Giusi et al. 2010). The use of behavioral endpoints has been slowly integrated into aquatic toxicology because, until recently, there was a poor understanding on how changes in behaviour may be correlated to ecologically-relevant concentrations of xenobiotics (Kane et al. 2005) and on their usefulness in ecological risk assessment (Weis et al. 2001; Amiard-Triquet 2009). Although acute bioassays are useful for generating guidelines to protect against physiological death

(i.e., mortality) of aquatic animals, they ignore the '*ecological death*' that may occur following exposure to much lower toxicant levels; even if animals are not clearly harmed by a contaminant, they may be unable to function in an ecological context if their normal behavior is altered (Scott and Sloman 2004). Indeed, environmental contamination measured in natural ecosystems often occurs at concentrations lower than those causing significant mortality. Because behavior links physiological function to ecological processes, behavioral indicators of toxicity appear ideal for assessing the effects of aquatic pollutants on anuran larvae populations.

In South America, crop production depends on genetically modified organisms (GMOs) (López et al. 2012). In this context, GMOs as the solution to world hunger is strongly questioned by the scientific community, since it is believed that if additional strips of farmland become available for agricultural use, the use of dangerous fertilizers and pesticides will be even more extended (Anonymous 2010). Pesticides and related chemicals originated from agriculture are discharged directly or indirectly into the receiving water bodies (Larson et al. 1997). Accordingly, pesticide use has triggered a large amount of research about amphibian decline (e.g. Sparling and Cowman 2003; Hayes et al. 2006). Amphibians are well-known to be vulnerable to pesticides that are ChEs inhibitors (Wang and Murphy 1982; Sparling and Fellers 2009; Lajmanovich et al. 2010). Glufosinate ammonium (GLA) is the ammonium salt of phosphinothricin (D,L-homoalanin-4-[methyl] phosphinate), an amino acid structurally related to glutamate that belongs to the organophosphorous family (Ferreira Nunes et al. 2010). GLA commercial formulations usually contain a sodium polyoxyethylene alkylether sulfate surfactant (Koyama and Goto 1997). The first weed species to develop resistance to the non-selective herbicide GLA has been recorded by researchers in Malaysia (Jalaludin et al. 2010) and recently Avila-Garcia et al. (2012) reported GLA resistance in a weed species that involves an altered target site. GLA is highly soluble in water (about 1,370 g L^{-1}), is hydrolytically stable within the range of environmental relevant pH (5–9) and is not degraded by photolysis in water (Royer et al. 2000; EFSA 2005). In some studies, GLA has been classified as a persistent contaminant with reported half-lives ranging from 3 to 42 days (MAFF 1990). The high risk of GLA contamination in aquatic systems is related to accidental overspray or indirect exposure to surface runoff, leaching, and erosion of contaminated soils (Faber et al. 1998a). Therefore, amphibians may be at ecological risk directly or indirectly due to the impact of contaminants on the plankton community (Faber et al. 1998a, 1998b).

Studies that integrate biochemical responses with higher levels of biological organization, such as behaviour, are scarce in amphibians (Robles-Mendoza et al. 2011). Thus, the aim of this study was to experimentally establish the toxicity of GLA herbicide contained in the commercial formulation Liberty®, and to examine the effects of exposure on ChE activity and the frequency distribution of swimming speed on *Hypsiboas pulchellus* tadpoles.

Materials and methods

Experimental design

Tadpoles of *H. pulchellus* were selected as model test organisms. This common anuran has an extensive neotropical distribution (IUCN 2010), and is frequently found in forests, wetlands, and agricultural lands (Peltzer et al. 2006). Premetamorphic larvae were collected in August 2012 from temporary ponds in natural floodplains (31°10′21.12″ S–60°15′31.75″ W, Cayastá, Garay Department, Santa Fe province, Argentina) where no pesticides were used. The average size (snout-tail tip) was 15 ± 0.2 mm and weight was 0.40 ± 0.05 g; Gosner stages: 26–30 (Gosner 1960). Tadpoles were acclimated for 48 h to a 12 h light/dark cycle with dechlorinated tap water (DTW), pH 7.5 ± 0.05, conductivity: 160 ± 12.5 μmhos cm^{-1}, dissolved oxygen concentration: 6.5 ± 1.5 mg L^{-1} and hardness: 50.6 mg L^{-1} of $CaCO_3$ at 22 ± 2 °C. All tadpoles were fed on boiled lettuce ad libitum throughout the experiments.

