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Combined effects of temperature and clomazone (Gamit®) on oxidative stress responses and B-esterase activity of *Physalaemus nattereri* (Leiuperidae) and *Rhinella schneideri* (Bufonidae) tadpoles

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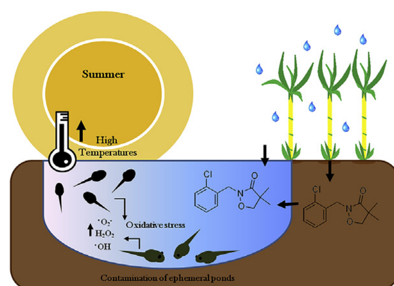
HIGHLIGHTS

- Clomazone effects at 28, 32 and 36 °C were evaluated in two tadpole species.
- Temperature enhanced oxidative stress in tadpoles exposed to clomazone.
- Carboxilesterase, but not acetylcholinesterase, was altered by clomazone.
- Neotropical tadpoles have different vulnerability to the heat stress and chemicals.
- Temperature effect was more evident than clomazone effects by the IBR analysis.

GRAPHICAL ABSTRACT



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ABSTRACT

Temperature is an important factor influencing the toxicity of chemicals in aquatic environments. Neotropical tadpoles experience large temperature fluctuations in their habitats and many species are distributed in areas impacted by agriculture. This study evaluated the effects caused by the exposure to clomazone (Gamit®) at different temperatures (28, 32 and 36 °C) on biochemical stress responses and esterase activities in *Physalaemus nattereri* and *Rhinella schneideri* tadpoles. Results evidenced that temperature modulates the effects of clomazone on biochemical response of tadpoles. Antioxidant enzymes, including catalase, superoxide dismutase (SOD), and glucose-6-phosphate dehydrogenase had their activities increased by clomazone in *P. nattereri* treated at higher temperatures. The biotransformation enzyme glutathione-*S*-transferase (GST) was also induced by clomazone at 32 and 36 °C. In *R. schneideri*, clomazone failed to alter antioxidant enzymes at 28 °C, but SOD and GST were increased by clomazone at higher temperatures after three days. All enzymes had their activities returned to the control levels after eight days in *R. schneideri*. Lipid peroxidation was induced in both species exposed to

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Oxidative stress
Esterase

clomazone at 32 and 36 °C, but not at 28 °C. Acetylcholinesterase was not sensitive to clomazone and temperature, while most treatments impaired carboxylesterase activity. Integrated biomarker response (IBR) was notably induced by temperature in both species, and a synergic effect of temperature and clomazone was mostly observed after three days of exposure. These findings imply that tadpoles from tropical areas may present differential responses in their physiological mechanism linked to antioxidant defense to deal with temperature fluctuations and agrochemicals presence in their habitats.

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1. Introduction

Temperature is an important variable that has direct impacts for aquatic ectotherms, such as amphibians. Changes on environmental temperature can alter behavior, metabolism, development, digestion and vision of amphibians, resulting in altered physiological performance in the environment (Murata and Yamauchi, 2005; Rome, 2007). Indeed, temperature can also interfere in the amphibian's responses triggered by environmental contaminants in natural systems (Freitas et al., 2017, 2016; Hooper et al., 2013; Noyes et al., 2009), and one of the current challenges in environmental toxicology is to understand how abiotic factors, such as temperature fluctuations, can interfere on the effects of chemical compounds in aquatic organisms.

Amphibians can experience large temperature fluctuations in their habitats, especially during the larval stages, in which they are restricted to small and ephemeral ponds, where the water occupies small volumes and then can be rapidly heated due to sun incidence (Wilbur, 1990). The process of water heating is also favored by deforestation and loss of vegetation in nearby areas of amphibian habitats. In tropical areas of the world, the water of these ponds can reach high temperatures (up to 40 °C) during the summer, which is the period of reproduction for many amphibian species (Freitas et al., 2016). Higher water temperature diminishes the availability of dissolved oxygen, accelerates ventilation and the metabolism, enhancing the oxygen demand of cold-blooded animals (Kaur et al., 2011), and possibly increasing the susceptibility to the effects of xenobiotics (Blaustein et al., 2010; Moe et al., 2013). Gradual change in temperature can be physiologically compensated by the organism, however a rapid change disturbs homeostasis and may become a stress (Kaur et al., 2011), especially in the presence of environmental pollutants.

In Brazil, agriculture has gradually invaded open areas used by amphibians for breeding (Da Silva et al., 2011). After intense periods of rain, soils of agriculture areas are flooded forming ephemeral ponds used by these animals to spawn. However, rainwater usually carries several agrochemicals, such as insecticides and herbicides, which leach from the soil to these water fractions, making them available to tadpoles (Bridges et al., 2004). Moreover, concentrations of agricultural compounds in these habitats are also usually higher than in other aquatic environments (e.g. rivers and lakes), due their smaller size. Considering these factors, the biological consequences caused by exposure to pesticides to anurans have a high potential to be significant (Mann et al., 2009).

