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RESEARCH ARTICLE

Toxicity of atrazine, glyphosate, and quinclorac in bullfrog tadpoles exposed to concentrations below legal limits

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Abstract This work sought to ascertain survival and possible changes in levels of glycogen, triglycerides, total lipids, cholesterol, protein, and lipid peroxidation in gills, liver, and muscle of bullfrog tadpoles (*Lithobates catesbeianus*) exposed to low concentrations of atrazine (2.5 μg L^{-1}), glyphosate (18 μg L^{-1}), and quinclorac (0.025 μg L^{-1}) at laboratorial conditions. Tadpoles showed a reduction of glycogen and triglyceride in all organs and an increase in lipid peroxidation (LPO) compared with control animals. Total lipid in gills and muscle increased in exposure to atrazine, and gills alone in exposure to glyphosate, but decreased in gills, liver, and muscle after quinclorac. Cholesterol increased in gills and liver after atrazine, in gills and muscle after glyphosate, and decreased in liver after quinclorac. Total protein in gills decreased after exposure to all herbicides, increased in muscle after atrazine, and in liver and muscle after quinclorac. These findings show that at concentrations of these herbicides tested can lead to an increase in energy expenditure to maintain homeostasis and survival of these animals despite the increase in lipid peroxidation levels in all organs analyzed. Responses observed can be one of the factors responsible for the decline in the number of amphibians around the world.

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Introduction

The way humans use the world's natural resources has led to significant impacts on other species that inhabit the planet. One example of this phenomenon is the decline in the number of amphibians over the last few decades, an extinction that has no precedent in any animal class in this period and which may be the result of an isolated action or interaction of different factors, such as habitat loss, ultraviolet radiation, global warming, diseases, over-harvesting, and/or the introduction of agrochemicals, especially pesticides—even at low levels—into the environment (Allran and Karasov 2000; Blaustein et al. 2003; Boone et al. 2005; David and Kartheek 2015; Davidson 2004; Gascon et al. 2005; Relyea 2003; Sayim 2008).

Approximately 1 % of agrochemicals used in the field reach their specific targets. The remaining 99 % can move through the different environmental compartments and may have an indirect effect on non-target organisms exposed to contaminants (Belluck et al. 1991).

Amphibians are among the animals that may be indirectly exposed to these agrochemicals, and the exposure may account for the great amphibian mortality that has been observed in recent years. Tadpoles appear to be more sensitive than adults, which is consistent with the greater fragility of these animals in the larval stage (Blaustein et al. 2003; David and Kartheek 2015; Johansson et al. 2006; Murphy et al. 2000; Sayim 2008; Wang et al. 2001).

The environmental changes induced by the use of agricultural chemicals can interfere with physiological and

biochemical parameters in non-target aquatic organisms, affecting functions such as growth, development, and reproduction (Upasani and Balaraman 2001; Venkataramana et al. 2006). Nwani et al. (2010) and Roy and Hänninen (1993) reported that the indiscriminate use of herbicides may have harmful effects on aquatic organisms, possibly leading to sublethal effects, such as biochemical and metabolic changes in the tissues of exposed animals.

According to Oba et al. (2009), some animals' responses on exposure to chemicals, such as pesticides, can include a variety of metabolic changes: mobilization of energy substrates, as expressed by depleted glycogen stores; lipolysis; inhibition of protein synthesis; increased muscle protein catabolism; and changes in the levels of fatty acids and cholesterol.

Dornelles and Oliveira (2014), working with bullfrog tadpoles exposed to the same pesticides as in this study, reported changes in the biochemical composition (glycogen, proteins, lipids, and triglycerides) of the animals. This response seems to be related to a depletion of energy reserves and increase in energy expenditure, possibly to detoxify and allow the animals to survive environmental change. A similar response pattern was observed by other authors in different species (El-Banna et al. 2009; Ganeshwade 2012; Moyes and Schulte 2010; Salbego et al. 2010; Sounderraj et al. 2011; Vutukuru 2005).

Different animals when exposed to pesticides have had their levels of lipid peroxidation (LPO) increased in different organs, which may lead to a chemical damage and possible cell death if the antioxidant and defense system were not enough to counteract this damage (Al-Othman et al. 2011; Champe and Harvey 2006; Dornelles and Oliveira 2014; El-Banna et al. 2009; Patil et al. 2009; Uchendu et al. 2012). The LPO is among the best predictors of systemic level of damage induced by reactive oxygen species (ROS) (Sayeed et al. 2003) and is one of the molecular mechanisms involved in the toxicity of pesticides to bodies (Kehrer 1993; Kavitha and Venkateswara Rao 2007).

It is known that the gill epithelium is responsible for breathing, elimination of nitrogen excreted, osmoregulation, and other functions directly related to animal survival; furthermore, the gills are in direct contact with the environment which can act as a site of absorption, magnifying the action of these herbicides when compared to other organs (Bernabò et al. 2008), making this an interesting organ for analysis after the exposure of the animals to these herbicides.

Of the organs widely used for toxicological studies, the liver stands out mainly for having a central role in major functions of the organism, being responsible for the biotransformation and the detoxification of exogenous substances, with its capacity to convert hydrophobic substances into water-soluble products that can be secreted readily from the body. Furthermore, the liver is a metabolically active organ responsible for many vital functions, such as bile production and excretion; excretion of bilirubin, cholesterol, hormones, and drugs; metabolism of fats, proteins, and carbohydrates; storage of glycogen, vitamins, and minerals; synthesis of plasma proteins, such as albumin, and clotting factors; and uptake of amino acids, lipids, carbohydrates, and vitamins and their subsequent storage, metabolic conversion, and release into the blood and bile (Guillouzo 1998; Vickers 1994). These important activities and the exposure to different substances make the liver one of the more susceptible organs to injury.

