Ecotoxicol. Environ. Contam.,v. 17, n. 1, 2022, 70-81
doi: 10.5132/eec.2022.01.07

Original Research

A preliminary approach to the Impact of a commercial formulation of glyphosate (Roundup®) in ecologically relevant concentrations on *Pseudis minuta* tadpoles

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Received April 24, 2022; Accept June 10, 2022

Abstract

Freshwater natural environments are suffering with human impacts and economic activities. Agriculture is one of the most environmental impactful activities that uses several toxic agents to ecosystems, polluting and poisoning non-target species. The goal of this study was to evaluate the Brazilian most utilized herbicide impacts in larval fase of an exclusively aquatic anuran species. We collected *Pseudis minuta* eggs mass in a Butiazal reservation, inside a soy and rice farm. In lab, we exposed 25 hatched tadpoles to 65µg/L, 250µg/L and 500µg/L of glyphosate and we had a control group that was kept in clean water. After exposition (seven days), we analyzed growing and morphometric patterns, oxidative balance (Superoxide Dismutase, Catalase and lipoperoxidation levels) and biotransformation (Glutathione S-transferase) parameters. We have not observed significatively differences about morphometric parameters, however, tadpoles grew above the expected in concentration 65 µg/L. There were not significatively differences between any oxidative balance marker, suggesting a tolerance or resistance level of this species. We do not discard this possibility because glyphosate is the most used pesticide in Rio Grande do Sul and in Brazil. It is possible that *Pseudis minuta*, in this developmental stage (larval), doesn't configure a good sentinel model organism. This work is a preliminary approach given the low number of tadpoles obtained from egg mass and consequently low number of replicates of exposure concentrations. Thus, future experiments should be designed to confirm these findings. The use of this type of assay may stimulate the development of new studies to assess the toxic risk of xenobiotics.

Key Words: Amphibians; Condition Factor; Herbicide; Oxidative Balance; Pollution; Resistance

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INTRODUCTION

What happens if all amphibians in the world disappear? According to West (2018), Amphibians are important not only for nature itself, but also for human population. As amphibians, especially Anurans, are generalists, they prey all kinds of insects, maintaining their population (West, 2018). That means, if all Amphibians disappear, possibly, there will be a substantial increase in insects populations from all types, including mosquitoes in all their stages of development and which are transmitters of many diseases such as Dengue, Leishmaniasis, Zika, Chikungunya, Malaria, Yellow Fever, or those transmitted by flies, such as Sleeping Sickness, but also diseases transmitted to large animals in agriculture, and which generate great economic losses in this sector. Therefore, a mass extinction of amphibians leads to an increase in diseases, like many zoonoses and to an economic loss in agricultural sector (West, 2018). Despite those direct harms in human health, a great lost in amphibians' population would also injury trophic food chain, generating an impact on ecosystems and its complexity. Amphibians occupy many trophic niches, they can be planktivory or carnivore, but they are also preyed upon by many animals, such as reptiles, birds, fish, and even many communities of human beings (Hopkins, 2007; West, 2018). Which means that if amphibians disappear it is possible to have a lost in biodiversity, as well as in energy and nutrition transfer throughout food chain (Hopkins, 2007). Moreover, several amphibians produce important peptides for pharmaceutical industry and the extinction of (still) unknown species also extinguishes possible important drugs (West, 2018).

In this context, the alarming scenario of amphibian's rapid decline stands out, whose reports, debates and concerns extend back to early 1970s (Stuart et al. 2004). What has been observed is that almost 50% of amphibian species described around the world are experiencing some level of population decline, and estimates suggest that about 160 species may already have been extinct (Baillie et al., 2004; Stuart et al., 2004; AmphibiaWeb, 2021).

It is assumed that there is no primary cause responsible for this great global decline, but a series of factors that interact with each other and act synergistically on these organisms (Mann et al., 2009; Hayes et al., 2010; Hopkins, 2007; Beasley, 2020). Among various factors that threaten amphibious biodiversity, advances in intensive agricultural practices may represent one of the disturbances with greatest impact on populations, since they configure activities that most use and convert proportions of land for planting, characterizing fragmentation and degradation of habitats, as well as being correlated with (often excessive and indiscriminate) use of pesticides, constituting a significant source of environmental contamination (Davidson, 2004; Hopkins, 2007; Mann et al., 2009). In addition, traditional agriculture has a great impact on aquatic systems, since it is the world's largest user of water resources, both surface and underground, as well as the large application of pesticides and fertilizers represent a high risk of contamination (Romagnoli, 2018; Shiklomanov and Rodda, 2004).

Although these substances benefit food production, many of them are placed directly in aquatic environments, or reach these locations, and then pollute, causing harm to human health and wildlife (Horak et al., 2021). Because of this aquatic pollution, organisms are exposed to those substances through food and/or through their surface areas (skin, gills) (Horak et al., 2021), which can generate bioaccumulation and biomagnification, reaching a very large number of individuals and even affecting terrestrial organisms (Katagi, 2010).

