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RESEARCH ARTICLE

Effects of ecologically relevant concentrations of Boral® 500 SC, Glifosato® Biocarb, and a blend of both herbicides on markers of metabolism, stress, and nutritional condition factors in bullfrog tadpoles

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Abstract

The aim of this study was to verify the effects of the isolated forms of Boral® SC 500, Glifosato® Biocarb herbicides, and a blend of both herbicides on metabolism and oxidative balance markers of *Rana catesbeiana* tadpoles and on their nutritional condition. Groups of tadpoles were divided into different treatments: control (no herbicides), Boral® 500 SC (sulfentrazone: 130 μg/L), Glifosato® Biocarb (glyphosate: 234 μg/L), and a blend of both herbicides. After 7 days, the liver, caudal muscle, and blood samples were taken to subsequently perform the biomarkers determination by spectrophotometry. The intestinal condition factor increased in animals exposed to glyphosate and herbicide blends, suggesting a hyperphagic effect. This hypothesis was confirmed by the rise of triglycerides and circulating very low–density lipoprotein (VLDL). There was a significant increase in the levels of uric acid in tadpoles exposed to the herbicide blend. Corticosterone levels reduced significantly in animals exposed to glyphosate and the herbicide blend. Oxidative stress markers had a tissue-dependent response. In the liver, glutathione S-transferase increased, and superoxide dismutase and catalase decreased in animals exposed to sulfentrazone and glyphosate. Lipoperoxidation was reduced in the glyphosate treatment. In the caudal muscle, superoxide dismutase and catalase activities were maintained, and there was a decline in the levels of glutathione S-transferase and TBARS only in the blend group.

Keywords *Rana catesbeiana* · Intermediate metabolism · Corticosterone · Antioxidative enzymes · Lipid peroxidation · Herbicides

Introduction

Currently, the contamination of water resources by agricultural chemicals is a worldwide problem and Brazil is the largest pesticide consumer (Pignati et al., 2017; UNICA 2015). Among the world's widely used agrochemicals, herbicides have great potential in contaminating hydric resources. The contamination occurs due to herbicide characteristics such as its high leaching potential, persistence in the soil, moderate adsorption by the soil organic matter, and fixation potential in soil and sediments. The potential of herbicides in reaching the groundwater and their moderate water solubility, depending on the physical and chemical conditions of the environment, also influence the soil contamination (Bailey et al. 2018; Moura et al. 2008).

Glyphosate-based herbicides are the world's widely sold agrochemicals which ubiquitously contaminate natural water bodies. The glyphosate-based commercial formulation (N-phosphometyl glycine) is frequently associated with a surfactant (polyoxyethylene), which has higher toxicity than the glyphosate salt (Brausch and Smith 2007; Gill et al. 2018). Glyphosate belongs to the substitute glycine group, classified as a non-selective herbicide of systemic action that is used mainly in coffee, sugarcane, cotton, rice, corn, and soy

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plantations. Glyphosate and glyphosate salts inhibit the action of EPSP synthase (5-enol-piruvilshiquimate 3-phosphate synthase) in the route synthesis of aromatic amino acids such as phenylalanine, tyrosine, and tryptophan. Blocking the synthesis of these amino acids in plants leads to the accumulation of shikimate. Glyphosate is highly soluble (11,600 ppm at 25 °C) (Kollman and Segawa 1995) and a non-persistent compound; it has soil adsorption capacity and no bioaccumulation potential. Depending on pH and temperature, glyphosate has an estimated half-life of 7–70 days in the water (Giesy et al. 2000). The toxic effects of glyphosate have been recorded from lower invertebrates to higher vertebrates (Gill et al. 2018).

Sulfentrazone, or 2′,4′-dichloro-5,4-difluoromethyl-4,5-dihydro-3-methyl-5-oxo-1H-1,2,4-triazol-1-yl methanesulfonanilide, is the active ingredient of Boral. It is a broad-spectrum herbicide used to control weeds in soybean, coffee, sugarcane, pineapple, and citrus fruits (Vidal 2002). (Vidal 2002). Boral belongs to the aryl triazolinone chemical group and has an estimated half-life of 110–280 days depending on the soil type and environmental conditions. In soils with low clay and organic matter contents, the contamination by Boral is more probable since it has high water solubility, discharge flow, and leaching potential towards underground waters (Hatzios 1998; Passos et al. 2013). Non-target vascular plants, phytoplankton, aquatic invertebrates, and fishes are highly susceptible to the effects of sulfentrazone (USEPA 2009).

In Brazil, the use of residual herbicides has become a common management practice to reduce the initial competition between weed species. In most cultures, this practice is likely to increase the range of controlled species beyond the control period (Damalas and Eleftherohorinos 2001). The use of sulfentrazone associated with glyphosate during pre-seeding in soybean crops is an excellent strategy to reduce the influence of meta-competition (Osipe et al. 2008). Armas et al. (2007), detected a wide variety of herbicides (acetochlor, ametryn, atrazine, clomazone, diuron, glyphosate hexazinone, isoxaflutole, pendimethalin, imazine, sulfentrazone, tebuthiuron, and trifluralin) in surface water and sediments of the Corumbataí River (State of São Paulo), in a region of sugar cane production.

During the last decade, the population of amphibians has been decreasing worldwide; the habitat loss, the introduction of exotic species, the presence of parasites and diseases, increasing UV radiation, and the indiscriminate use of herbicides are few of the causes (Kiesecker et al. 2001). There are few gaps in the space-temporal information about the concentrations of contaminant to which amphibians are exposed, mainly concerning the synergetic actions of different chemicals (Carey et al. 2001). The contamination is more critical since herbicides may change biochemical, functional, endocrine, and behavioral parameters of several amphibian species (Blaustein and Johnson 2003; Bókony et al. 2017; Coltro et al. 2017; Howe et al. 2004; Mesléard et al. 2016; Rissoli et al. 2016; Solomon et al. 2008; Van Der Kraak et al. 2014). These contaminants can also impact amphibians' survival, growth, and reproduction (Gill et al. 2018; Relyea 2005).

Dornelles and Oliveira (2014, 2016), evaluated the exposure of bullfrog tadpoles to ecologically relevant concentrations of the herbicides Facet® (active ingredient—quinclorac), Roundoup® (active ingredient—glyphosate), and Primoleo® (active ingredient—atrazine). The authors demonstrated that energetic reserves (glycogen, lipids, triglycerides, and proteins) of gills, liver, and muscles were completely depleted. Freitas et al. (2017), performed one of the few studies about the effects of Boral® 500SC (active ingredient—sulfentrazone) on amphibians. The authors evaluated the influence of different temperatures (28, 32, and 36 °C) and concentrations of sulfentrazone (10, 50, and 100 μg/L) in *Physalaemus nattereri* and *Rhinella schneider* tadpoles in oxidative stress biomarkers.

Herbicides may cause an imbalance in the cellular redox status, leading to oxidative stress of the organism through the excessive formation of reactive oxygen species (ROS). Oxidative stress may cause enzymatic inactivity, DNA/RNA damage, macromolecules oxidation, cellular apoptosis, mutagenesis, carcinogenesis, among other alterations (Costantini 2014; Jones et al. 2010; Lushchak and Bagnyukova 2006). Cases of oxidative stress caused by herbicide exposure have been recorded in different larval stages of amphibians (Coltro et al. 2017; Costa et al. 2008; Dornelles and Oliveira, 2014; Freitas et al. 2017; Gripp et al. 2017; Lajmanovich et al. 2011; Yin et al. 2014).