The muscle tissue is composed mainly of proteins and is responsible for providing the contractile force to movement (Hill et al. 2012). Moyes and Schulte (2010) state that in muscle, ATP is produced by three major mechanisms: by high-energy transfer from creatine phosphate to ADP, by glycolysis, and by oxidative phosphorylation, characterized as a low metabolic rate process.

Since our experimental model has generalist eating habits and voracious predatory behavior, analyses of the liver and the muscle provides information about not only the storage of nutrients but also the action of these herbicides in a long period (7 days of exposure) in an organ with a high (liver) and another with a low (muscle) metabolic rate.

According to Massoud et al. (2011), there is a paucity of information on the toxicity of pesticides at sublethal concentrations and sensitive biomarkers related to the metabolism of these animals. Therefore, research on the impact of these contaminants in exposed communities of aquatic animals—even at low concentrations—is justified.

Taking this lack of information into account, and the results obtained in the laboratory with tadpoles exposed to concentrations within the range permitted by law (Dornelles and Oliveira 2014), the present work sought to assess potential changes in biochemical parameters, levels of lipoperoxidation, and survival in bullfrog tadpoles exposed to concentrations below legal limits of atrazine, glyphosate, and quinclorac. Our hypothesis states that these herbicides, even at realistic concentrations found in the natural environment, are able to introduce changes in the energy reserves and oxidative status in different organs in prometamorphic tadpoles.

Material and methods

Chemicals All chemical reagents used were obtained from Merck and Sigma-Aldrich. For toxicity testing, three herbicides in concentrations below legal limits—atrazine (3 μg L^{-1}) (US Environmental Protection Agency 1985), glyphosate (65 μg L^{-1}) (Brasil and Meio Ambiente 2005), and quinclorac—were used. For the latter, we used a concentration below that found in natural bodies of water (Marchezan et al. 2007; Silva et al. 2009), considering that no specific legislation for maximum allowable concentrations in natural

water bodies is available for quinclorac (Marchezan et al. 2007). All pesticides were used as commercial formulations: atrazine (A): Primóleo® 400 g L^{-1} (Syngenta); glyphosate: Roundup Original® 306 g L^{-1} (Monsanto); and quinclorac: Facet® 500 g kg^{-1} (Basf). The use of these herbicides is justified by their wide use in different types of crops, not only in Brazil but in several countries (Dornelles and Oliveira, 2014).

The concentrations chosen for the present study were 2.5 μg L^{-1} for atrazine, 18 μg L^{-1} for glyphosate, and 0.025 μg L^{-1} for quinclorac. The pesticides were applied only once, at nominal concentrations according to Dornelles and Oliveira (2014) in respect to the time of permanency in water of these chemicals: between 34.8 and 742 days at pH between 2.9 and 6.0 for atrazine, between 7 and 10 weeks for glyphosate (Montgomery et al. 2008), and between 21 and 30 days for quinclorac (Barceló and Hennion, 2003; Rodrigues and Almeida 1998).

Experimental model For this study, 76 bullfrog (*Lithobates catesbeianus*) tadpoles were procured from a frog farm in the municipality of Imbé, state of Rio Grande do Sul, Brazil. All the tadpoles were 3 months old and had no visible limbs (Landis and Yu 2003), and they were in the larval stage 25, according to Gosner (1960).

The animals were transported in air-filled plastic bags to the Conservation Physiology Laboratory at Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS), where they were individually measured, weighed, and randomly divided into groups.

Experimental design The tadpoles were distributed to a control group (Co 7=only water, without herbicides) and three exposure groups (A 2.5=atrazine 2.5 μg L^{-1}; G 18=glyphosate 18 μg L^{-1}; Q 0.025=quinclorac 0.025 μg L^{-1}). Each experiment was performed using 8 or 10 tadpoles for treatment, and in duplicate, being 16 the final number of animals in the control group and 20 in the herbicide groups. All aquariums contained 12 L of water each, with constant aeration, a water temperature of around 22±2 °C, pH 6.2±0.3, and a 12-h light/dark cycle. The animals were fed once daily (with approximately 5 % of the early biomass in each aquarium) with the same fish feed used at the frog farm, with a crude protein content of 38 %.

A static exposure system was used in accordance with a Standard Guide for Conducting the Frog Embryo Teratogenesis Assay—Xenopus (FETAX, ASTM 1998). The levels of ammonia in the water were monitored daily by a commercial kit (LabCom) and showed no toxic values (variation between 0 until 0.5). The levels of pH and dissolved oxygen were monitored too (Sanxin SX721 Portable pH/ORP Meter) and remained within the established by FETAX guide (ASTM 1998), with the treatment pH between 6.5 and 9. The conductivity and dissolved oxygen do not have values established by the FETAX guide. Our values of dissolved oxygen remained between 3.5 and below 5 mg L^{-1} (ASTM 1998).

The total duration of the experiment was 14 days: 7 days for acclimation and 7 days of exposure to herbicides. Herbicides were introduced in the aquariums on the eighth day after the beginning of the experiment—the commercial formulations, both liquid (atrazine and glyphosate) and powder (quinclorac), were diluted in distilled water and added only once to the aquariums at a particular concentration. The time of exposures for 7 days was based on the half-life of atrazine, glyphosate, and quinclorac in water (Solomon et al. 1996; Giesy et al. 2000; Rodrigues and Almeida 1998, respectively). In the control group, the animals remained for seven more days under the same acclimation conditions. At the end of the experiment, all animals were euthanized by the freezing method, and the left and right gills, liver, and muscle were removed by dissection.

All research protocols used in this work were authorized by the Pontifícia Universidade Católica do Rio Grande do Sul Animal Research Ethics Committee with registration number CEUA 11/00250, as set forth in approval letter number 157/11-CEUA, December 2011.