Amphibians have peculiar characteristics that make them more susceptible to pollutants than other groups of animals, such as their permeable and highly vascularized skin, the potential bioaccumulation of contaminants, their climate-sensitive reproductive cycle, and the fact that many species live, throughout its life span, both in aquatic and terrestrial environments (Costa et al., 2017; Degarady and Halbrook, 2006; Meredith et al., 2016). According to Hua (2014), amphibians, however, can plastically respond to various environmental stressors by altering their physiology, behavior, and morphology. These responses, nevertheless, will depend on the organism and of the pollutant and the exposure time (Billet and Hoverman, 2020).

In this context, *Pseudis minuta*, Günter 1858, is an amphibian specie of Hylidae family, which can be considered particularly susceptible to aquatic pollutants, since it spends a large part of its life in this environment, being considered exclusive to this system, and lives, preferably, in more anthropic places (Huckembeck et al., 2012; Rodrigues et al., 2008). These animals have the most generalized reproductive mode, that is, eggs are laid in lentic environments and larvae develop there (Melchiors et al., 2013). *P. minuta* spends much of its life floating in water waiting for its prey, being especially exposed to aquatic pollution, being abundant in a variety of aquatic environments of the Pampa domain. Huckembeck et al. (2013) examined the spatial distribution pattern of amphibians in terms of microhabitats and the influence of abiotic factors (relative humidity, rainfall, and temperature) on seasonal fluctuations in the abundance of this species. The species showed seasonal fluctuation in abundance, and it was most abundant in months with higher temperatures (spring-summer). Among the abiotic parameters analyzed, only the monthly mean temperature showed a significant correlation ($p < 0.05$; $r = 0.67$) with the abundance of *P. minuta* (Huckembeck et al., 2013).

It is in this place that the Pampa, a specific biome in south of South America, occurs and which contains a unique ecosystem, called Butiazal. Butiazal is composed of native palm trees of the *Butia* genus and performs several extremely necessary ecological functions that are interdependent with the large southern grasslands (Sosinki et al., 2019). They are protected by law in Brazil, as they are essential for conservation of grassland ecosystems, as they provide gene flow, seed dispersal, animal displacement, recolonization of degraded areas, etc. Unfortunately, like amphibians, this system is in decline, mainly due to the advance of agribusiness. Thus, bearing in mind that amphibians are fundamental to ecosystem

balance, their decline can portray an important indicator of the real threat that these activities can pose to Butiazal and other natural systems (AmphibiaWeb, 2021; Hopkins, 2007).

Among the ten most used pesticides in Brazil, glyphosate leads the ranking of the Brazilian Institute for the Environment of Renewable Natural Resources (IBAMA), with 217,590 tons of this substance being sold throughout the country (IBAMA, 2020a), and Rio Grande do Sul, the state where *P. minuta* occurs, according to the same institute, is the second state that most uses this herbicide, configuring, in 2019, 31.4 thousand tons of this active ingredient sold in the state (IBAMA, 2020b).

Glyphosate is more popular for one of its commercial formulations, Bayer's Roundup®. Known for being a defoliant that acts to control post-emergent weeds, this herbicide is rarely used without adjuvants, which allow an increase in its activity, promoting its toxicity. However, these adjuvants are considered inert diluents because they are not seen as directly responsible for glyphosate activity as a pesticide (Annett et al., 2014). Even so, the adjuvants of each commercial formulation are confidential even to regulatory agencies (Mesnage et al., 2014). al., 2015). Concentration of glyphosate in spray, which can be sprayed by various ground or aerial machinery, or applied by direct jet, will depend on the plant to be exterminated.

In Brazilian legislation, glyphosate is allowed in concentrations of up to 65µg/L in Class 1 Freshwater natural environments, that is, water intended for human consumption, protection of aquatic communities, primary contact recreation, such as swimming, water skiing and diving, according to the resolution of National Council for the Environment (CONAMA) nº 375, of 2005 (CONAMA, 2005). It is worth to note that, at the national level, Ministry of Health Ordinance No. 2,914, of December 12, 2011, which provides for water quality control procedures for human consumption standards, establishes a limit of 500µg/L the sum of glyphosate and AMPA in drinking water (BRASIL, 2011; FIORI et al., 2018).

Despite being designed to exterminate weeds in crops, several studies scientifically prove this pesticide is not safe for amphibian species, especially in the aquatic phase, with teratogenic effects being identified in *Xenopus laevis* embryos (Bonfanti et al., 2018), genotoxic and mutagenic effects in *Dendropsophus minutus* tadpoles (Carvalho et al., 2018), histopathological lesions in *Leptodactylus latrans* tadpoles (Bach et al., 2018) and delays in oocyte maturation in *Xenopus laevis* tadpoles (Slaby et al., 2018). al., 2019). In addition, effects of glyphosate on tadpoles may also induce changes in proportions of energy and structural reserves, such as glycogen, total lipids and proteins, as well as in oxidative balance, such as increased production of Reactive Oxygen Species (ROS) (Coltro et al., 2017; Dornelles and Oliveira, 2016, 2014; Gripp et al., 2017; Silva et al., 2020; Wilkens et al., 2019), leading to alterations of tadpole anti-predation responses, with potential negative consequence for its population (Browne and Moore, 2014).

The objective of this work was to understand effects of a commercial formulation of glyphosate on oxidative balance and body condition in *Pseudis minuta* tadpoles, exposed to sublethal and legally relevant concentrations, to generate information for society and, with that, obtain subsidies for decision-making, by the competent government, regarding the use and release of these chemicals in native biota.