Rana catesbeiana Shaw, 1802, popular known as bullfrog, is a Ranidae species endemic to North America. The species introduction in Brazil during the 1930s had a commercial appeal due to the quality of its meat, skin, and sub-products (Cunha and Delariva 2009). It has been previously shown that *R. catesbeiana* is resistant to external stressful factors and can adapt very well to laboratory conditions. Considering the species' potential to occupy a wide variety of habitats, it is considered one of the invasive species with the highest potential to impact native species diversity worldwide (Jones et al. 2010). To date, *R. catesbeiana* is known to populate natural ecosystems of eleven Brazilian states, in biomes considered biodiversity hotspots (Both et al. 2011; Santos-Pereira e Rocha 2015; Preuss 2017). Thus, it is relevant to understand the pesticide resistance potential of this species, especially to herbicides (the most commonly used pesticide in Brazil). Their resistance potential and their possible impact on native species (Both et al. 2014) may lead to a loss of Brazilian amphibian diversity.

Glucocorticoids action, such as corticosterone, is crucial to the survival of vertebrates, including amphibians. These hormones have a wide range of metabolic effects such as gluconeogenesis stimulation, inhibition of glucose uptake by peripheral tissues, and insulin secretion suppression, causing the mobilization of stored energy and their redirection to the muscle. The energy intake, growth, organ differentiation, and

control of stressful situations are also mediated by glucocorticoids (Costantini et al. 2011). There is an association between the increase of the circulating glucocorticoid levels and the reactive oxygen species formation; the intensity of the response is time- and tissue-dependent (Costantini et al. 2011; Costantini 2014). Denver (2009) states that the activation of the hypothalamus-hypophysis axis in tadpoles is crucial to coordinate their responses during stressful situations, modulating the corticosterone secretion by the inter-renal gland. Besides the metabolic effects, the increased circulating corticosterone levels may raise the metamorphosis speed in tadpoles, which is a stage-dependent effect (Denver 2009; Hayes 1997).

Even though amphibian species inhabit polluted environments and/or have declining populations, ecotoxicological studies on these populations are scarce in comparison with other vertebrate groups (Lau et al. 2015). Xenobiotic toxic effects seem to be more efficiently neutralized in the most advanced developmental stages of amphibians (Bucciarelli et al. 1999) than during embryonic and larval stages (Bókony et al. 2017; Coltro et al. 2017; Dornelles and Oliveira 2016).

Considering the great diversity of amphibians in Brazil and the current great Brazilian potential in the agroindustry, the use of chemicals is alarming. The conduction of studies establishing the biochemical and functional markers is imperative to understand the action of agrochemicals in the environment, mainly those in ecologically relevant concentrations. It is important to stress that the responses to the chemicals depend on the organ analyzed, species, contaminant concentration, and exposure time (Jones et al. 2010; Persch et al. 2017).

The aim of this study was to assess the effect of the herbicides Boral® SC 500 and Glifosato® Biocarb and their blend on the metabolism, stress, and oxidative balance markers of bullfrog tadpoles (*Rana catesbeiana*). The following parameters were analyzed: metabolism markers—glucose, triglycerides, proteins, and uric acid; stress marker—corticosterone; and oxidative balance markers—liver and caudal muscle superoxide dismutase, catalase, glutathione S-transferase, and lipoperoxidation. Tadpole nutritional status parameters were also evaluated. The oxidative stress status of the liver and caudal muscle was estimated without any exposure to herbicide; after the exposure, the organs' responses were evaluated.

Material and methods

Experimental design

Sixty *R. catesbeiana* tadpoles were purchased from Rasanul ranarium (Imbé, RS, Brazil, 29° 56′ 57.5″ S, 50° 07′ 52.0″ W). The 3-month-old tadpoles were classified between the premetamorphic stages 26 and 30 according to Gosner's classification (Santos et al., 2016) and had no apparent limbs. Bullfrog tadpoles have a few features that have enabled their use as models in this study. They are easy to purchase and larvae from different broods can be used, their large body size allows few individuals to obtain suitable quantities of tissues and/or organs, increasing the variety of analyses (Costa et al. 2008).

In the laboratory, the animals were measured (total length) with a digital caliper (0.01-cm precision) and weighed on an electronic scale (0.001-g precision). All procedures followed the Conselho Nacional de Controle de Experimentação Animal (CONCEA 2015) guide and were approved by the Comitê de Ética para Uso de Animais da Universidade (protocol no. 15/00471—CEUA/PUCRS).

During the experimental period, tadpoles were randomly distributed in 20 aquariums (maximum capacity of 3–5 L) filled with a variable volume of water depending on the number of animals in each one (two to three animals per aquarium). The density of 1 individual at each 1.5 L of water was maintained in all treatments, as well as the total animal weight in each aquarium. The aquariums were filled with dechlorinated water, kept with constant aeration at a temperature of 23 ± 1 °C and controlled photoperiod (12-h light:12-h dark). The animals were acclimated to these conditions for 7 days; at day 8, herbicides were added to the exposed experimental groups for another 7 days.

After the acclimation period (7 days), three aquariums were randomly selected (acclimation group). A blood sample of each animal was taken by cardiac puncture using a heparinized syringe. Tadpoles were stunned in an ice bath and killed by spinal transection, weighed, and measured. The intestine, liver, and part of the caudal muscle were removed and immediately frozen in liquid nitrogen for the determination of biochemical parameters and nutrition.

The tadpoles were separated into four experimental groups: (C) control group, with no herbicide exposure; (B) boral group, exposed to 130 μg of sulfentrazone/L; (G) glyphosate group, exposed to 234 μg glyphosate/L; and (BG) blend group, exposed to a mixture of Boral®500 SC (130 μg a.i./L) and Glifosato® Biocarb (234 μg a.i./L). The number of animals and replicates used in each experimental group is shown in Table 1.

We used the commercial formulations of Boral® 500 SC (an FMC product composed of Sulfentrazone—50% *w/v* plus other ingredients—72.16% *w/v*, registered at the Ministry of Agriculture, Livestock and Supply—No. 07495), and Glifosato® Biocarb (a Biocarb Agroquímica product composed of Glyphosate 480 *w/v* plus solvent and inerts). Agrochemicals were diluted in distilled water to create stock solutions of Boral® 550 SC (1000 mg/L) and Glifosato® Biocarb (1000 mg/L). Aliquots of stock solution were mixed with aquarium water to obtain herbicide concentrations for the experiments with exposed groups.

Table 1 Organization of the number of animals in aquariums, time of experiment, and relation of agrochemicals for each group

	Number of animals (beginning of the experiment)	Number of animals (end of the experiment)	Acclimation time (days)	Exposure time (days)
Acclimation (replicates = 3)	6	6	7	–
Control (replicates = 4)	12	9	14	–
B (Boral® 500SC) (replicates = 5)	13	12	7	7
G (Glifosato® Biocarb) (replicates = 5)	13	12	7	7
BG (Boral® 500SC + Glifosato® Biocarb) (replicates = 6)	16	16	7	7
Total number of animals	60	55	–	–

Mortality of 8.33% (5 animals), being 3 tadpoles of group C, 1 of group B, and 1 of group G

The experiment was performed in a static exposure system; herbicides were added only once at the beginning of the experiment at nominal concentrations (determined mathematically only). Animals were fed a commercial diet (38% crude protein), comprising 5% of the total biomass of each aquarium during the entire 14-day experimental period (nearly 0.146 g/animal/day/aquarium).