Biochemical analysis All biochemical analyses of tissue specimens were performed in quadruplicate, by spectrophotometric methods, and the respective results were expressed in milligrams per gram of tissue. Glycogen was extracted by Van Handel's method (1965) and quantified as glucose after acid hydrolysis (HCl) and neutralization (Na_2CO_3) (Geary et al. 1981), using a commercial Glucose Oxidase Kit (Labtest). The total protein concentration was determined by the colorimetric biuret method: the sample is added to the reagent kit, vortexed, placed in a bath, cooled, and read in a spectrophotometer. A commercial kit was used (Total Protein Kit, Labtest); lipid, triglyceride, and cholesterol extraction was performed using the chloroform/methanol method (2:1) (Folch et al. 1957). Lipid content was determined by the sulfo-phospho-vanillin reaction (Frings and Dunn, 1970), and triglycerides, by the lipoprotein lipase method, using the commercial Triglycerides GPO-ANA Kit (Bio-Diagnostic). Cholesterol was determined with the Liquiform Kit (Labtest). Measurement of lipid peroxidation was carried out by the TBARS (TBA-reactive substances) method: 150 μL of 10 % trichloroacetic acid (TCA), 50 μL of tissue homogenate, 100 μL of 0.67 % thiobarbituric acid (TBA), and 50 μL of distilled water are added to a test tube (total volume 350 μL). The tube is shaken, incubated at 100 °C for 15 min, and cooled for 10 min. Then, 300 μL of *n*-butyl alcohol is added to the sample for extraction of the colored product from aqueous solution. Tubes are shaken for 45 s and centrifuged for 10 min at 3000 rpm. The supernatant was added to the

spectrophotometer cuvette and read at 535 nm. Results were expressed as nanomoles per milligram of protein.

Statistical analysis Comparisons between the experimental and control group were made using the Statistical Package for the Social Sciences 12.0 (SPSS) environment, using the Kolmogorov-Smirnov test for normality. The data was compared with control group for Student's *T* test to the independent sample. The results were expressed as mean±standard deviation. The level of significance was set at $p<0.05$.

Results

Gills There were significant reductions in glycogen levels in the gills of animals when exposed to atrazine ($\bar{x}=0.044$ mg glycogen/g wet weight), glyphosate ($\bar{x}=0.027$ mg glycogen/g wet weight), and quinclorac ($\bar{x}=0.032$ mg glycogen/g wet weight) as compared with animals in the control group ($\bar{x}=0.197$ mg glycogen/g wet weight) (Fig. 1(A1)). In comparison to the control group ($\bar{x}=0.166$ mg cholesterol/g wet weight), total lipid levels were increased in animals who were exposed to atrazine ($\bar{x}=0.539$ mg total lipids/g wet weight) and glyphosate ($\bar{x}=0.511$ mg total lipids/g wet weight), but reduced in those who were exposed to quinclorac ($\bar{x}=0.071$ mg total lipids/g wet weight) (Fig. 1(A2)). Triglyceride levels decreased in animals exposed to atrazine ($\bar{x}=0.059$ mg triglycerides/g wet weight) and glyphosate ($\bar{x}=0.134$ mg triglycerides/g wet weight) in relation to the control group ($\bar{x}=0.232$ mg triglycerides/g wet weight) (Fig. 1(A3)). Cholesterol levels were increased in those exposed to atrazine ($\bar{x}=0.238$ mg cholesterol/g wet weight) and glyphosate ($\bar{x}=0.439$ mg cholesterol/g wet weight) as compared with control animals ($\bar{x}=0.095$ mg cholesterol/g wet weight) (Fig. 1(A4)). Quinclorac exposure had no significant effect on triglyceride or cholesterol levels. Total protein level decreased in the animals who were exposed to glyphosate ($\bar{x}=0.093$ mg protein/g wet weight) and quinclorac ($\bar{x}=0.091$ mg protein/g wet weight) in relation to the control group ($\bar{x}=0.155$ mg protein/g wet weight), but there was no significant difference in atrazine-exposed animals (Fig. 1(A5)). All herbicides increased LPO levels in relation to the control group ($\bar{x}=9.025$ nmol TBARS/mg protein): levels were $\bar{x}=57.753$ nmol TBARS/mg protein in animals exposed to atrazine, $\bar{x}=47.601$ nmol TBARS/mg protein in those exposed to glyphosate, and $\bar{x}=57.849$ nmol TBARS/mg protein in those exposed to quinclorac (Fig. 1(A6)).