2 MATERIALS E METHODS

All gathering and experimental procedures were approved in accordance with Brazilian laws, having been authorized upon obtaining the following licenses: Chico Mendes Institute for Biodiversity Conservation (ICMBio: nº. 71255-2); National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SisGEn- nº. A321D14) and Ethics Committee in the Use of Animals (CEUA: nº 9480).

2.1. Collection, Cultivation and Exhibition

In early September 2019 (pre-planting) we collected egg masses from about 60 adults between male and female *Pseudis minuta*, which spawned approximately 300 eggs over three consecutive nights. Adults were collected using the active search method at different points in an area of native Butiazal, located in Tapes (-30.516952˚, -51.378308˚), Rio Grande do Sul, Brazil. The gathering site belongs to a private farm divided into two large areas: a farming area, where soybeans and irrigated rice are planted alternately; and a conservation area where the presence of *Butia odorata* predominates, composing a native Butiazal with about 750 hectares of extension, where only cattle have access under controlled management (Figure 1).

Figure 1 Satellite image of adult's males and females' collection sites. Google Earth Image.

We placed up to two pairs (males and females) in gathering bags filled with local water and air. The couples remained in these conditions for two consecutive nights for spawning. In the following morning we placed all eggs found in plastic containers, containing water from the breeder collection site under constant aeration. During adults gathering, at the reproductive sites, we verified the abiotic parameters (Table 1) using a specific multiparameter probe (AK87), so that they could be used as a basis for the cultivation of eggs and tadpoles in the laboratory.

Table 1 Abiotic parameters measured through multiparameter inside three *Pseudis minuta* reproductive sites. Values are represented by three expedition days media ± standard error.

Sites	pH	Dissolved O2 (mg/L)	Water conductivities (µs/200µs)	Water temperature (˚C)	Air temperature (˚C)
1	6.93 ± 0.05	7 ± 0.89	20.6 ± 3.9	19.2 ± 2.19	19.4 ± 3.15
2	6.44 ± 0.46	6.83± 2.29	39.9 ± 11.34	19.4 ± 2.01	20.1 ± 1.28
3	6.65 ± 0.21	6.66± 1.70	32.9 ± 5.71	20.6 ± 1.55	20.23 ± 3.07

After the three days, we transported the eggs to the Center for Biological Models (CEMBE) at the Pontifical Catholic University of Rio Grande do Sul. We accommodated them all together in a single 5L aquarium with dechlorinated water and some of the water from the collection site. After five days at CEMBE, 28 tadpoles hatched from the eggs and we left them in a separated aquarium to develop until they reached Gosner stage 25, when they begin to feed (Gosner, 1960). The rest of the eggs showed development of fungi, probably coming from water collection site, being discarded. After 15 days in the laboratory, three subjects died (21%) and 25 animals (78%) reached Gosner stage 25. So, our final n was 25 tadpoles.

We randomly separated the animals into four aquariums of 5L, with 7 animals for the control and 6 for each one of the concentrations of glyphosate in commercial formulation (Roundup® Original). The culture room was maintained at a controlled temperature of 20˚C ±2˚C, circadian cycle 12h light/12h dark. We kept the aquariums under constant aeration and monitored the pH levels (ranging from 6.6 to 7.2) and ammonia daily. We quantified ammonia using a commercial kit (Labcon®) and during pre-exposure, every time the levels exceeded 2.0 mg/L, we changed part of the aquarium water. We fed tadpoles fish food (38% crude protein) once a day, considering 5% of the average biomass present in

each aquarium. After 26 days in laboratory, we weighed the animals on a precision scale (0.001g) and measured them with a digital caliper (0.01 mm).

After 10 days of acclimatization, counting from the moment they reached Gosner's stage 25, we exposed the animals to three different concentrations of the commercial formulation Roundup® Original, in which the active ingredient is glyphosate and which contains the following specifications: isopropylamine and n-(phosphonomethyl) glycine in proportion of 480g/L (48% m/v), acid equivalent of N-(phosphonomethyl) glycine (Glyphosate) in proportion of 360g.L^{-1} (36% m/v) and inert ingredients in a proportion of 684 g.L^{-1} (68.4% w/v). Finally, we established the concentrations considering the maximum allowed by the National Council for the Environment (CONAMA) for class 1 freshwater (CONAMA, 2005) and the concentration found in freshwater environments in the region where the place of collection, municipality of Tapes (Peruzzo et al., 2008).

For the preparation of the stock solution, the concentration of active ingredient described in the package insert of the commercial formulation was observed to calculate a necessary volume, to reach a known concentration (1000 mg.L^{-1}) and, from this, the nominal concentrations were calculated for each aquarium volume. Thus, the animals were exposed to 65µg.L^{-1}, 250µg.L^{-1} and 500µg.L^{-1} of glyphosate (Roundup® Original).

To confirm the nominal concentration of active ingredient added to the aquarium water, glyphosate was quantified in the stock solution (1000 mg.L^{-1}) before mixing an aliquot of it into the aquarium water. Ion chromatography was used, according to the methodology proposed by Marques et al. (2009) in which fluoride (F-), chloride (Cl-), nitrate (NO3-), phosphate (PO4-3) and sulfate (SO4-2) anions were determined simultaneously with glyphosate (Marques et al., 2009). All anions were below the maximum allowed by Brazilian legislation for surface water, the concentration of glyphosate in the mother solution was 1253 mg.L^{-1}. After 7 days of exposure, we cryoeuthanized, weighed and measured the animals from each group, including the control, and stored them at -80˚C until biochemical analysis.