The assayed product in short-term (48 h) static toxicity tests was the GLA herbicide contained in the commercial formulation Liberty®. According to Material Safety Data Sheet (Bayer CropScience®, Argentina, number 1/AR-102000000577), this herbicide contains GLA (CAS Registry Number 77182-82-2) [18 %] and the following hazardous components: phosphinic acid (CAS Registry Number 6303-21-5) [>0.10 < 2.5 %], alkylethersulfate, sodium salt (CAS Registry Number 68891-38-3) [>10.0 %] and 1-metoxi-2-propanol (CAS Registry Number 107-98-2) [>1.0 %]. In this study, the herbicide was tested as a complex commercial mixture because this is how it is applied in cultivated fields and introduced into the environment. Moreover, some studies demonstrated that other inert ingredients contained in formulations may contribute to amphibian pesticide toxicity (e.g., Relyea and Jones 2009; Lajmanovich et al. 2010).

Glass aquariums (12.5 cm diameter and 13.5 cm height) containing 1 L of test solution were used in the experiments. Tests were conducted at 22 ± 2 °C and at 12 h light/dark cycle. Because of the lack of information in the literature about the effects of GLA exposure on amphibians, particularly on native species, the first step was to elucidate the direct toxicity of the herbicide on *H. pulchellus* tadpoles. Range-finding toxicity tests consisted in exposing larvae of each species to GLA solutions to

estimate the median lethal concentration (LC_{50}), the lowest-observed-effect concentration (LOEC), and the no-observed-effect concentration (NOEC). The nominal concentrations used to test single toxicities of herbicide were: 3.55, 4.74, 6.32, 8.43, 11.25, 15, 20, 26.6, and 35.5 mg GLA L^{-1}, plus negative controls with DTW. Both control and test solutions were carried out in triplicate with seven tadpoles per aquarium ($N = 210$). Treatments were randomly assigned to the recipients and to the sampling order. Larval mortality was monitored once every 24 h, and dead larvae were removed from the test vessels.

To test sublethal effects of GLA, enzymatic determinations and behavioral assessment were conducted at 48 h, based on LC_{50} and ChEs values previously determined in amphibian tadpoles for the tested herbicides (Lajmanovich et al. 2013).

Enzymatic determinations

Control and treated animals (at all concentrations) that had a survival rate >85 % at 48 h (Lajmanovich et al. 2010) were killed according to the criteria reported in ASIH (2004) with the approval from the animal ethics committee of the Faculty of Biochemistry and Biological Sciences. Whole tadpoles were homogenized (on ice) in 20 % (w/v) buffer containing 0.1 % t-octylphenoxypolyethoxy ethanol (triton X-100) in 25 mM tris (hydroxymethyl) aminomethane hydrochloride (pH 8.0) using a homogenizer. The homogenates were centrifuged at 14,000 rpm at 4 °C for 15 min, and supernatants were collected. Total protein concentrations in the supernatants were determined according to the Biuret method (Kingsley 1942). Vertebrates possess two types of ChEs, acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). AChE and BChE activities were measured according to Ellman et al. (1961). The reaction mixture consisted of 890 µl 25 mM Tris–HCl containing 1 mM $CaCl_2$ (pH = 7.6), 50 µl 2 mM dithio bis 2-nitrobenzoic acid, 10 µl 20 mM acetylthiocholine, and butyrylthiocholine iodide (AcSCh and BuSCh, respectively) and 50 µl of extract. Enzyme kinetics assays were performed in duplicate and all assays were conducted at 25 °C. The variation in optical density was recorded at 410 nm for 1 min at 25 °C using a Jenway® 6405 UV–VIS spectrophotometer. AChE and BChE activities were expressed as nmol min^{-1} mg^{-1} protein using a molar extinction coefficient of 14.15 10^3 M^{-1} cm^{-1} for AChE and 13.6 10^3 M^{-1} cm^{-1} for BChE (Sánchez-Hernández et al. 2004).