Liver Statistically significant reductions in glycogen and triglyceride levels in the liver were noted in animals that were exposed to atrazine, glyphosate, and quinclorac. Glycogen levels declined in animals exposed to atrazine ($\bar{x}=0.047$ mg glycogen/g wet weight), glyphosate ($\bar{x}=0.047$ mg glycogen/g wet weight), and quinclorac ($\bar{x}=0.057$ mg glycogen/g wet weight) in relation to the control group ($\bar{x}=0.113$ mg glycogen/g wet weight) (Fig. 2(B1)). Total lipid level decreased after exposure to atrazine ($\bar{x}=0.429$ mg total lipids/g wet weight) and quinclorac ($\bar{x}=0.171$ mg total lipids/g wet weight) than to the control group ($\bar{x}=0.723$ mg total lipids/g wet weight) (Fig. 2(B2)), but there was no significant difference in lipid level after glyphosate exposure. Triglyceride levels declined after exposure to atrazine ($\bar{x}=0.263$ mg triglycerides/g wet weight), glyphosate ($\bar{x}=0.229$ mg triglycerides/g wet weight), and quinclorac ($\bar{x}=0.180$ mg triglycerides/g wet weight) in relation to the control group ($\bar{x}=0.345$ mg triglycerides/g wet weight) (Fig. 2(B3)). Atrazine exposure led to an increase in cholesterol levels ($\bar{x}=0.262$ mg cholesterol/g wet weight), but animals exposed to glyphosate ($\bar{x}=0.032$ mg cholesterol/g wet weight) and quinclorac ($\bar{x}=0.053$ mg cholesterol/g wet weight) showed a reduction in cholesterol levels as with controls ($\bar{x}=0.209$ mg cholesterol/g wet weight) (Fig. 2(B4)). Total protein level decreased in the glyphosate group ($\bar{x}=1.013$ mg protein/g wet weight) but increased in the atrazine ($\bar{x}=1.683$ mg protein/g wet weight) and quinclorac ($\bar{x}=1.935$ mg protein/g wet weight) groups in relation to the control group ($\bar{x}=1.454$ mg protein/g wet weight) (Fig. 2(B5)). In the liver, as well as in the gills, all herbicides were associated with an increase in lipid peroxidation levels in relation to the control group ($\bar{x}=14.212$ nmol TBARS/mg protein): $\bar{x}=22.656$ nmol TBARS/mg protein in animals exposed to atrazine, $\bar{x}=22.419$ nmol TBARS/mg protein in those exposed to glyphosate, and $\bar{x}=23.855$ nmol TBARS/mg protein in those exposed to quinclorac (Fig. 2(B6)).

Muscle In muscle tissue, there were statistically significant decreases in glycogen levels on the exposure of glyphosate ($\bar{x}=0.007$ mg glycogen/g wet weight) and quinclorac ($\bar{x}=0.007$ mg glycogen/g wet weight), but there was no significant decrease for atrazine in relation to the control group ($\bar{x}=0.009$ mg glycogen/g wet weight) (Fig. 3(C1)). Total lipid levels increased after exposure to atrazine ($\bar{x}=0.075$ mg total lipids/g wet weight) but decreased in animals exposed to glyphosate ($\bar{x}=0.006$ mg total lipids/g wet weight) and quinclorac ($\bar{x}=0.004$ mg total lipids/g wet weight) in relation to the control group ($\bar{x}=0.008$ mg total lipids/g wet weight) (Fig. 3(C2)). Triglyceride levels decreased after exposure to atrazine ($\bar{x}=0.021$ mg triglycerides/g wet weight), glyphosate ($\bar{x}=0.039$ mg triglycerides/g wet weight), and quinclorac ($\bar{x}=0.058$ mg triglycerides/g wet weight) in relation to the control group ($\bar{x}=0.139$ mg triglycerides/g wet weight) (Fig. 3(C3)). Regarding cholesterol levels, only atrazine ($\bar{x}=0.030$ mg cholesterol/g wet weight) was associated with a significant increase in relation to