2.2. Condition factors

The condition factor Kn provides information about recent dietary conditions and can be affected by several factors, both biotic and abiotic. This quantitative parameter makes it possible to compare populations that live under different environmental and feeding conditions by providing data on the physiological state of the animals (Gomiero et al., 2008). Although the Fulton condition factor ($K = W/L^3$) is widely used in fish, it can lead to distortions in the results, since it assumes isometry (b= 3). Thus, Kn was estimated from the following equation:

$$Kn = W/L^b$$

Which W is the individual's total mass, L is the total length and b is the fit coefficient of the weight/length ratio equation ($y = a.x^b$). From the result of b, we were able to calculate not only the Kn, but also the relationship between the observed and expected body mass value (K). For K, we used as "standard population" the measurements of all animals before exposure.

2.3. Oxidative Balance

We homogenized animals using an ultra-turrax, in phosphate buffer (20 mM), added potassium chloride (140 mM) and a protease inhibitor (PMSF at 1 mM), we centrifuged homogenate at 6000 rpm for 10 min. at 4°C. Afterwards, we aliquoted supernatant into four eppendorf tubes and frozen at -80ºC for Lipoperoxidation (TBARS) analyzes, Superoxide Dismutase (SOD), Catalase (CAT) and Glutathione S-Transferase (GST) (Coltro et al., 2017; Wilkens et al., 2019). Concentration of total proteins in the homogenate of tadpoles was quantified using Kit BioTécnica, which has as active principle the copper ion reaction with the peptide bonds of serum proteins, forming a purple liquid that turns read at 545 nm; this result was used for standardization of oxidative parameters.

Lipid peroxidation was quantified using the method described by Buege and Aust (1978), in which biotic material is incubated in an acidic medium, heated to 100°C in the presence of thiobarbituric acid (TBA) (Buege and Aust, 1978). Condensation of thiobarbituric acid reactive substances (TBARS) forms products that can be measured by a spectrophotometer (CARY 3E-UV-Visible Spectrophotometer VARIAN), in visible light at 532 nm (Lima and Abdalla, 2001). Concentrations were expressed as µmoles TBARS.mg $protein^{-1}$.

We measured total SOD using Boveris et al. (1982) method, which measures enzyme reaction at three different sample concentrations by an indirect pathway, in which the enzyme inhibits reaction of superoxide radical with epinephrine in a known time interval. This reaction changes medium color as the seconds pass, thus being measured by spectrophotometry at 480 nm (Boveris and Cadenas, 1982). Quantification of SOD activity was expressed in units of SOD.mg of $protein^{-1}$.

CAT activity was quantified by enzymatic kinetics using Boveris and Cadenas (1982) method, which measures consumption of hydrogen peroxide offered in a known time (Boveris and Cadenas, 1982). This reaction is measured at 240 nm. For results expression, sample proteins were quantified using a commercial kit (LabTest) and results were expressed in pmoles CAT.mg $protein^{-1}.min^{-1}$.

GST activity was determined in homogenate supernatant by enzymatic kinetics, in a reaction quantified by spectrophotometry at 340 nm, as described by Boyland and Chasseaud (1969). In this reaction, reduced glutathione (GSH) conjugates with 1-chloro 2,4dinitrobenzene (CDNB) proportionally to conjugated compound production rate,

generating a yellowish color (Boyland and Chasseaud, 1969). GST activity was expressed as µmoles of CNDB.min^{-1}.mg protein^{-1} conjugate.

2.4. Statistics

We tested data distribution obtained through Shapiro Wilk (due to sample n less than 30). Afterwards, we used Kruskal-Wallis test with Lillieferos significance correlation, for independent samples, followed by Pairwise. Significance level adopted for this study was 5% with Bonferroni correction. All statistical tests were performed using SPSS program, version 26 (IBM 2019).

3. RESULTS AND DISCUSSION

It should be noted that during the cultivation of the egg mass an expressive number of eggs sniffled, preventing the hatching of the animals. This fact may be related to the mixing of water from the collection site with dechlorinated water. Thus, our final number of tadpoles that reached Gosner stage 25 was only 25 animals, which limited the number of replicates per concentration, which determines a preliminary character to this set of results. After tadpoles reached Gosner stage 25, we did not observe mortality among animals either during acclimatization period (10 days) or during exposure (7 days). Other studies with tadpoles of *Rana catesbeiana* (Dornelles and Oliveira, 2014; Wilkens et al., 2019) also showed none or very low mortality (1.66%) after seven days of exposure to a commercial formulation containing glyphosate in strips. Concentration like the one used in this study.