The herbicide concentrations used in our experiments were based on the levels found in superficial waters in Brazil (Iguchi 2012; Queiroz et al. 2011). Queiroz et al. (2011) recorded glyphosate concentration range of 100–700 μg a.i./L. Iguchi (2012) reported a variable glyphosate concentration range of 360–2160 μg a.i./L in agriculture. There is little information about the sulfentrazone concentration in surface waters of Brazilian rivers. Therefore, we used the recommended sulfentrazone concentration for its use in the field (Martinez et al. 2010). Studies report possible herbicide concentrations that may be available in aquatic habitats after leaching (Thorngren et al. 2017). Freitas et al. (2017) used Boral® 500 SC—sulfentrazone concentrations of 10, 50, and 100 μg/L in exposure experiments using *Rhinella schneideri* and *Physalaemus nattereri* tadpoles.

In order to maintain the water quality during captivity, water parameters were recorded twice a day, every 3 days, throughout the experimental period (14 days). Ammonia and pH levels in the aquarium were estimated by commercial kits; oxy-reduction potential, conductivity, and dissolved oxygen levels were measured using SANXIN model SX751.

After the 14-day experimental period, blood samples were taken from the tadpoles between 9 and 11 a.m. through cardiac puncture using heparinized syringes. The animals were stunned in ice, weighed, measured, and euthanized by total medullary transection. Afterward, the liver, intestine, and caudal muscle were removed; the intestine was used to estimate the nutritional condition parameters. Blood, caudal muscle, and liver were frozen in liquid nitrogen and stored at − 20 °C immediately after the removal.

Nutritional condition factors determination

Fulton's condition factor was used to calculate the animals' nutritional condition factor (K) by the equation $K = W/L^b$ (where $b = 3$). The relationship between mass and body length was assumed to be isometric. We choose Fulton's factor since *R. catesbeiana* has a mass increase in the same proportion as length in the period that precedes the metamorphosis.

The specific condition factors of organs ($K_{hepatic}$ and k-$_{intestinal}$) and the hepatosomatic index (HSI) followed Nunes et al. (2011): K_{index} = (total weight/total length^3) × 1000; $K_{hepatic}$ = (liver weight / total length^3) × 1000; k-$_{intestinal}$ = (intestine weight / total length^3) × 1000; and HSI (%) = (liver weight / total weight) × 100.

Plasmatic metabolic markers

Blood samples were centrifuged at 4 °C and 1000×*g* for 5 min to separate the erythrocytes from the plasma. The determination of plasmatic metabolites (expressed as metabolite milligram/plasma deciliter) was estimated in duplicates by spectrophotometric methods (CARY 3E—UV–Visible Spectrophotometer Varian) using commercial kits.

Triglycerides were quantified using Biotécnica® commercial kit. The protein lipase promotes the hydrolysis of triglycerides, releasing glycerol which is converted into glycerol-3-phosphate by the glycerol kinase action. In the presence of glycerol-phosphate oxidase, glycerol-3-phosphate is oxidized into dihydroxyacetone and hydrogen peroxide. Afterward, there is a coupling reaction between hydrogen peroxide, 4-aminoantypirine, and 4-chlorophenol, catalyzed by peroxidase, producing a quinonimine with maximum absorption at 505 nm. The intensity of the resulting red color is directly proportional to the concentration of triglycerides in the sample. The very low–density lipoprotein (VLDL) cholesterol was estimated by the mathematical relationship between the triglycerides results (VLDL = TGL/5).

The total protein levels were estimated using the Biotécnica® kit based on the copper ion reaction with the serum protein

peptide bonds in an alkaline medium (Biuret Reagent). This determination created a purple liquid with a maximum absorbance at 545 nm. The uric acid levels were estimated using Biotécnica® commercial kit based on the uric acid oxidation into allantoin and hydrogen peroxide using uricase (UOD).

The glucose levels were estimated by the oxidase glucose method using Biotécnica® kit. Glucose oxidase catalyzes the reaction of glucose, forming hydrogen peroxide that reacts with 4-aminoantipyrine to create phenol through the action of a peroxidase via an oxidative coupling reaction, forming a red antipyrilquinone. The color intensity was proportional to the concentration of glucose in the sample and had absorbance at 500 nm.

The corticosterone levels were estimated by the enzyme-linked immunosorbent assay (ELISA) using Enzo Life Science kit (ADI-900-097). The detection limit was 4 pg/mL, and the assay extension was in a range of 160–100,000 pg/mL; absorbance at 405 nm was detected in an ELISA reader. This kit can be used for the corticosterone quantification in the plasma of different vertebrate species according to the manufacturer information. We previously standardized the method for bullfrog tadpoles (Coltro et al. 2017). The results were logarithmized and expressed in pg corticosterone/mL plasma based on a standard curve established for known concentrations of the hormone (160–100,000 pg corticosterone/mL plasma). The corticosterone quantification assay was performed on the same day, using the same kit. The intra-assay variation coefficient was 3.9%, calculated in replicates obtained from the standard assay and part of the samples.

Oxidative balance markers

To estimate the antioxidant system parameters, liver and caudal muscle parts were removed, weighed, and immediately frozen. For each gram of tissue, 6 mL of solution was added; the solution was comprised of phosphate buffer (20 mM), potassium chloride (140 mM), and phenylmethyl sulfonyl fluoride (PMSF) in the concentration of 1 mM (protease inhibitor). The tissues were homogenized in an Ultra-Turrax (IKA-WERK) homogenizer at a temperature of 4 °C and centrifuged for 10 min at 1000×*g* in a centrifuge cooled. The resultant precipitate was despised, and the supernatant was removed, fractionated, and frozen at − 20 °C to estimate the following parameters: superoxide dismutase (SOD); catalase (CAT); glutathione S-transferase (GST); and the lipoperoxidation levels. The concentration of proteins from the homogenized supernatant was determined according to the method previously described.

Quantification of SOD activity was based on the inhibition of the reaction of the superoxide radical with adrenaline, using the relative unit quantification; where a unit of SOD is defined as the amount of enzyme that inhibits the detector's oxidation rate (adrenaline) by 50%. In this method, the adrenaline oxidation leads to the formation of a colorful product, the adrenochrome, which was detected through spectrophotometry. The SOD activity was determined by measuring the adrenochrome formation speed (at 480 nm) in a reaction containing NAOH-glycine (50 mM, pH = 10) and adrenaline (1 mM) (Boveris and Cadenas 1982). The enzyme was expressed as units of SOD mg proteins^{-1}.

CAT is a highly specific enzyme, acting only on hydrogen peroxide, methyl, and ethyl hydroperoxides (Webster and Nunn 1988). Therefore, CAT's activity can be measured by evaluating the hydrogen peroxide consumption following pseudo-first-order kinetics. Aliquots of phosphate buffer (955 μL, 50 mM, pH = 7.4) and sample (10 μL of the homogenate supernatant) were mixed into a quartz bucket and read against a white background containing the phosphate buffer. Afterward, 35 μL of hydrogen peroxide (0.3 M) was added and observations were performed every 2 or 4 min depending on the tissue; the absorbance was recorded every 10 s at a wavelength of 240 nm. Results were expressed in pmol H_2O_2 mg proteins^{-1} min^{-1} (Boveris and Chance 1973).

GST activity was estimated according to Boyland and Chasseaud (1969) through the measurement of the conjugated 1-chlore 2,4 dinitrobenzene by the reduced glutathione (GSH). The enzymatic assay was performed during 2.5 min in 100 mM potassium phosphate buffer (KPi), 1 mM EDTA at pH 7.0, containing 1 mM GSH, and 1 mM CDNB. The basal absorbance was deducted after the reading of the assay reaction in the absence of the sample. A quantity of 25 μL of the sample was used as the reaction initiation agent. The activity of the enzyme was expressed as mmol conjugate CNDB × mg proteins^{-1} × min^{-1}.