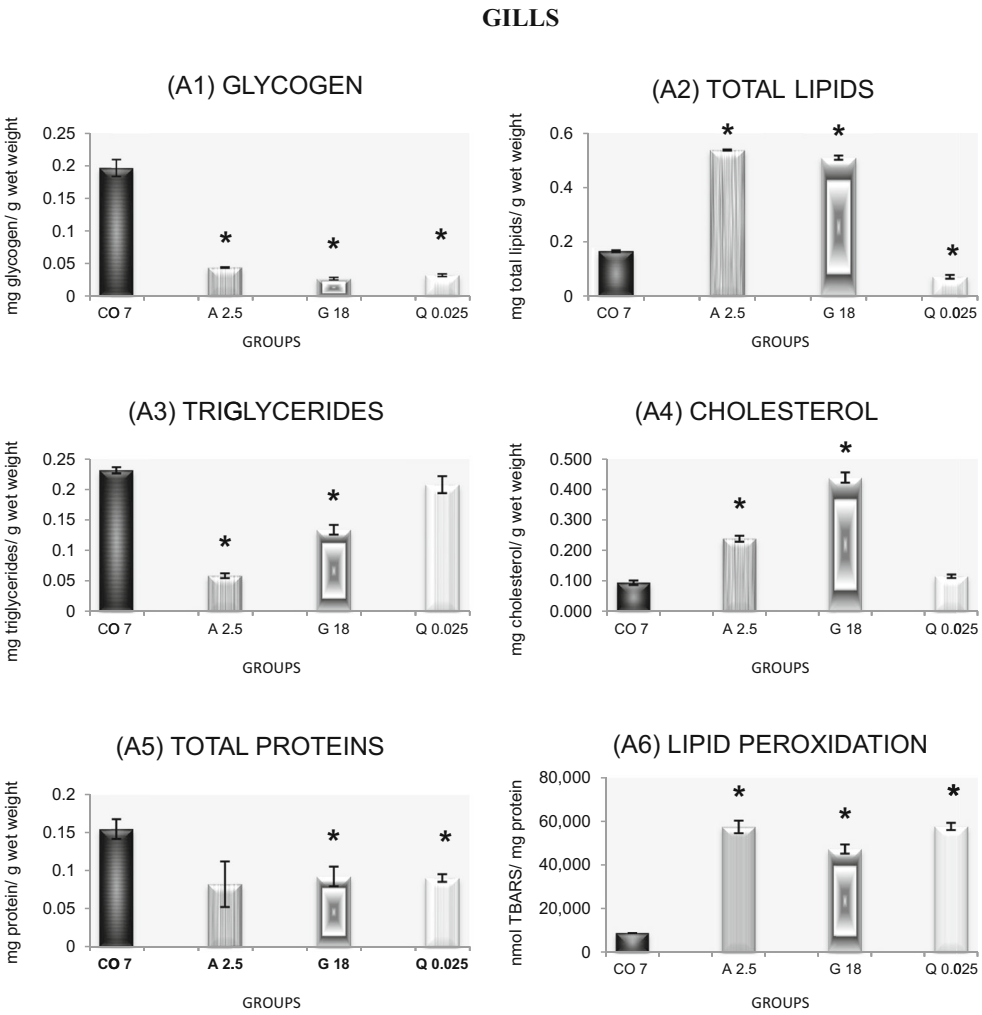

Fig. 1 Graphics *A1*, *A2*, *A3*, *A4*, *A5*, and *A6*: Levels of glycogen, total lipids, triglycerides, cholesterol, total proteins, and lipid peroxidation in the gills of *Lithobates catesbeianus* tadpoles exposed to the herbicides atrazine, glyphosate, and quinclorac (*Co 7* control 7 days, *A 2.5* atrazine 2.5 μg L^{-1}; *G 18* glyphosate 18 μg L^{-1}, *Q 0.025* quinclorac 0.025 μg L^{-1}). The results are expressed as the mean ±standard deviation. The *asterisk beside the bars* indicates a significant difference compared to the control group 7 days, being $p<0.05$

the control group ($x=0.022$ mg cholesterol/g wet weight) (Fig. 3(C4)). All herbicides were associated with increases in total protein levels: $x=0.424$ mg protein/g wet weight after exposure to atrazine, $x=0.391$ mg protein/g wet weight after exposure to glyphosate, and $x=0.525$ mg protein/g wet weight after exposure to quinclorac, versus $x=0.258$ mg protein/g wet weight in the control group (Fig. 3(C5)). As in the gills and the liver, lipid peroxidation levels in muscle tissue increased after exposure to herbicides in relation to the control group ($x=20.543$ nmol TBARS/mg protein): levels were $x=55.646$ nmol TBARS/mg protein after atrazine exposure, $x=69.186$ nmol TBARS/mg protein after glyphosate exposure, and $x=52.411$ nmol TBARS/mg protein after quinclorac exposure (Fig. 3(C6)).

Weight, size, and survival All animals experienced increases in weight (Fig. 4(D1)) and size (Fig. 4(D2)) during the experiment. There was no mortality (Fig. 4(D3)), even among the animals exposed to herbicides.

Discussion

The findings of our research were consistent with the hypothesis that there was no mortality, significant change in weight gain, and growth of these animals after exposure to herbicides in low concentrations. However, changes occurred in biochemical parameters in the animal tissues. According to Johansson et al. (2006), exposure to pesticides at levels as low as those found in nature usually does not cause mortality. A study was conducted in our laboratory with tadpoles exposed to higher concentrations of these pesticides, and the results also showed increased energy demands and no significant mortality in the animals (Dornelles and Oliveira 2014).

Pollutants such as herbicides are environmental stressors, which can induce an adaptive response in exposed animals due to changes in their metabolic balance, thus producing a physiological response in an attempt by the animal to reestablish the homeostasis (Roy and Hänninen 1993; Sasikala et al. 2011; Wendelaar Bonga 1997). The responses induced by this stress can occur as a result of metabolic changes, due to an increase in