We did not observe significant differences for body mass and length between experimental groups, nor between exposure control and exposed animals (Figure 2A and B). However, at the end of experimental period, all animals showed an increase in length and body mass when compared to animals before exposure. This pattern suggests a greater feeding activity and thus, allocation of part of the nutrients for growth maintenance and another part for survival. This is reinforced when we analyze values found in lowest concentration of herbicide (65µg.L^{-1} of glyphosate) that seems to allow tadpoles a greater investment in growth, while in the other exposed groups (250µg.L^{-1} and 500 µg.L^{-1} of) we observed a predominance of this investment in survival. It is well known that the cost of resistance to a toxic compound implies increased energy demand to repair adverse effects caused by stress (Wang at el. 2019). Dornelles and Oliveira (2014) also observed an increase in body mass and total length of bullfrog tadpoles exposed for seven days to concentrations of 36 to 144 µg.L^{-1} of glyphosate; however, percentages of increase verified in these groups were smaller than those of the control group.

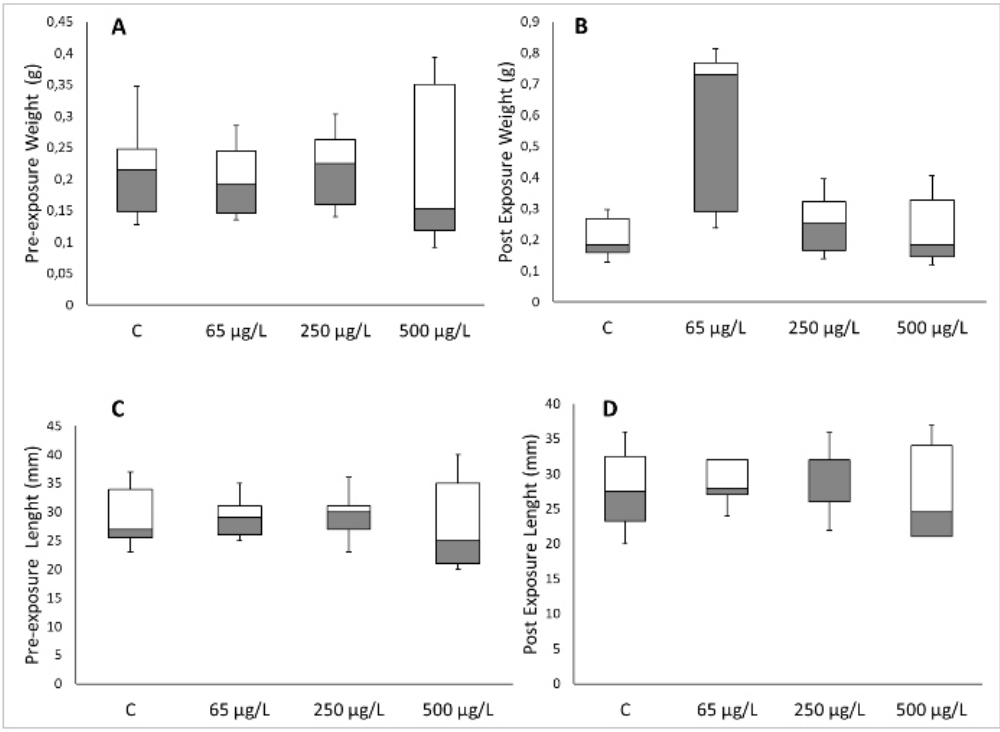

Figure 2 Weight and length of Pseudis minuta tadpoles before exposure (Pre-exposure: A and C) and after seven days of exposure (Post exposure: B and D) to different glyphosate concentrations. Vertical bars represent standard deviation, and absence of letters on the bars denotes the absence of significant differences.

Estimated weight-length ratio for *Pseudis minuta* tadpoles was W= 0.0006.L$^{1.7462}$. Thus, the value of b coefficient was 1.7462, indicating negative allometric growth, that is, weight increases more slowly than animal length (Figure 3A). From this equation, body condition factors K and Kn were calculated (Figure 3B and C, respectively). These factors present the same

variation pattern, with a significant increase in these markers' values (p= 0.028 and p= 0.044, respectively) in tadpoles exposed to 65 µg.L^{-1} of glyphosate, when we compared them to control group animals, as well as to the acclimatization period. This increase in K and Kn values again allows us to suggest an increase in food intake due to higher energy expenditure to metabolize the herbicide. Aquatic pollutants such as pesticides can affect several aspects of animal behavior, including foraging behavior, both stimulating and inhibiting (Pavlov et al., 1992; Semlitsch et al., 1995).