Effects on swimming activity

Swimming speed (s cm^{-1}) and mean distance (cm) were considered to characterize general movement at each concentration (3.55–15 mg GLA L^{-1}) and control (DTW). Tadpoles of *H. pulchellus* are not gregarious, consequently each tadpole was treated as independent experimental units (Van Buskirk and McCollum 2000). A given test tadpole was used once only, and three replicates were conducted for each concentration (Widder and Bidwell 2008). A glass petri dish (15 cm diameter, 2 cm depth) was used as a test recipient and before each trial it was washed thoroughly and replenished with solution. One test larva (Gosner 26–30) was released at the center of the dish and allowed to acclimate for 5 s; then swimming behavior endpoints were recorded during 60 s with a digital camera (Genius® HD 2.0 mega pixel). Videos (avi format) were analyzed automatically using MedeaLab 5.9 (trial version) Tracking System (1994–2010; Medea AV GmbH, Erlangen, Germany).

Data analyses

LC_{50} values and their respective 95 % confidence intervals (CI) were estimated by the Trimmed Spearman-Karber method (Hamilton et al. 1977). Mortality data were statistically evaluated by the Dunnett's test for post hoc comparison of means to determine the NOEC and LOEC (USEPA USEPA (US Environmental Protection Agency) 1989).

Data of enzymatic activity were expressed as the mean ± standard error of the mean (SEM). The influence of GLA concentrations on the activity of both enzymes was analyzed using general linear models (GLMs) followed by Dunnett post hoc comparisons. Data were tested for variance homogeneity and normality (Kolmogorov–Smirnov test and Levene test). Correlations among control and GLA concentrations and their respective specific enzyme activities were tested using a Spearman's correlation test.

Swimming speed frequency distribution was analyzed for each GLA concentration to characterize unimodal or bimodal speed (shorter <3 s cm^{-1}; medium >3.25) based on the value that appears most often in the data set (mode). GLMs were used to determine if there were significant overall differences in swimming endpoints among GLA concentrations (Denoël et al. 2010). Subsequent univariate analyses of variance (ANOVA) followed by the Dunnett's test for post hoc comparisons were done to determine the concentrations that differed with respect to the control. Data were checked for normality using the Kolmogorov–Smirnov test and for homoscedasticity using the Bartlett's test, and appropriate data transformations were made when necessary (Zar 1999). In addition, to assess the relationship between enzymatic activity (AChE and BChE) and swimming endpoints, Spearman correlation analyses were used, separately, for GLA concentration.

These statistical analyses were performed using SPSS 10.0 (SPSS, Chicago, IL). A significance level of 0.05 was used in all analyses and descriptive statistics were expressed as mean ± SEM.

Results

Acute toxicity tests

No mortality was recorded in control treatment. Results of the acute toxicity bioassays are summarized in Table 1. Remarkably, LC_{50}, LOEC and NOEC values were similar at 24 and 48 h of GLA exposure.

Effect of glufosinate amonium on enzymatic activity

The mean value of the AChE activity in control tadpoles was 56.74 ± 4.69 nmol min^{-1} mg^{-1}protein at 48 h. The GLA concentrations assayed affected AChE activities significantly (GLM F = 5.84; df = 6; $p < 0.01$, $r^2 = 0.46$), differing significantly in all cases from the control AChE activity (Dunnett's post hoc test $p < 0.05$; $p < 0.01$, Fig. 1). Control BChE activities were 7.45 ± 0.6 nmol min^{-1} mg^{-1} protein at 48 h. Likewise, BChE activities were affected significantly by GLA concentrations (GLM F = 7.72; df = 6; $p < 0.01$, $r^2 = 0.53$). BChE inhibition at each concentration differed significantly with respect to the control (Dunnett's post hoc test $p < 0.05$; $p < 0.01$, Fig. 2). ChEs showed a tendency toward GLA concentration-dependent inhibition (AChE: r Spearman = −0.91; BChE: r Spearman = −0.97; $p < 0.01$). The maximum percentages of inhibition of AChE and BChE activity in treated tadpoles after 48 h of exposure with 15 mg GLA L^{-1} were 50.86 and 53.02 %, respectively.