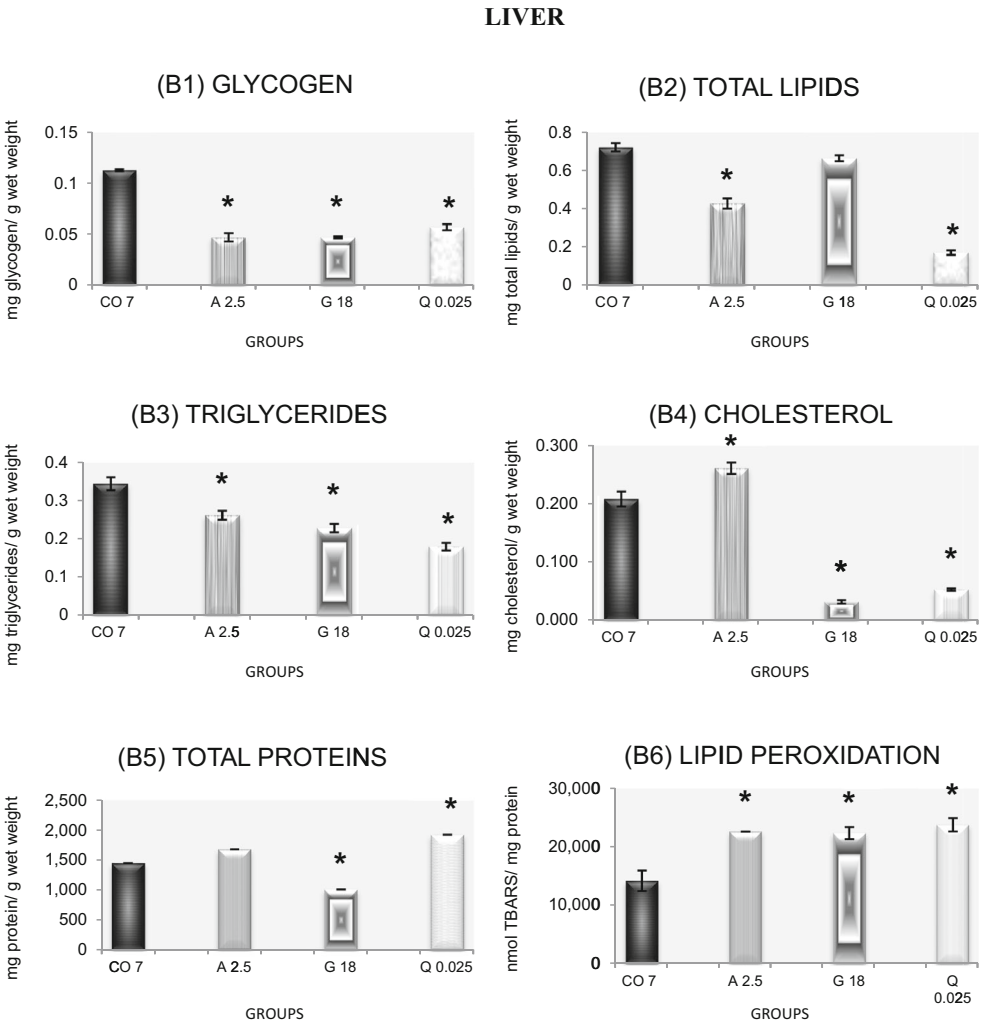

Fig. 2 Graphics *B1*, *B2*, *B3*, *B4*, *B5*, and *B6*: Levels of glycogen, total lipids, triglycerides, cholesterol, total proteins, and lipid peroxidation in the liver of *Lithobates catesbeianus* tadpoles exposed to the herbicides atrazine, glyphosate, and quinclorac (*Co 7* control 7 days, *A 2.5* atrazine 2.5 μg L^{-1}, *G 18* glyphosate 18 μg L^{-1}, *Q 0.025* quinclorac 0.025 μg L^{-1}). The results are expressed as the mean ±standard deviation. The *asterisk beside the bars* indicates a significant difference compared to the control group 7 days, being $p<0.05$

metabolic processes and a consequent increase in energy expenditure as a function of the stressor (Weissman 1990).

The decrease in glycogen concentrations in all tissues observed after exposure to the three tested herbicides is an expected response and similar to that found by other researchers, where this polymer acts as an immediately available energy reserve. This response may be due to the fact that pesticides usually affect energy metabolism, leading to an increase in energy expenditure, and glycogen can act as a rapid source of fuel—through the mobilization of stored glycogen—in times of stress exposure (Dornelles and Oliveira 2014; Dua et al. 2010; Oba et al. 2009; Tiwari and Singh 2003; Triebskorn et al. 1998; Venkataramana et al. 2006; Weissman 1990). So this polymer shows a protective function during periods of high energy demand, such as those caused by the presence of xenobiotics, acting as an immediately accessible energy reserve.

The effects of some pesticides on the lipid profile may be more pronounced in the skeletal muscles and liver and can cause a decrease in tissue lipid levels (Adamu and Kori-Siakpere 2011; Triebskorn et al. 1998). In our experiment, we observed a reduction in lipid levels in the liver after exposure to all herbicides and in the muscle after exposure to glyphosate and quinclorac, which may suggest mobilization of stored lipids, a common response in cases of exposure to chemical stressors (Adamu and Kori-Siakpere 2011). An increase of total lipids was observed in gills and muscle after exposure to atrazine, and it increased too in the gills after exposure to glyphosate. This response profile may be related to the sensitivity of each pesticide, the function, and the amount of metabolites in each organ. The liver has the highest values of total lipids and triglycerides followed by gills and muscle. Already, the glycogen content was higher in the gills followed by the liver and muscle, and total proteins were highest in the liver, followed by muscle and gills.

Little information is available about the effects of pesticides on lipid metabolism, but it is known that the liver has many functions such as synthesizing and processing of multiple substances, as lipoproteins, fatty acid, and