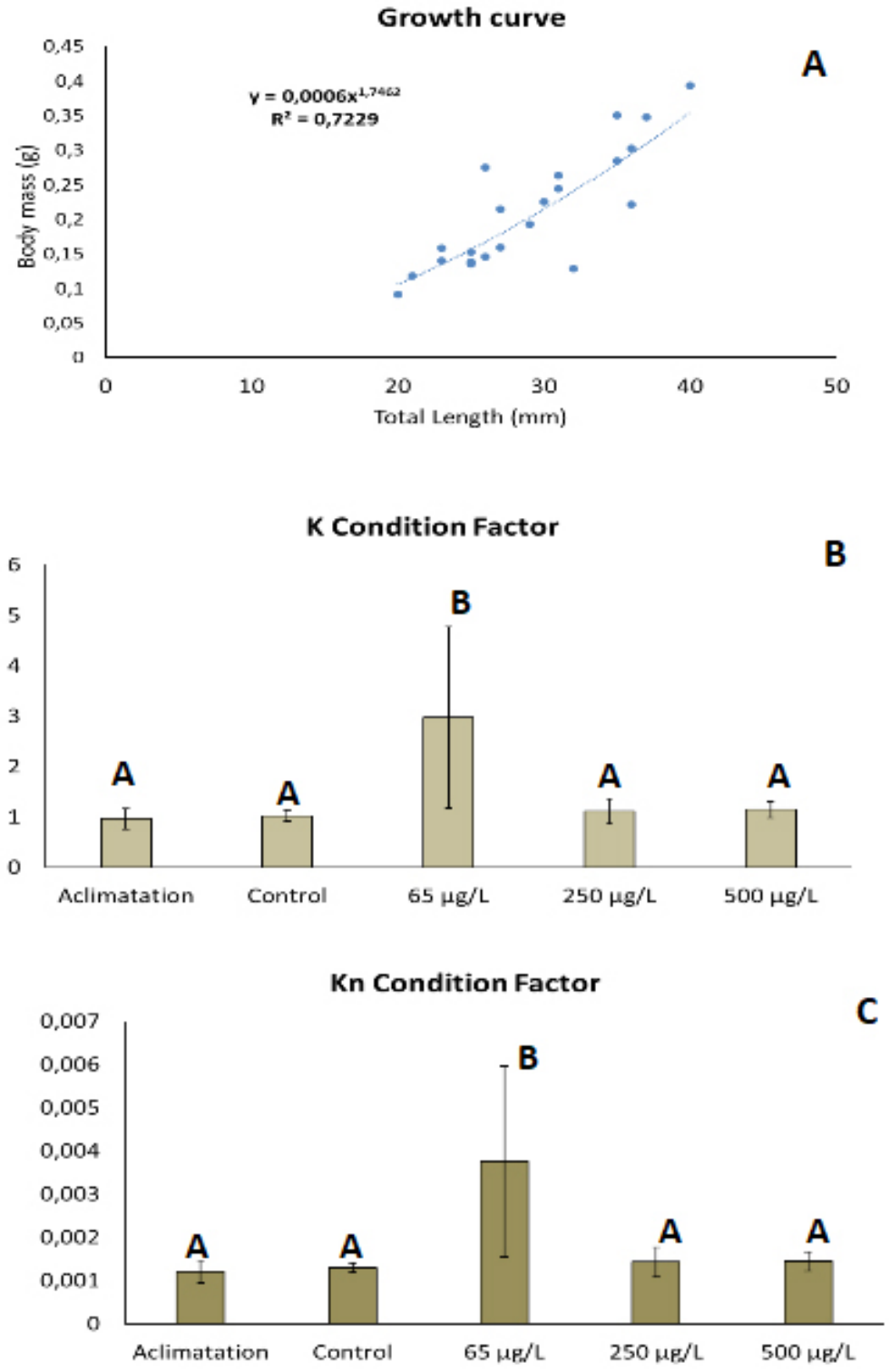

Figure 3 Corporal and growth condition factors K and Kn comparing animals before and after exposition and between exposition groups. Vertical lines represent standard error of each group, and different letters represent significant differences (p< 0,05).

Wilkens et al. (2019) attributes body mass gain and increased Fulton K index in bullfrog tadpoles exposed to glyphosate (230 µg.L^{-1}) to increased feeding activity, management of metabolism, and use of reserves to ensure growth and survival of exposed tadpoles. However, at higher glyphosate concentrations there is no increase in growth (mass or length) when compared to control. This suggests that exposure to xenobiotic possibly led to a greater allocation of energy reserves to support increase in biotransformation pathways and antioxidant defenses, making it impossible for animals to maintain energy investment for growth.

Yousefi et al. (2021) demonstrated that carp (*Cyprinus carpio*) have a developmental impairment when exposed to glyphosate at concentrations ranging from 0.25 to 2 mg.L^{-1}, over 96 h. Authors also conclude that glyphosate affects lipid metabolism in these animals, reducing cholesterol, triglycerides, LDL and HDL. In addition, glyphosate increased concentration of aminotransferases in blood plasma, which indicates damage to organs such as liver and kidneys (Yousefi et al., 2021). Dornelles and Oliveira (2014) observed a drastic decrease in glycogen triglycerides and protein levels in liver and muscle of bullfrogs exposed for seven days to glyphosate (36, 72 and 144 µg.L^{-1}). Likewise, Da Silva et al. (2020), showed a decrease in glycogen when *Melanophriniscus admirabilis* tadpoles were exposed for 96 h to 234 µg.L^{-1} of glyphosate.

This trade off was demonstrated by Rowe *et al.* (1998), after observing polluted environment, where tadpoles compromised growth and development. Authors hypothesized that this energy, normally directed towards growth and development, may have been allocated to sustain the energy costs of survival in face of exposure to a pesticide, which would result in smaller individuals (Bridges, 2000; Rowe et al., 1998). Despite this, Bridges et al. (2000) found no effect of an insecticide, carbaryl, on tadpole metamorphosis; however, if population constantly affected by pesticide, starts to invest energy of growth for survival, resulting in smaller individuals, consequently, adult females would lay fewer eggs. Thus, a reduction in next generation would cause a decrease in population (Bridges, 2000).