Table 1 Summary of median lethal concentrations (LC_{50}), lowest-observed-effect concentrations (LOEC), and no-observed-effect concentrations (NOEC) (mg L^{-1}) of GLA on anuran tadpoles after 24 h exposure

Time (h)	LC_{50}	LOEC	NOEC
24	23.07 (20.98–25.36)[a]	20	15
48	21.47 (19.67–23.44)[a]	20	15

Toxicity endpoints were calculated based on nominal concentrations

[a] Values in parenthesis correspond to the 95 % confidence interval of each estimate

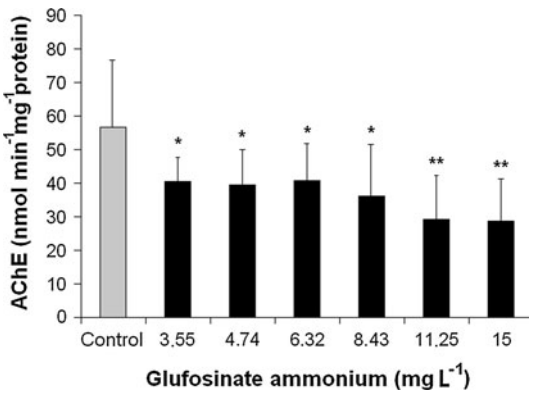

Fig. 1 Acetylcholinesterase (AChE) activity in *Hypsiboas pulchellus* tadpoles exposed (48 h) to GLA herbicide. Data are expressed as mean ± SEM. Significant differences were $*p < 0.05$ and $**p < 0.01$with respect to the control (Dunnett's post hoc test)

Effect of GLA on swimming

Hypsiboas pulchellus swimming endpoints varied at sublethal GLA exposures (Fig. 3). *H. pulchellus* tadpoles showed a bimodal speed distribution in the control individuals, characterized by short and medium-frequency peaks at 0.25 and 3.75 cm s^{-1}, a mean speed of 1.85 cm $s^{-1}$and a mean distance of 4.21 cm (Figs. 3, 4). The mean speed values after exposure are shown in Fig. 3. In general, tadpoles showed a trend in unimodal speed distribution, characterized by short movements (frequency peaks 1.87 cm s^{-1} at 3.55; 1.84 cm s^{-1} at 4.74; 1.56 cm s^{-1} at 6.32 mg GLA L^{-1}) and medium movements (frequency peaks 3.55 cm s^{-1} at 8.43, 3.11 cm s^{-1} at 11.25 and 3.67 cm s^{-1} at 15 mg GLA L^{-1} (Fig. 4). In addition, mean distances were greater than 5 cm at all GLA concentrations, except at 8.43 mg GLA L^{-1}.

The analysis of swimming endpoints showed significant multivariate effects in the GLA treatments (Wilks'lambda 0.23, F = 2.46, df = 26; $p < 0.05$). Univariate analyses indicated that swimming speed was significantly affected by GLA concentrations (F = 4.39; df = 6; $p < 0.05$, $r^2 = 0.65$). Compared to the control, swimming speed varied significantly only at 15 mg GLA L^{-1} (Dunnett's post hoc test $p < 0.05$, Fig. 4). Similarly, distance was affected by GLA concentrations (F = 3.81; df = 6; $p < 0.05$, $r^2 = 0.62$), and at 15 mg GLA L^{-1} mean distances differed from mean distance of the control (Dunnett's post hoc test $p < 0.05$, Fig. 4).

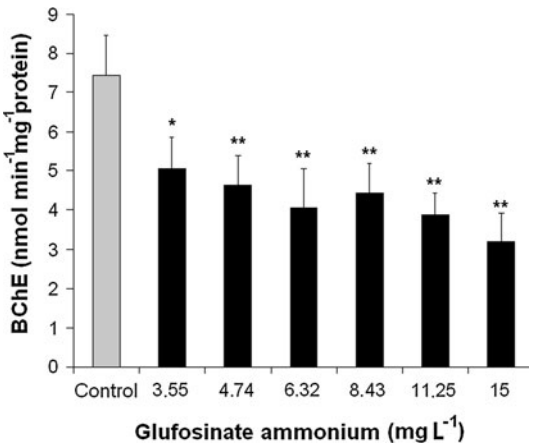

Fig. 2 Butyrylcholinesterase (BChE) activity in *Hypsiboas pulchellus* tadpoles exposed (48 h) to GLA herbicide. Data are expressed as mean ± SEM. Significant differences were $*p < 0.05$ and $**p < 0.01$with respect to the control (Dunnett's post hoc test)