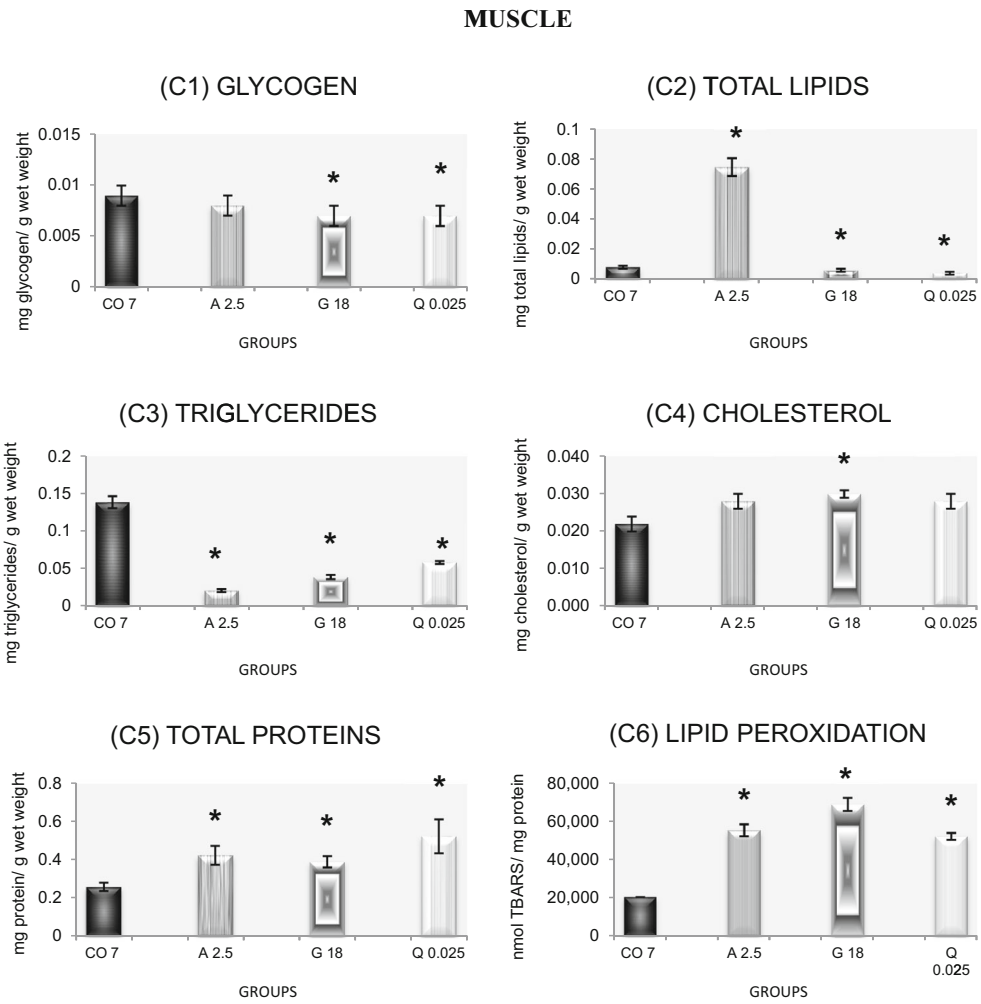

Fig. 3 Graphics *C1*, *C2*, *C3*, *C4*, *C5*, and *C6*: Glycogen levels, total lipids, triglycerides, cholesterol, total proteins, and lipid peroxidation in the muscle of *Lithobates catesbeianus* tadpoles exposed to the herbicides atrazine, glyphosate, and quinclorac (*Co 7* control 7 days, *A 2.5* atrazine 2.5 μg L^{-1}, *G 18* glyphosate 18 μg L^{-1}, *Q 0.025* quinclorac 0.025 μg L^{-1}). The results are expressed as the mean ±standard deviation. The *asterisk beside the bars* indicates a significant difference compared to the control group 7 days, being $p<0.05$

glucose, to be transported to other areas of the body. Also, it is responsible for the maintenance of blood glucose, in addition to endo- and xenobiotic detoxification of substances that are chemically altered or excreted by the liver (Hill et al. 2012; Lasram et al. 2009; Moyes and Schulte 2010). This functional profile requires a high energy demand which can, together with the stress caused by the chemical, lead to the use of a larger amount of metabolites; this response also is related to the toxic potential of each herbicide to which the animal is exposed. This hypothesis is reinforced by the reduction in glycogen, lipid, and triglyceride reserves in the hepatic tissue of animals exposed to herbicides—and the reduction of protein levels in tadpoles exposed to glyphosate—in this study. After a stressful event, fat is used as the primary fuel source, in a process whereby triglycerides are metabolized into fatty acids and glycerol and then metabolized as fuel (Weissman 1990).

In this study, the herbicide atrazine led to an increase of cholesterol levels in all organs studied, the glyphosate determined a decrease of this metabolite in the liver and an increase in muscle, and quinclorac has an effect in the liver only, showing a decrease in cholesterol levels. The results showed that cholesterol levels were altered in different ways in the different types of tissues and showed a specific responsiveness and more sensitivity of the liver, which suggests that this organ acts in metabolizing these herbicides and that atrazine had a more powerful action on the cholesterol metabolism in relation to other herbicides.

Some authors, such as Weissman (1990), have shown that aquatic animals can alter their cholesterol levels on exposure to pesticides accordingly. Cholesterol levels may increase due to the stress induced by exposure to these agents, indicating an induced chemical disruption of lipid metabolism (Weissman 1990). The hypercholesterolemia observed in frogs exposed to pesticides can be the result of a decrease in the ratio of cholesterol conversion to bile acids or an impaired liver function. Conversely, pesticide exposure also can decrease cholesterol levels, leading to an increase in food intake (Adamu and Kori-Siakpere 2011; Sounderraj et al. 2011).

According Yeagle (1989) the cholesterol has been shown to be an ion pump regulator, and is some cases show an absolute

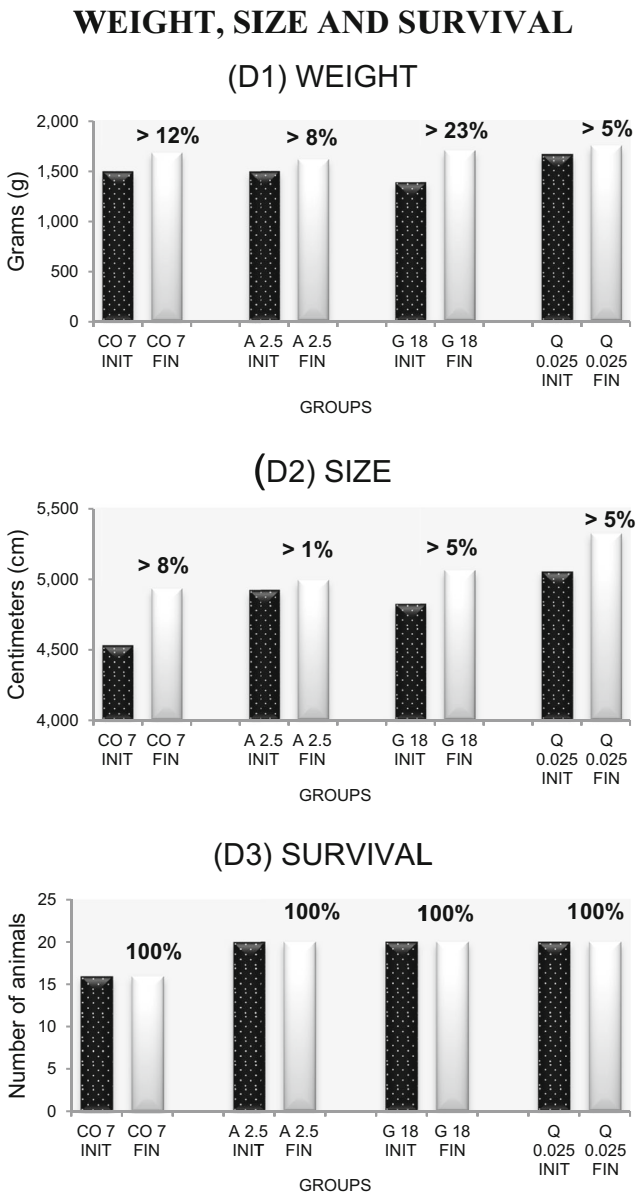

Fig. 4 Graphics *D1*, *D2*, and *D3*: Weight gain during the experiment compared to the initial weight of the groups (the *value beside the bar* indicates the percentage of weight gain), size at the beginning and end of the experiment (the *value beside the bar* indicates the percentage of increase in size during the experiment), and survival of *Lithobates catesbeianus* tadpoles until the end of the experiment (the *value beside the bar* indicates the percentage of survival) (*Co 7* control 7 days, *A 2.5* atrazine 2.5 μg L^{-1}, *G 18* glyphosate 18 μg L^{-1}, *Q 0.025* quinclorac 0.025 μg L^{-1}, *INIT* initial, *FIN* final)