LPO was estimated by the thiobarbituric acid–reactive substances (TBARS) method. Aliquots of 0.67% thiobarbituric acid (TBA) (0.2 mL), distilled water (0.1 mL), 10% trichloroacetic acid (TCA) (0.3 mL), and homogenized (0.1 mL) were sequentially added into a test tube. TBA reacts with lipoperoxidation products to form a Schiff base in which TCA acts on denaturing proteins and acidifying the reaction medium. Then, the tubes were shaken and heated for 15 min at 100 °C. After cooling, 0.6 mL n-butyl alcohol was added into the tubes to extract the formed pigment. The tubes were shaken for further 45 s and centrifuged for 10 min at 1000×*g*. The colored product was removed and read at a 535 nm wavelength. The TBARS concentration was expressed in μmol/mg proteins (Lima and Abdalla 2001).

Statistical analysis

Results were expressed as mean ± standard error. The distribution of data was analyzed using the Kolmogorov-Smirnov test. Since all results demonstrated a non-parametric distribution, we used the Mann-Whitney test to compare acclimation (7 days) and control (14 days) groups.

Animals from the control group and animals exposed to herbicide remained the same time in the experimental conditions and had a similar lifespan. Therefore, the exposed groups were compared between one another and between the control groups. Comparisons between the control and exposed groups were performed using Kruskal-Wallis with the Student-Newman-Keuls complement. Tests were performed in BioEstat software v. 5.0 and confirmed in SPSS 20.0 software; values of $p < 0.05$ were considered significant.

Table 2 Mean values of oxidation redox potential (ORP), conductivity, and dissolved oxygen throughout the experimental period

Group	ORP (mV)	Conductivity (mS)	Dissolved oxygen (mg/L)
C_7	193.666 ± 2.60	308.000 ± 11.84	6.220 ± 0.01
C	207.000 ± 2.23	323.710 ± 18.56	6.591 ± 2.12
B	201.055 ± 2.39	318.555 ± 9.936	6.525 ± 2.28
G	193.388 ± 2.57	322.611 ± 10.96	6.597 ± 2.17
BG	187.178 ± 2.32	279.746 ± 14.79	6.680 ± 1.71

Results and discussion

In Brazil, the use of residual herbicides in weed management has become a common practice in order to reduce the initial competition between weed species. Osipe et al. (2008) recommended the use of sulfentrazone associated with glyphosate, during pre-seeding in soybean crops, as an excellent strategy to reduce the influence of meta-competition. This association may result in larger plasticity during weed management practices and a better culture selectivity.

This was the first study to evaluate the adverse effects of a mixture of Boral® 500 SC and Glifosato® Biocarb on bullfrog tadpoles in laboratory conditions. These herbicides, containing sulfentrazone and glyphosate, are widely used in soy fields, where anuran species are often found. Amphibians play an essential role in aquatic and terrestrial ecosystems, acting as secondary consumers and on the balance of many food chains.

The survival rate of tadpoles throughout the experiment was 91.7%. Three animals died in the control group (4.98%), one in the boral group (1.66%), and one in the glyphosate group (1.66%). No mortality was seen in the blend group. The low mortality rate in the different groups indicates a good quality of the experimental conditions, and the mortality rate in the exposed groups confirms the sub-lethal concentrations of herbicides (Table 1). The ammonia levels remained stable, below the toxic values of 0.5 ppm, the pH ranged from 7.1 to 7.3, and the other water quality parameters did not vary significantly between the groups nor throughout the experimental period (Table 2).

Dornelles and Oliveira (2014) found no mortality in experiments using tadpoles exposed to ecologically relevant concentrations of three herbicides (atrazine, glyphosate, and quinclorac). The experimental conditions and animals' body size and developmental stage were the same used in our experiments. The lack of mortality in bullfrog tadpoles exposed to low herbicide concentrations has been acknowledged in other studies (Coltro et al. 2017; Costa et al. 2008; Dornelles and Oliveira 2016). Some authors have associated this survival to intrinsic characteristics of *R. catesbeiana* such as its high adaptive capacity and physiological plasticity (Jones et al. 2010; Vieira 1993).

Nutritional condition factors

The nutritional condition factor parameters (K_{index}, $K_{hepatic}$, $K_{intestinal}$, and hepatosomatic index) did not show significant variations between the acclimation (A) and control (C) groups (Table 3). However, after 7 days of acclimation, the body mass of animals (A) decreased 30% in comparison with the initial body mass. The body length increased by 8.4% after 7 days of acclimatization (Table 4). The permanence of animals for another 7 days in the herbicide exposure experiment (control group) determined the recovery of body mass (15.5%) and length (4.5%) when compared with the acclimation group (Table 4). These results reinforce the good conditions of the experimental conditions and the adaptation of animals to this system.

Comparing tadpoles from the control and exposed groups, animals from the boral group had an average total body length similar (only 0.4% larger) to those of the control group. Animals from the glyphosate and blend groups had a 7.2% and 7.6% increase in the body length when compared with the control group, respectively. However, these differences were not statistically significant (Table 4).

Throughout the experimental period, the body mass of animals increased 14.4% in the boral group, 49.8% in the glyphosate group, and 51.9% in the blend group when compared with the control group, which increased 15.5% in relation to the acclimation group (Table 4). The presence of glyphosate seems to be a determining factor for a body mass gain, suggesting that this herbicide influences the food intake. This pattern was not observed with the same intensity in animals exposed to sulfentrazone. Similar percentages (43, 32, and 23%) were observed by Dornelles and Oliveira (2014) when exposing bullfrog tadpoles to different glyphosate concentrations (36, 72, and 144 μg a.i./L, respectively).

The K_{index} and $K_{hepatic}$ levels were similar in all studied groups (Fig. 1a and b, respectively). Although there were no differences between the $K_{intestinal}$ levels of the exposed groups and the control group, there were differences in the comparison between the boral and the other exposed groups. In the glyphosate group, the $K_{intestinal}$ level was 1.37 times higher, and in the blend group, it was 1.44 times higher (Fig. 1c).

Table 3 Results obtained for the different parameters analyzed in the plasma and hepatic and muscular tissues of animals from acclimation (7 days) and control (14 days) group

	Acclimation	Control
Nutritional condition factors		
K_{index}	5.998 ± 0.37 g/mm	6.743 ± 0.51 g/mm
$K_{hepátic}$	0.196 ± 0.02 g/mm	0.297 ± 0.05 g/mm
$K_{intestinal}$	0.935 ± 0.25 g/mm	0.874 ± 0.1 g/mm
HSI	3.35 ± 0.42%	4.555 ± 0.75%
Plasma		
Triglycerides (TGL)	42.179 ± 5.67 mg/dL	39.34 ± 2.36 mg/dL
Very low–density lipoprotein (VLDL)	14.9601 ± 6.58 mg/dL	7.7996 ± 0.41 mg/dL
Total proteins	1.414 ± 0.62 mg/g	2.272 ± 0.79 mg/g
Uric acid	0.267 ± 0.03 mg/dL	0.293 ± 0.01 mg/dL
Glucose	4.862 ± 0.68 mg/dL	14.181 ± 3.15 mg/dL*
Corticosterone	51.04 ± 14.69 pg/mL	68.073 ± 16.92 pg/mL
Liver		
Superoxide dismutase (SOD)	0.509 ± 0.03 U/mg protein	1.241 ± 0.08 U/mg protein*
Catalase (CAT)	3.328 ± 0.55 pmol/mg protein min	5.589 ± 0.71 pmol/mg protein min*
Glutathione S-transferase (GST)	3.951 ± 1.05 mmol/mg protein min	7.088 ± 0.63 mmol/mg protein min*
Lipoperoxidation (LPO)	3.274 ± 0.56 μmol/mg protein	4.145 ± 0.43 μmol/mg protein
Muscle		
Superoxide dismutase (SOD)	6.175 ± 1.4 U/mg protein	8.181 ± 1.38 U/mg protein
Catalase (CAT)	4.955 ± 1.84 pmol/mg protein min	4.52 ± 0.78 pmol/mg protein min
Glutathione S-transferase (GST)	4.228 ± 1.3 mmol/mg protein min	3.037 ± 0.22 mmol/mg protein min
Lipoperoxidation (LPO)	18.075 ± 6.32 μmol/mg protein	13.478 ± 2.89 μmol/mg protein

Asterisks (*) indicate significant differences between groups C7 and C14 at $p < 0.05$

Coltro et al. (2017) exposed bullfrog tadpoles to quinclorac and observed significant variations of the K_{index} and $K_{hepatic}$ index at the lowest and highest herbicide concentration, respectively. However, the $K_{intestinal}$ was not quantified by the authors. The authors attributed such variations to a metabolism modulation and use of energy reserves to ensure the survival of the animals.