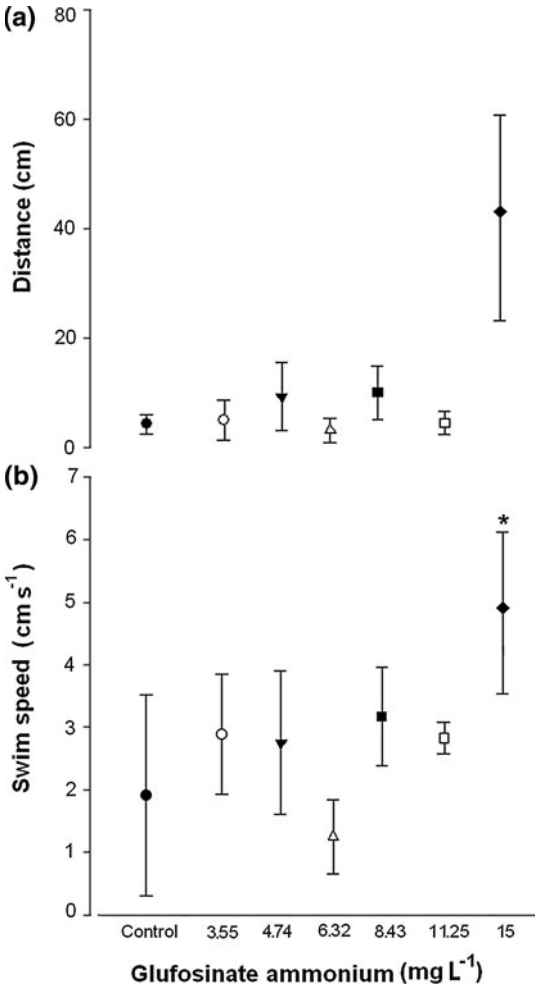

Fig. 3 Swimming endpoints (speed and distance) of *Hypsiboas pulchellus* tadpoles at different GLA herbicide concentrations. Data are expressed as mean ± SEM. Significant differences were $^{*}p < 0.05$ with respect to the control (Dunnett's post hoc test)

Swimming speed and distance showed a significantly positive correlation with BChE activity in the control tadpoles (r Spearman = 0.16; $p < 0.01$). On the other hand, swimming speed was negatively correlated with BChE activity at 15 mg GLA L^{-1} (r Spearman = −0.16; $p < 0.01$). No correlation was found between AChE activity and swimming endpoints at any GLA concentrations or control treatment.

Discussion

In many South American countries, the expansion of agricultural land has caused extensive habitat loss and degradation, which are among the greatest current and future threats to biodiversity (Dirzo and Raven 2003). Thus, a great mixture of herbicides and their residues are present in a wide variety of aquatic habitats of agricultural lands (Wharfe 2004).

Very little information is available on the acute toxicity of GLA-based herbicides on amphibians (see Dinehart et al. 2009, 2010). The similar values of 48 h LC_{50} (21.4 mg GLA L^{-1}) and LOEC-48 h (20 mg GLA L^{-1}) are likely to relate to specific features of the concentration–response curve that constrained the ability of the Spearman-Karber basic procedure to deliver feasible estimates, e.g., mortality proportions were not monotonically increasing, zero response in the lowest concentration, and 100 % response in the highest concentrations (Hamilton et al. 1977). The similarities between equivalent endpoints estimated following 24 and 48 h of exposure may relate to the relatively low mortality observed during the final 24 h of the test, which indicates that most of the pesticide toxicity occurred mostly during the first 24 h of the assay. Such a pattern was already observed in the literature. For example, Lajmanovich et al. (2011) observed that the mortality of *R. arenarum* tadpoles was recorded only during the first 24 h of exposure to four herbicides formulations (i.e. glyphosate) in an acute-toxicity assay, which is typical of surfactant toxicity (Mann 2000). 48 h-LC_{50} values of Liberty® obtained in this study for *H. pulchellus* tadpoles were greater than those observed in other amphibian species exposed to the same herbicide. For instance, according to Dinehart et al. (2010), Ignite® (Bayer CropScience) 48 h-LC_{50} values for *Spea bombifrons* and *S. multiplicata* tadpoles were 3.55–5.55 mg GLA L^{-1}, respectively. The variability in toxicity of pesticides to different amphibian species is a known phenomenon worldwide (Relyea and Jones 2009; Jones et al. 2009; Lajmanovich et al. 2010; Junges et al. 2012). Moreover, another study determines that one commercial formulation is more toxic than GLA technical grade. For example, for the aqueous formulation, the 96 h-LC_{50s} for fish (*Oncorhynchus mykiss*) were between 12.3 and 79 mg L^{-1} and for the active ingredient they were between 320 and 1,000 mg L^{-1} (MAFF 1990). These estimates reflect that toxicity of GLA herbicide may be strengthened by the presence of "hazardous components" (e.g., surfactants) contained in formulated products.