Regarding oxidative balance, we did not verify significant differences in any of analyzed biomarkers (SOD, CAT, GST, and TBARS), when compared to control, nor between exposed groups (Figure 4). However, we cannot rule out that other components of antioxidant system, both non-enzymatic component (e.g., glutathione, melatonin, and vitamins) and enzymatic (e.g., glutathione peroxidase), are mobilized to prevent the formation of ROS, high levels of lipoperoxidation and carbonyl proteins. Reichert (2021) also did not observe significant changes in oxidative balance markers (SOD, CAT, TBARS, GST, and Carbonyl Proteins) in *Rhinella icterica* tadpoles exposed to glyphosate concentrations (100, 250 and 500 µg/L), but this author verified a change in metamorphosis time of exposed animals.

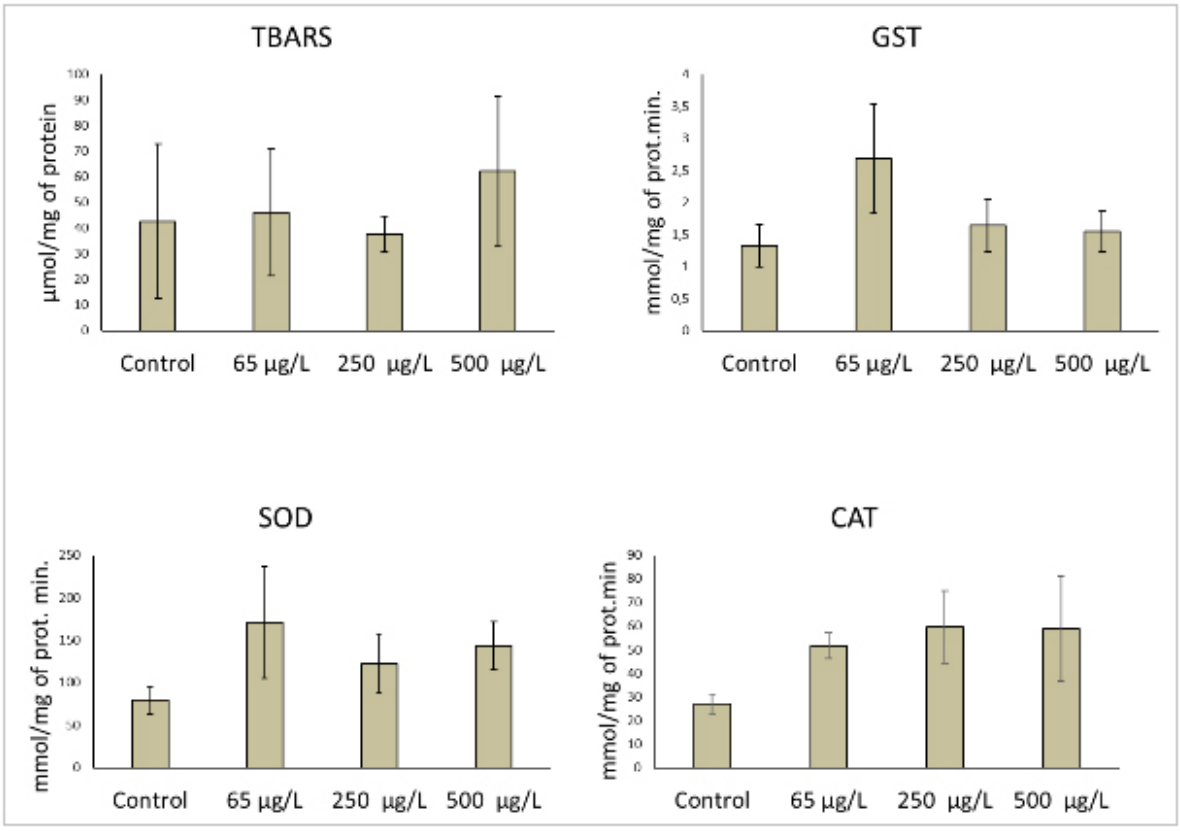

Figure 4 P. minuta oxidative balance exposed to three Roundup® Original different concentrations. Vertical lines represent standard error to each group, and absence of letters on the bars denotes the absence of significant differences.

In addition, the egg collection site was close to a farm that uses glyphosate in its plantations (personal communication). Therefore, frequent exposure to this herbicide for several generations could confer a tolerance to this pesticide in animals. Therefore, the frequent exposure to this herbicide for several generations, could grant a tolerance to this chemichals in animals. There is some evidence that populations that are frequently exposed to pesticides can acquire tolerance through natural selection (Billet and Hoverman, 2020). Tiffon (2018) refers to environmental exposures that cause changes in DNA conformation as "environmental epigenetics" and this change in genetic material conformation could give organisms better aptitude to react positively to a toxic agent and still pass this change on to their descendants (Vandegehuchte and Janssen, 2014).