This set of results, represented by the increase in body mass associated to the increase of the $K_{intestinal}$ in the animals exposed to glyphosate and to the herbicide blend, suggests a higher feeding activity of these tadpoles, possibly in search of a replenishment of their energy reserves order to ensure their survival in this situation. This strategy may lead to a change in the energy balance aiming at the adequate metabolization of xenobiotics. This hypothesis is reinforced by results found by Dornelles and Oliveira (2014, 2016). In this work, the authors showed that abdominal fat was also higher in animals exposed to herbicides atrazine, glyphosate, and quinclorac, as well as the abdominal fat index for the individuals of the atrazine and glyphosate groups, when compared to the control, which may be related to an increase in energy demand due to the of the agrochemical stressor, leading the animal to increase food intake and this store fat as an energy stock. We cannot rule out the role of glyphosate as a modulator of the feeding behavior of these animals since only the groups exposed to glyphosate had this response in our experiments. Dallegrave et al. (2003) observed that rats exposed to the glyphosate herbicide also presented an increase in body mass as well as an increase in food intake, thus corroborating our results.

Therefore, this finding probably evidences an increase in the hepatic activity due to the digestive tract nutrient allocation and/or to the substrate supply from other tissues to the liver. These processes would keep the herbicide detoxification potential and guarantee the tadpoles survival (91.7%) since no differences were seen when the HIS was compared between the experimental groups (Fig. 1d).

Changes in fat and/or glycogen amounts stored in the liver significantly influence the organ's mass, altering the hepatosomatic relation (Nunes et al. 2011, 2015; Tavares-Dias et al. 2000). For instance, other factors, such as feeding and infections, might also be responsible for eventual changes in the biometric features of teleost fishes (Nunes et al. 2011, 2015; Tavares-Dias et al. 2000). *Pelophylax ridibundus* adults demonstrate a slight increase in the HSI when exposed via intraperitoneal to 0.138×10^{-3} mL Roundup®/g per body

Table 4 Mean body mass and length of animals measured in arrival (baseline), after a 7-day acclimation period, and at 14 days (experimental period, control) in animals exposed to Boral® (B), Glyphosate® Biocarb (G), and both herbicide blend (BG)

Group	Body mass (g)	Length (mm)
Baseline (0 days) ($n = 60$)	2.924 ± 0.328	62.613 ± 1.255
Acclimation (7 days)	$2.046 \pm 0.231^{*}$	$67.845 \pm 2.984^{*}$
($n = 6$)	$A_0 = -30.0\%$	$A_0 = 8.4\%$
Control (14 days)	2.364 ± 0.209^{a}	70.892 ± 2.106^{a}
($n = 9$)	$A_A = 15.5\%$	$A_A = 4.5\%$
	$A_0 = -19.2\%$	$A_0 = 13.2\%$
B	2.658 ± 0.181^{a}	71.171 ± 1.59^{a}
($n = 12$)	$A_A = 29.9\%$	$A_A = 4.9\%$
	$A_0 = -9.09\%$	$A_0 = 13.7\%$
G	3.382 ± 0.364^{b}	75.774 ± 2.244^{a}
($n = 12$)	$A_A = 65.3\%$	$A_A = 11.7\%$
	$A_0 = 15.7\%$	$A_0 = 20.1\%$
BG	3.426 ± 0.228^{b}	76.086 ± 1.605^{a}
($n = 16$)	$A_A = 67.4\%$	$A_A = 12.1\%$
	$A_0 = 17.17\%$	$A_0 = 21.5\%$

A_0 and A_A, percent change in body weight or length over the corresponding period of 14 days and 7 days in culture respectively. Asterisks (*) denote significant differences in baseline values, whereas different letters denote significant differences between the experimental groups and control group

mass (Păunescu and Ponepal 2011). The authors suggested that the liver cells were affected, and the herbicide increased the production rate of endoplasmic reticulum for the protein synthesis. This process would have a deleterious effect on the frogs' liver. The herbicide concentrations used in our experiments did not increase the HSI, which probably allowed the herbicide metabolization without any hepatic damage, ensuring the survival of animals. Future experiments aimed at elucidating such peculiarities are required.

Metabolic markers

Most metabolic markers parameters did not significantly change in comparisons between the acclimation (A) and control (C) groups (Table 3). However, the plasma glucose levels changed significantly, with an increase of 2.92 times in group C in comparison with group A. Similarly, the superoxide dismutase, catalase, and glutathione S-transferase activity levels also increased (2.44, 1.68, and 1.79, respectively) in group C in comparison with group A. Such differences may indicate that during the acclimation period tadpoles went through a stressful situation that led to a decrease in body mass (first 7 days). In the following 7 days, there was an increase in the body mass that may be associated to the increment of metabolic markers, suggesting an increase and/or better use of food supply, leading to an adaptation to the culture system.

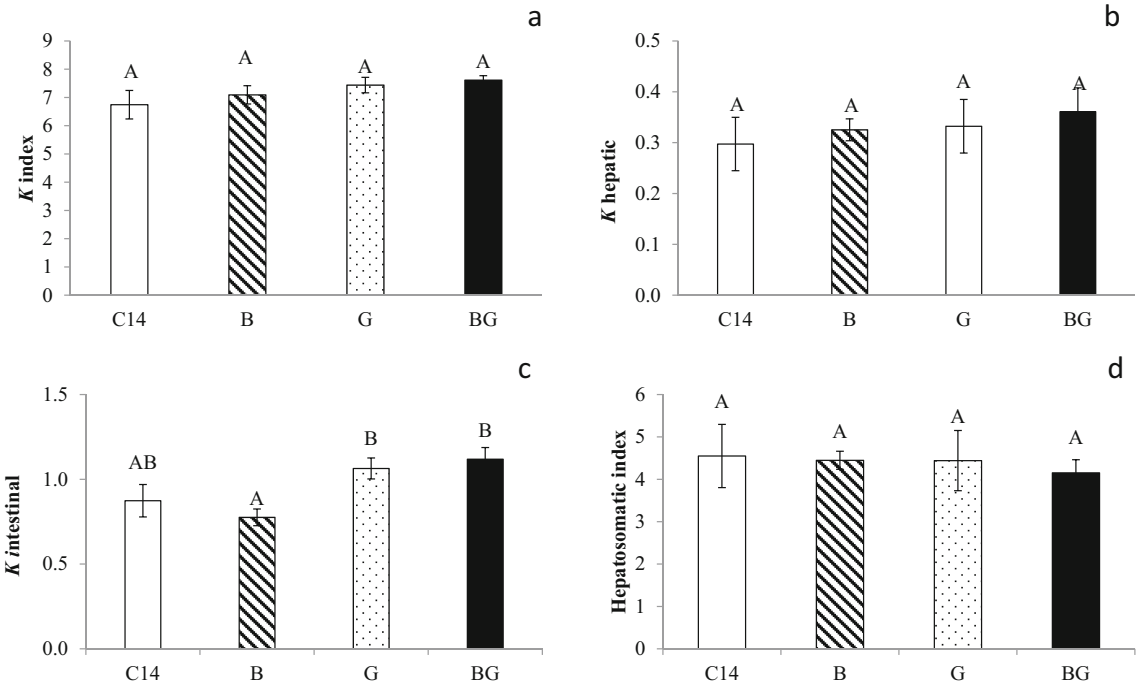

Fig. 1 Variations in the nutritional factor conditions and hepatosomatic index. **a** K level, **b** $k_{hepatic}$ level, **c** $k_{intestinal}$ level, and **d** hepatosomatic index obtained for the control group (C), boral group (B), glyphosate group (G), and herbicide blend group (BG). The results are expressed as mean ± standard error. Different letters above the histograms represent significant difference at $p < 0.05$