BChE and AChE activities were significantly inhibited in *H. pulchellus* tadpoles after 48 h of exposure to all the sublethal concentrations of GLA tested. More than 50 % inhibition of ChEs was detected at the maximum concentration measured (i.e., NOEC for lethal assay). Some authors have suggested that GLA, a phosphorus-containing amino acid, could inhibit ChE activity, as we have seen after organophosphate poisoning (e.g., Watanabe and Sano 1998). In pure chemical terms, GLA herbicide is an organophosphate compound derivative of phosphinic acid with potential anti-ChEs activity (Gupta 2006); thus, both AChE and BChE could be inhibited by GLA. However, recent studies toxicological screening of acute human GLA

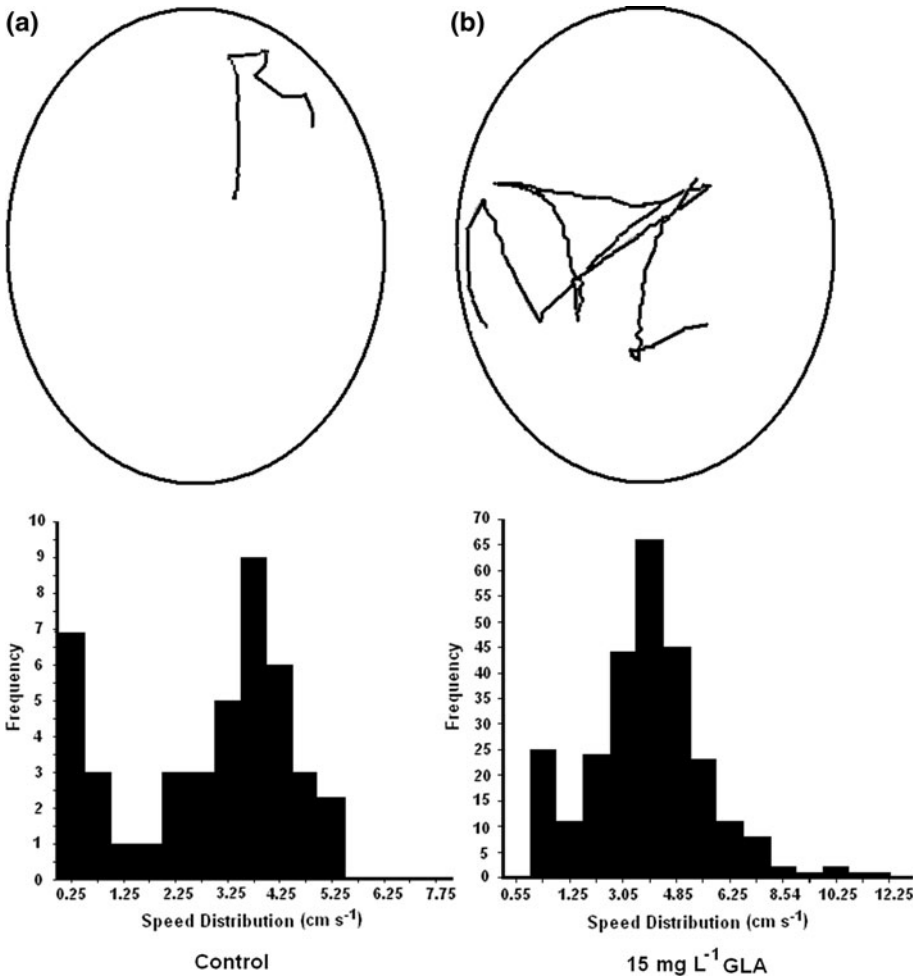

Fig. 4 Representative video tracks (*up*) and examples of swimming speed distribution frequencies (*down*) of *Hypsiboas pulchellus* tadpoles. **a** control, **b** highest GLA concentration (15 mg L^{-1})

poisoning revealed that ChE activity was normal (Mao et al. 2011, 2012, 2012). According to Mao et al. (2012) the electronegativity of the phosphorus atom (essential for ChE inhibition) in GLA is probably much weaker than the one in the phosphate esters (e.g., organophosphate insecticides). Therefore, further studies are needed to examine the potential mechanisms of GLA action in amphibians.