Furthermore, plasticity of an organism, which is the ability of a single genotype to produce multiple phenotypes under different environmental conditions (Billet and Hoverman, 2020), can also help organism to exhibit tolerance and/or resistance. These phenotypic changes include morphological, behavioral, and physiological changes (Billet and Hoverman, 2020). It is adversity and environment that will shape what organisms are, by virtually impacting epigenome and health of individual (Tiffon, 2018). Although we have made a lot of progress trying to understand ecological consequences of pesticide exposure, evolutionary consequences are poorly understood (Cothran et al., 2013). Moreover, resistance in target species of chemical is known, however, little is known about resistance in non-target organisms (Cothran et al., 2013; Hua et al., 2014, 2015). Therefore, it is important to consider that xenobiotics can induce critical genetic variations, which can encode adaptive traits in a single generation and allow population to persist (Hua et al., 2015), or not, and can also induce disease (Vandegehuchte and Janssen, 2014).

When an animal faces an environmental challenge, it needs to maximize its survival and adapt under adverse conditions. Natural selection has given organisms this phenotypic plasticity, where a single genotype can produce different phenotypes under challenging environmental conditions, considering both: those pertinent to life cycle of species and unexpected ones, such as the presence of a pollutant (Auld et al., 2010). In addition to plasticity, organisms rely on epigenetics to overcome environmental adversities (Tiffon, 2018; Vandegehuchte and Janssen, 2014). Thus, resistance, or tolerance, can be defined as a reduction in negative impact of glyphosate on amphibians, in *P. minuta* case, because of genetic changes in population (Dunlop et al., 2018). Considering that, this species inhabits anthropogenic locations and close to crops throughout Rio Grande do Sul, and as already told, the second state that most uses glyphosate in the entire country, hence, we can suggest the presence of tolerance of this organism against this pesticide.

Other authors have reported tolerance of *Lithobates sylvaticus* tadpoles to insecticides, such as carbaryl and chlorpirifos, as well as to the herbicide glyphosate, testing a variety of concentrations of these pesticides and combinations of stressors, such as pesticide + predator, etc. (Billet and Hoverman, 2020; Cothran et al., 2013; Hua et al., 2015, 2014). Hua et al. (2014) analyzed carbaryl-induced tolerance to other different insecticides such as malathion, chlorpyrifos, cypermethrin and permethrin, of which the first two have the same mechanism of action as carbaryl and the last two work differently. Author found cross-tolerance between pesticides that share the same mechanism of action, as carbaryl induced tolerance in *L. sylvaticus* embryos, and tadpoles to malathion and cyperemethrin. On the other hand, Cothran et al. (2013) found no evidence for increased resistance associated with living costs in environmental context when *L. sylvaticus* tadpoles were exposed to chlorpyrifos and glyphosate. But authors observed that when closer to a crop, as the presence of predators, the greater the resistance to both herbicides, which led to a lengthening of larval development period and slower metamorphosis (Cothran et al., 2013).

This tolerance can occur through organism plasticity itself, but it can also be passed from one generation to another through epigenetics, or even as defenses passed from the parent to offspring, through antioxidant molecules acquired from food or metabolism from the first generation. In fact, Da Silva et al. (2020) who exposed tadpoles of *Melanophriniscus admirabilis* to different concentrations of sulfentrazone and glyphosate, observed a decrease in lipid peroxidation at highest concentrations of these herbicides and attributed this event to the possibility of previous resistance, due to contact with alkaloids acquired from the diet and which protect from the poisonous substances of this genus (Da Silva et al., 2020). Therefore, it is possible that exposure to toxic substances in most recent life stages leads to a strengthening of antioxidant system and, thus, a tolerance during larval development period. However, Figueró (2021) working with *P. minuta* adults collected at the same reproductive sites (conserved area) and at other sites inside crop area, observed an increase in nuclear anomalies (micronuclei and lobed nuclei) in erythrocyte cells; thus, evidencing an impact of pesticides used in soybean and irrigated rice crops on this stage (adults) of the life cycle.

4 CONCLUSION

This work is a preliminary approach given the low number of tadpoles obtained through the cultivation of egg masses. However, the use of this type of assay using eggs of native Brazilian species can stimulate the development of new studies to assess the toxic risk. There was no mortality during the experimental period, which indicates good cultivation conditions. This also indicates sublethal herbicides concentration for these animals at this life cycle stage. In addition, we have an impact of glyphosate at lowest concentration tested on the K and Kn index, suggesting a modulation of this herbicide on foraging behavior. Considering lifestyle of *Pseudis minuta*, which is exclusively aquatic, and collection site, where animals lived in a conserved area, but close to a field of rotational cultivation of soybeans and

irrigated rice, we cannot rule out the possibility of tolerance of these organisms to glyphosate. It is important to have more studies to understand which evolutionary and physiological mechanisms lead to resistance and/or tolerance of some amphibians to pesticides.

ACKNOWLEDGEMENTS

We thank Fazenda São Miguel, in the person of Carmem Barros for all the support in the field, as well as Professor Dr. Alexandro Tozetti for supporting the collection of animals. This study was supported by the National Council for Scientific and Technological Development (CNPq) and for the financial support granted by Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS: 19/2551-0001818-5) to G.T.O.

Credit author statement N.M.A.W. and G.T.O. conceived the study; N.M.A. and G.C.K. collected the animals; N.M.A.W. and G.C.K. performed the biochemical analyzes and produced the results; N.M.A.W., G.C.K. and G.T.O. analyzed and produced the data.; N.M.A.W. and G.T.O. wrote the paper. All Authors revised the paper.

Conflicts of interest Authors declare that there are no conflicts of interest.

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