The plasmatic levels of triglycerides (TGL) and cholesterol (VLDL) differed significantly between the exposed groups (B, G, and BG) and the control (Fig. 2a and b). In comparison with the control group, the plasmatic levels of TGL and VLDL increased 1.5 times in the boral and blend groups and 1.4 times in the glyphosate group. The rise of $K_{intestinal}$ might intensify the food ingestion, digestion, and absorption, which consequently might be reflected in the increasing plasmatic lipid series. This pattern corroborates previous studies performed in our laboratory. Bullfrog tadpoles exposed to a glyphosate concentration range of 36, 72, and 144 μg a.i./L had a decrease in the total lipid and triglycerides levels in gills, liver, and caudal muscle (Dornelles and Oliveira, 2014). Similar results have been described by other authors who evaluated the total lipid and/or triglyceride levels in muscle and/or liver of animals exposed to xenobiotics (El-Banna et al. 2009; Persch et al. 2017; Salbego et al. 2010; Sounderraj et al. 2011).

Total plasma protein levels did not vary between the experimental groups (Fig. 2c). However, the uric acid levels increased by 2.7, 2, and 9.5 times in the exposed groups, respectively (Fig. 2d). Therefore, we believe that the exposure to the respective concentrations of sulfentrazone (130 μg/L), glyphosate (234 μg/L), and the herbicide blend induces an increase of uric acid production. The uric acid, along with its derivates, would act as an antioxidant, especially in animals exposed do the blend. Oppositely, Coltro et al. (2017) found a negative-dependent relationship between the plasmatic uric acid levels and the Facet® concentration (quinclorac active formulation) in exposed bullfrog tadpoles. The authors suggested the use of uric acid as an antioxidant in low quinclorac concentrations.

In amphibians, the uric acid is considered an intermediate product of the metabolism of nitrogen compounds, in which the main nitrogen excreta is urea (Nelson and Cox 2014). The uric acid and its derivates seem to act as natural antioxidants in different animal groups such as the barnacle *Balanus improvisus* (Zanette et al. 2015) and insects (Hermes-Lima 2004). In birds, the uric acid antioxidant properties, along with its high plasmatic concentrations, suggest a possible protection mechanism to oxidative damages (Tsahar et al. 2006).

The plasmatic levels of glucose did not vary significantly between the exposed groups, nor in comparison with the

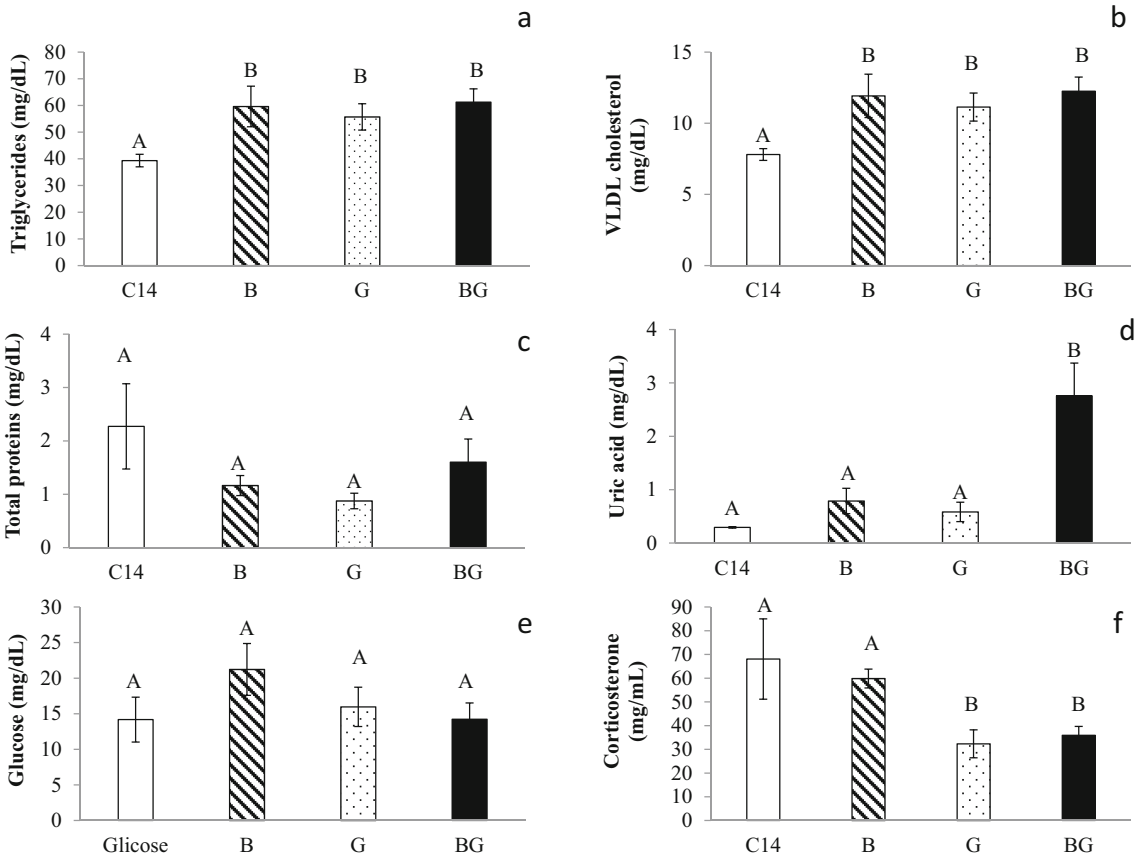

Fig. 2 Plasma levels of TGL (**a**), VLDL (**b**), total proteins (**c**), uric acid (**d**), glucose (**e**), and corticosterone (**f**) in the control group (C), boral group (B), glyphosate group (G), and herbicide blend group (BG). Different letters above the histograms represent significant difference for $p < 0.05$

control group (Fig. 2e). The constant circulating glucose levels have been demonstrated in bullfrog tadpoles exposed to different concentrations of quinclorac, variating only between the concentration of herbicide (Coltro et al. 2017). Bullfrogs exposed to different concentrations of glyphosate demonstrate an intense decrease in the glycogen levels in different organs (mainly liver) (Dornelles and Oliveira 2016). This polysaccharide had a central role in the energetic balance of different vertebrate species, providing quick energy in stressful situations such as herbicide exposure (Barton and Iwama 1991). Therefore, the glucose resultant from the glycogenolysis may also be used in the glycemia stability, glycoprotein synthesis, and essential cell components (Vutukuru 2005).