dependence on cholesterol for activity. The author suggests that an essential role that cholesterol plays in mammalian cell biology is to enable crucial membrane enzymes to provide function necessary for cell survival. Studies of Na^+K^+ ATPase in human erythrocyte membranes showed inhibition of activity by high levels of membrane cholesterol. Similar results have been found in other membranes: in rabbit erythrocyte membranes, guinea pig erythrocyte membranes, rat liver membranes, and kidney basolateral membranes (Yeagle 1989). The variations found in the cholesterol levels may be a result of the disruption of the plasma membrane of cells after lipid peroxidation. We must also consider the possibility of using this molecule as a regulator of the electrogenic pump activity and indirectly in the ATP demand by the cells.

Proteins can also be involved in compensatory mechanisms in response to animal stress (Dornelles and Oliveira 2014). The reduction in protein levels in the gills is observed after the exposure to all herbicides, as well as the reduction in liver protein levels after exposure to glyphosate, which suggests that this decline in protein content can be due to a reduction in protein synthesis and/or an increase in proteolysis, and use of proteins in metabolic processes with respect to pesticide toxicity. These reductions in tissue protein content suggest that there are some pathways triggered after an attempt to restore depleted energy by breaking down protein to yield energetic fuel (Adamu and Kori-Siakpere 2011; Khan et al. 2002; Susan et al. 2010; Tiwari and Singh 2003; Venkataramana et al. 2006).

However, other responses may occur in the liver and muscle of aquatic animals exposed to pesticides, such as an increase in levels of protein. This may be due to a decrease in protein catabolism and/or to an increase in protein synthesis, as verified by Sahib et al. (1984) in the tissues of fish exposed to malathion. A similar response was observed in muscle tissue of tadpoles exposed to all herbicides (atrazine, glyphosate, and quinclorac) and in the liver of animals exposed to atrazine and quinclorac in the present study.

In the present work, the pattern of glycogen response to herbicide exposure was the same in all the tissues, which demonstrates the importance of polysaccharides to the energy homeostasis and survival of these animals. The other metabolites analyzed showed different responses according to the tissue and the herbicide. The intense depletion of energy reserves observed in this study may be associated with the high survival rate of tadpoles exposed to low concentrations of the tested herbicides (atrazine, glyphosate, and quinclorac) (Fig. 4(D3)).

Lipid peroxidation can provide important evidence of the toxicity of environmental pollutants. In this study, an increase in LPO levels after exposure to all herbicides and in all tissues analyzed was observed. These responses were more marked in gill tissue (5.2-fold to 6.3-fold increases) than in muscle (2.7-fold to 3.4-fold increases) and liver (1.6-fold increase), allowing us to suggest a higher antioxidant capacity in other organs, mainly the liver, compared to gills. Despite the increase in the lipid peroxidation levels, there was no mortality, which may be related to an antioxidant defense capacity both in enzymatic and non-enzymatic terms (Ballesteros et al. 2009). Farombi et al. (2008), Gultekin et al. (2000), and Brocardo et al. (2005) found similar results in their investigations.

Organisms respond to changes in the external environment by activating their mechanism of regulation of the homeostasis. In complex animals, homeostasis is maintained particularly in organs that serve as sites of exchange with the external environment, such as the gills (Hickman et al. 2001). As the gills are the first tissue to be exposed in amphibians, greater uptake of pesticides may occur and the response to exposure may be more intense (Streit 1992). The results of the present study reinforce this hypothesis, as glycogen, triglyceride, and total protein levels in gill tissue decreased and lipid peroxidation was markedly increased in response to all pesticides.

In animals, responses to stress are an adaptive mechanism that allows survival in the presence of stressors by maintenance of homeostasis. These responses can occur at the tissue level, which includes the mobilization of energy substrates. Depending on the intensity of the stressor, animals may be unable to tolerate the ensuing changes and exhibit reactions such as inhibition of growth, reproduction, or immune response (Lima et al. 2006).

Although there had been research on exposure of frogs to the herbicides such as atrazine, glyphosate, and quinclorac, our investigations have focused on behavioral effects, morphological deformities, changes in the developmental stages, and metabolic alterations; however, we were unable to find any similar research involving quinclorac.

Conclusion

The realistic concentrations of atrazine, glyphosate, and quinclorac used in this study had no effect on mortality, weight gain, or size in *L. catesbeianus* tadpoles exposed to the herbicides for 7 days. However, even using concentrations below the legal limits, the herbicides induced significant alterations in biochemical parameters, mainly reductions in the levels of glycogen, triglycerides, and proteins, and an increase in levels of lipid peroxidation in all organs studied, which are expected to have longer term effects. The intensity of the observed responses was dependent on the studied organ and herbicide to which the animals were exposed.

The use of energy reserves to maintain homeostasis and the survival of animals may influence other biological parameters, such as development and reproductive success. It is important to stress that the responses found in the present study and the aforementioned studies may differ depending on the species of animal, concentration of herbicide, and duration of exposure to the contaminant.

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