In amphibian tadpoles, the use of swimming activity as a behavioral endpoint is a well established sensitive measure of sublethal OP pesticide exposure (e.g., Richards and Kendall 2003; Widder and Bidwell 2006). Our results showed that swimming speed for *H. pulchellus* tadpoles was a sensitive endpoint at a NOEC of 15 mg GLA L^{-1}. Although several studies report inactivity in tadpoles exposed to pesticides (Bonfanti et al. 2004; Broomhall 2004; Punzo 2005; Brunelli et al. 2009; Denoël et al. 2012, 2013), the average swimming speed (cm s^{-1}) of tadpoles at different GLA concentrations revealed that swimming speed and distance were also considerably increased (Fig. 3). These effects may be associated with inhibition of ChEs, since the disruption of the normal nervous system function could affect locomotor performance and other critical parameters, such as muscular function of tadpoles (Payne et al.1996; Ballesteros et al. 2009). Accordingly, Widder and Bidwell (2008) determined that swimming speed did not correlate with the measured ChE activity in *Lithobates sphenocephala* tadpoles. They argued that the inhibition levels of ChE activity were generally not sufficient to exert an effect on swimming speed, which is consistent with our observations.

Another possible explanation for the neurotoxicity symptoms detected in *H. pulchellus* tadpoles exposed to GLA (increased swimming speed and ChEs inhibition) may be related to some effects of neurotransmitters and enzymes. For instance, in animal models, administration of GLA induces several behavioural changes, such as aggressiveness, impairment in respiration and circulatory failure (Watanabe and Sano 1998). In addition, Ebert et al. (1990) reported neurological symptoms in rats following injection of GLA. These authors posited that, because GLA is structurally similar to glutamate, neurotoxic symptoms may result from GLA interfering with the neurotransmitter role of glutamate. Kutlesa and Caveney (2001) found that *Calpodes ethylius* caterpillars fed with leaves coated with

GLA displayed symptoms consistent with neurotoxicity (e.g., convulsions, tremors, and paralysis prior to death) related to a competitive inhibitor of glutamine synthetase, an enzyme converting glutamate to glutamine in both plants and animals. More recently, Ferreira Nunes et al. (2010) reported that in animal models GLA toxicity is related to dopamine, a neurotransmitter related to motor functions; thus, dopaminergic neurotransmission in the nigrostriatal pathway is regulated by some neurotransmitters, including ACH.

Ecotoxicological data of GLA are too scarce to estimate the ecological significance of our results, especially at the highest concentration evaluated (15 mg GLA L^{-1}). However, the ecological significance of these results may include ethological disturbances caused by sub-lethal levels of pesticides disrupting interactions between amphibian species (Junges et al. 2012) and the ecological functioning of communities (Boone and Semlitsch 2002; Reeves et al. 2010; Relyea and Edwards 2010). For example, Relyea and Hoverman (2006) found that pesticides can have positive effects on prey survival by modifying their activity in the presence of predators. Moreover, Cooke (1971) determined that DDT caused hyperactivity in tadpoles, increasing the risk of predation of exposed tadpoles. GLA can be used in a spray-to-wet application at 1,410–3,750 mg a.i. L^{-1} of water for weed control (Ware and Whitacre 2004) and reaching concentrations of up to 8,000 mg GLA L^{-1} for herbicide tolerance evaluation (Zeldin et al. 2002). According to the toxicological endpoint calculated in our study, this would result in more than 50 % of the tadpoles dead by GLA.

In conclusion, the current study evidenced that GLA is toxic and had a detrimental impact on the ChE activities and behavioral responses of *H. pulchellus* tadpoles at sublethal concentrations. Under the experimental conditions evaluated, esterase activity in *H. pulchellus* tadpoles is a more sensitive endpoint than swimming activity. Thus, endpoints that integrate the behavioural and biochemical perspectives may provide a more sensitive indication of sub-lethal toxicity, and should hence be considered in the future. However, further studies are needed to elucidate the mechanism of toxicity in aquatic organisms exposed to GLA formulations, especially the role of surfactants and other hazardous components. Finally, we emphasize the importance of carrying out behavioral studies that can be used as a tool in biomonitoring programs to assess the ecotoxicological risk of pesticides in nontarget organisms, such as amphibians from agricultural habitats. Therefore, observations of behavioral changes provide a unique toxicological perspective, linking both biochemical and ecological consequences of environmental pollution.

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Conflict of interest The authors declare that they have no conflicts of interest.

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