The corticosterone (CORT) levels in the glyphosate group reduced 2.1 times in comparison with the control group. Among the exposed groups, CORT levels were 1.85 and 1.66 times higher in the boral group compared with the glyphosate and blend groups, respectively (Fig. 2f). In amphibians, corticosterone regulates the metamorphosis induction (Wright et al. 2003). Based on this, we suggest that the decrease in the CORT levels in the tadpoles exposed to glyphosate and herbicide blend might lead to a delay of the metamorphosis process probably due to the deviation of energetic compounds towards the detoxification process.

Coltro et al. (2017) observed a peak in the circulating levels of corticosterone in bullfrog tadpoles exposed to the lowest experimental concentration of quinclorac; the levels decreased as the exposure concentration increased. The authors suggested the occurrence of hormesis linked to the hypothalamic-pituitary-inter-renal axis. Pandey and Rudraiah (2015) investigated the effect of Roundup on the adrenal gland steroidogenesis and on the signaling pathway associated with steroid production in rats. Adult animals were exposed to 10, 50, 100, and 250 mg/kg bw/d Roundup concentrations for 2 weeks. The circulating levels of corticosterone decreased at the 10 mg/kg bw/d concentration but did not alter food intake and body weight. The results suggest that Roundup® may inhibit the hypothalamic-pituitary axis activity, leading to the reduction of StAR phosphorylation and corticosterone synthesis in the adrenal tissue. This inhibition may also lead to the reduction of the cyclic adenosine monophosphate (cAMP)/PKA pathway (Pandey and Rudraiah 2015).

High circulating and constant CORT levels in the initial period of stress can lead to a negative feedback, reducing the corticosterone axis responsiveness and its secretion, altering the functioning of cellular glucocorticoid receptors (Costantini et al., 2011; Costantini, 2014). The occurrence of Hormesis mechanism in the tadpoles exposed to glyphosate and the herbicide blend cannot be ruled out since the CORT levels in these experimental groups decreased after 7 days of exposure. After prolonged exposure, this decrease would make the tadpoles less responsive to stressful synergetic situations such as habitat fragmentation, UV radiation, pollutants, pathogenic agents, invasive species, and predators. All these factors, among others, would decrease the bullfrog populations.

In aquatic animals, the homeostasis stability as a response to stressful situations is an adaptive mechanism to the species survival (Lima et al. 2006). These responses might occur in the tissue level, including the mobilization of energy substrates. Depending on the stressor severity, the response mechanism could be dysfunctional and impact negatively the animal's physiology. This would often lead to the inhibition of growth or the immune response. The decline of amphibian populations may lead to long-lasting effects on the ecosystem, increasing the algal population and affecting the primary production of the ecosystem (Whiles et al. 2006).

Oxidative balance markers

There was a different pattern in the response of oxidative balance markers levels between the liver and caudal muscle. The GST levels were higher in the liver (Fig. 3c) than in the caudal muscle (Fig. 4c) in all experimental groups, including the control group. This finding reinforces the role of the liver as a site to herbicide metabolization. Similar results were found in bullfrog tadpoles exposed to quinclorac (Coltro et al. 2017); however, the authors found GST values 35–60 times lower in the liver and 1–12 times lower in the caudal muscle.

The SOD activity levels were lower in the hepatic tissue (Fig. 3a) than in the caudal muscle (Fig. 4a) in all experimental groups. The CAT (Figs. 3b and 4b) and TBARS (Figs. 3b and 4b) activity levels did not have a clear pattern regarding the tissue, ranging from low to high depending on the experimental group. Costa et al. (2008) demonstrated that Original Roundup increased the SOD and CAT activities in the liver but decreased their activities in the skeletal muscle after a 48-h exposure at 8 ppm. The SOD activity levels were higher in the muscle in comparison with the liver; the CAT levels were higher in the liver, regardless of the experimental group. Therefore, we confirmed the hypothesis proposed by Jones et al. (2010) and Persch et al. (2017) in which the oxidative balance would respond differently depending on the organ or tissue evaluated.

In the liver, the activity levels of SOD, CAT, and GST were significantly different among the herbicide groups and between them and the control group (Fig. 3a, b, and c). The

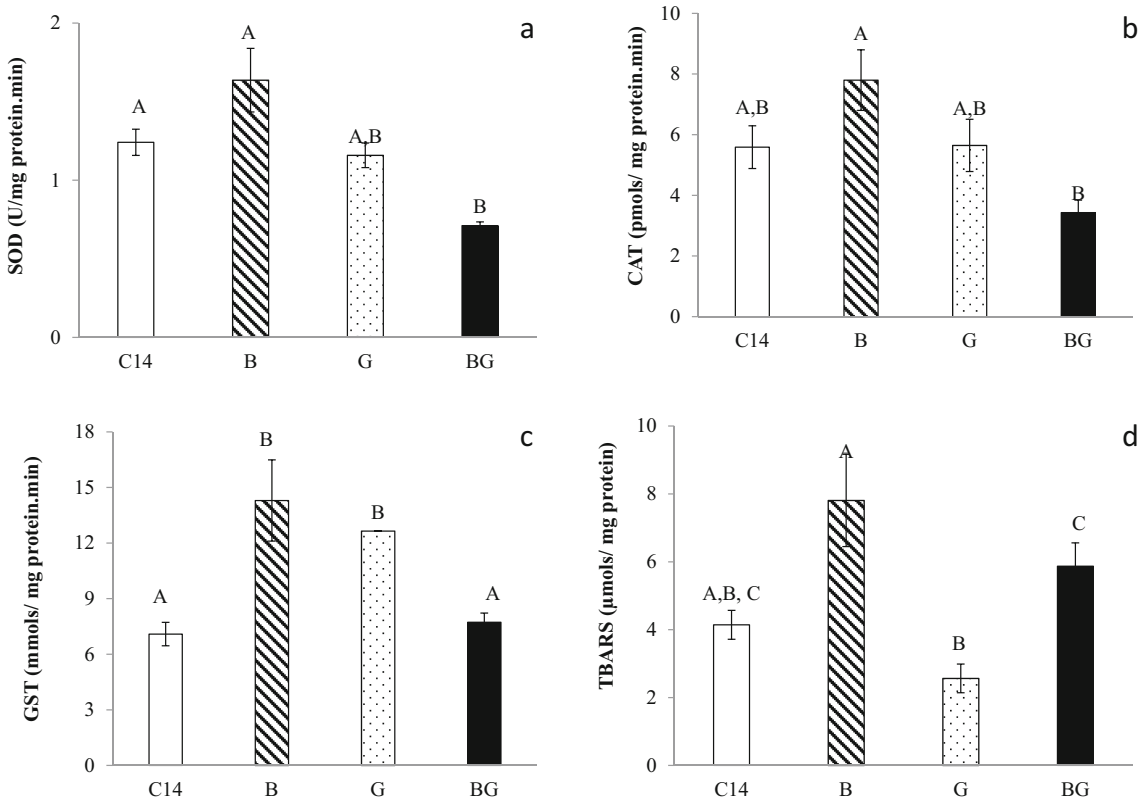

Fig. 3 Liver variations in the level of **a** superoxide dismutase (SOD), **b** catalase (CAT), **c** glutathione S-transferase (GST), and **d** TBARS in the control group (C), boral group (B), glyphosate group (G), and herbicide blend group (BG). The results are expressed as mean ± standard error. Different letters above the histograms represent significant difference at $p < 0.05$

SOD and CAT activities increased approximately 1.7 and 2.3 times more in the blend group in comparison with the control and boral groups, respectively. The GST activity was 2 and 1.8 times higher in the blend and glyphosate groups in comparison with the control group, respectively. The GST levels were also 1.85 higher in the blend group and 1.63 times higher in the glyphosate group when compared with the blend group, which had a similar level to that of the control. The TBARS levels were 3 and 2.3 times lower in the glyphosate group in comparison with the boral and blend groups, respectively (Fig. 3d).

The preservation of the SOD and CAT activities in the boral and glyphosate groups and the increased GST levels seem to be determinant to prevent the lipid peroxidation augmentation in the first and reduction in the latter group. The exposure to xenobiotics might be associated with the enlarged expression and/or activity of biotransformation enzymes such as glutathione S-transferases (Hermes-Lima and Storey 1993). The biotransformation of organic components can be briefly categorized in two main phases. In the phase I reactions, there is the epoxidation/hydroxylation of the molecules; in phase II, there is the conjugation of xenobiotics with GSH endogenous components catalyzed by GST (Nunes et al. 2015). The effects of glyphosate in aquatic organisms have been already evaluated in fishes (Cattaneo et al., 2011; Rossi et al. 2011).

According to Banerjee et al. (1999), the increase of GST activity seems to be an adaptive mechanism to restrain oxidative stress situations in animals. Our results reinforce this statement; the preservation of GST levels in animals exposed to the herbicide blend was enough to restrain the TBARS rise despite SOD and CAT reduction. The exposure to glyphosate increases the GST hepatic activity and other enzymes involved in the antioxidant defense of the fish *Prochilodus lineatus* (Modesto and Martinez 2010). It is worth mentioning that the tadpoles in the blend group had high levels of circulating uric acid in our experiment, suggesting an antioxidant role for this molecule.

In the caudal muscle, the activity levels of SOD and CAT did not change significantly in both exposed and control groups, nor in the comparison among them (Fig. 4a and b). The GST levels reduced in the animals exposed to the herbicide blend in comparison with all the experimental groups (Fig. 4c). This reduction was 15, 13, and 12.5 times lower than the control, boral, and glyphosate groups, respectively. The TBARS levels were 4.5, 2.6, and 3.6 times lower in the blend group than in the control, boral, and glyphosate groups, respectively (Fig. 4d).

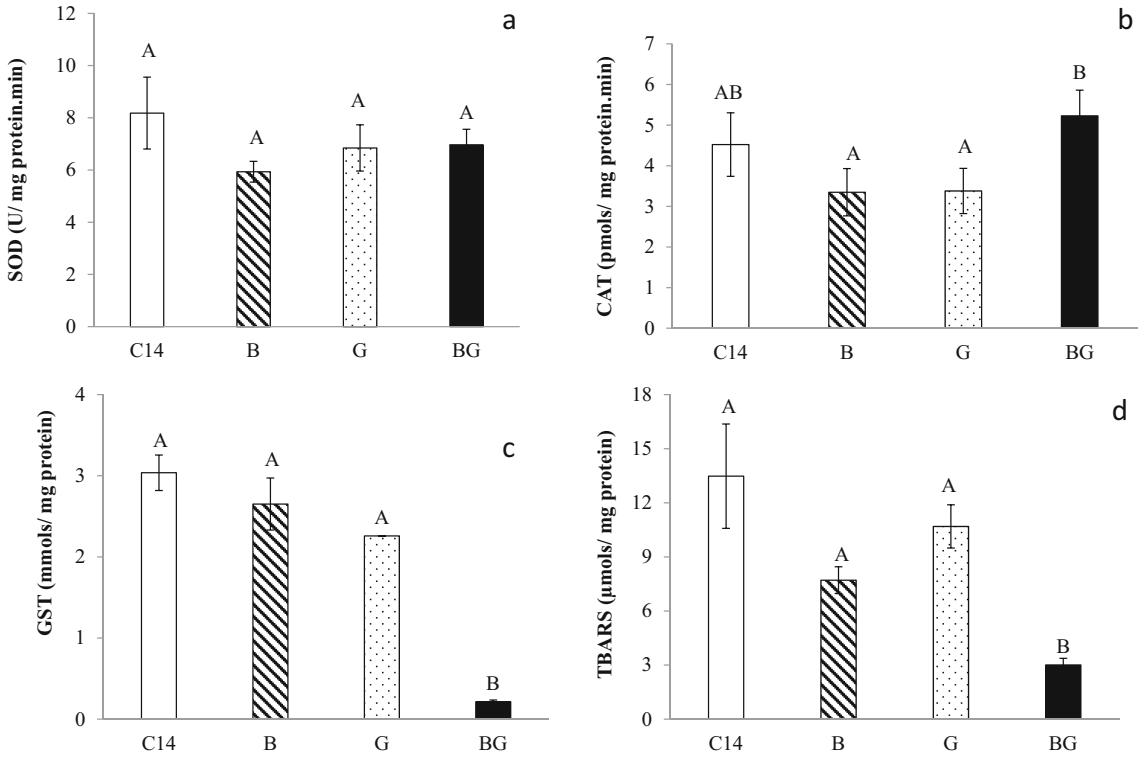

Fig. 4 Caudal muscle variations in the level of **a** superoxide dismutase (SOD), **b** catalase (CAT), **c** glutathione S-transferase (GST), and **d** TBARS in the control group (C), boral group (B), glyphosate group (G), and herbicide blend group (BG). The results are expressed as the mean ± standard error. Different letters above the histograms represent significant difference at $p < 0.05$

The herbicide blend drastically reduced the GST activity in the caudal muscle. Although there was no increase in the TBARS levels, this finding suggests that the blend seems to be more harmful to the tadpoles when compared with the isolated herbicides. It is important to highlight that the tadpoles from the blend group had high levels of circulating uric acid, suggesting the action of the non-enzymatic antioxidant system in the detoxification process. Coltro et al. (2017) suggested that the antioxidant system of *R. catesbeiana* tadpoles has a high constitutive capacity besides the participation of uric acid in the process. Jones et al. (2010) proposed that the enlarged activity of oxidative stress enzymes might be a result of the increase of transcriptional genes responsible for their codification and of the high constitutive enzymatic antioxidant capacity that would impair the oxidative damage in bullfrogs.

There is an association between the intensification of the circulating glucocorticoids levels and the formation of oxygen reactive species, and the intensity of this response is time- and tissue-dependent (Modesto and Martinez 2010). In the present study, none of the exposed groups presented increased levels of circulating corticosterone or levels of hepatic or muscular TBARS; in the G group, we observed a significant decrease in both hormones and TBARS values, which reinforces the hypothesis proposed by these authors.

Conclusions

In a 7-day experiment with a commercial formulation of sulfentrazone (130 μg/L), glyphosate (234 μg/L), and their blend, 92% of the tadpoles remained alive and apparently strong. This finding suggests that the bullfrog tadpoles were tolerant to the herbicide toxicity in ecological relevant concentrations.

These xenobiotics (sulfentrazone and glyphosate) induced changes in the analyzed parameters, mainly in the $K_{intestinal}$, triglycerides, uric acid, and plasmatic corticosterone levels. There were also changes in the SOD, CAT, and GST activities and modulation of the lipid peroxidation levels in the liver and caudal muscle. We can also suggest that the herbicide blend containing boral and glyphosate seems to be more harmful to the animals. Although there was no lipid peroxidation, this is probably due to the preservation and drastic rise in the GST levels in liver and caudal muscle, respectively.

Therefore, we propose that the tolerance to herbicide is associated with an increase in the energy demand to keep the homeostasis and ensure the animal's survival. This would reduce the energy available to growth and metamorphosis. Corticosterone levels reduced significantly in animals exposed to glyphosate and the herbicide blend. This finding suggests that the herbicides act on the endocrine homeostasis.

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Compliance with ethical standards All procedures followed the Conselho Nacional de Controle de Experimentação Animal (CONCEA 2015) guide and were approved by the Comitê de Ética para Uso de Animais da Universidade (protocol no. 15/00471—CEUA/PUCRS).

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