The rapid expansion of sugarcane plantations due to the increased biofuel demand has contributed significantly to the use of pesticides (Kissmann, 1991) in Brazil. Clomazone (2-(2-clorofenil) metil-4-dimetil-3-isoxazolidinone) is an herbicide widely used in sugarcane crops, but also widely applied in rice, potato and cotton plantations. In 2009, clomazone ranked among the top ten herbicides most widely used in Brazil (IBAMA, 2010). Although very effective, clomazone causes extensive environmental contamination due to its high solubility in water (1100 mg.L^{-1}), besides a half-life that can reach 84 days (Zanella et al., 2002). This herbicide is often applied in sugarcane culture from September to March, corresponding to the spring/summer period of the year. This period coincides with the higher rain incidence in some areas, such as the northwest region of the Sao Paulo state, in which 36 species of anuran were registered (Provete et al., 2011). Therefore, the intensive use of clomazone in sugarcane during the reproductive period of anurans in the northwest of Sao Paulo State would represent a risk for their populations due to this herbicide runoff to the aquatic environment, especially considering higher temperatures influence recorded in this period of the year.

Environmental contaminants can lead organisms to oxidative stress, a condition that is also triggered by increases of temperature (Freitas and Almeida, 2016; Gripp et al., 2017; Madeira et al., 2013; Stefani Margarido et al., 2013; Vinagre et al., 2012). Studies have indicated that changes on environmental temperature can alter physiological stress response in aquatic organisms, and that ROS production is usually increased at higher temperatures as a consequence of an increased metabolism which leads to higher oxygen consumption (Almeida and Mascio, 2011; Freitas and Almeida, 2016). Moreover, it is also known that temperature can also modulate neurotoxicity of acetylcholinesterase (AChE) inhibitors (Beauvais et al., 2002; Durieux et al., 2011). Previous studies have shown that clomazone cause suppression of the antioxidant enzyme catalase (CAT) and enhance lipid peroxidation levels in fish (*Rhamdia quelen*) (Crestani et al., 2007), besides activating the activity of biotransformation enzyme glutathione-*S*-tranferase (GST) in *Prochilodus lineatus* (Pereira et al., 2013). Moreover, clomazone was also reported as an inhibitor of AChE in brain and muscles of *P. lineatus* (Pereira et al., 2013), suggesting a neurotoxic effect of this herbicide despite at relatively high concentration (10 mg.L^{-1}). Yet, the implication of temperature elevation on oxidative stress parameters and AChE inhibition caused by clomazone remains unknown, especially for amphibians. In fact, the toxic effects of clomazone for amphibians still need clarification, especially considering studies with field species. There is only a recent study showing that acute exposure (96 hrs) to clomazone changed the hepatic response such as, melanomacrophages contents, accumulation of eosinophils and occurrence of lipidosis in the hepatocytes in tadpoles of the American bullfrog (*Lithobates catesbeianus*) at environmental concentrations (0.5 mg.L^{-1}) (Oliveira et al., 2016).

Considering these points, this study aimed to investigate the effects of a commercial formulation of the herbicide clomazone (Gamit® 360CS) in two species of tadpoles, *Physalaemus nattereri* and *Rhinella schneideri,* under different thermal conditions (28, 32 and 36 °C). Both species have wide geographical distribution in Brazil, including sites of intense agriculture practice and sugar cane plantation (Provete et al., 2011). *P. nattereri* has the current population trend designed as decreasing and its major threats is the spread of intensive agriculture in the Cerrado biome. *R. schneideri* is generally a common and increasing species, although its range appears to be contracting in some areas from South America (Stuart

et al., 2008). We evaluated oxidative stress parameters, such as the antioxidant enzymes superoxide dismutase (SOD), CAT, glucose-6-phosphate dehydrogenase (G6PDH), and the levels of total glutathione (*t*GSH) and lipid peroxidation in whole organism. Biotransformation enzyme GST and activities of AChE and carboxylesterase (CbE) analyzes were also conducted in this study. An integrated approach was applied to show the overall and summarized effects of clomazone on tadpoles under different temperatures. Specifically, the different biomarker responses were combined into an integrated biomarker response (IBR) framework (Beliaeff and Burgeot, 2002). Considering that biochemical and physiological responses of organisms provide an early warning of contamination and thermal stress (Colin et al., 2015), we predict that the effects of clomazone and temperature will interact causing changes on individual biomarkers and IBR.

2. Methodology

2.1. Tadpoles' collection and acclimatation

We collected spawns of both species in temporary ponds in non-agricultural areas in the region of Sao Jose do Rio Preto, Sao Paulo, Brazil (20°47′07.05″ S, 49°02′42.09″ W). After hatching, larvae were acclimatized and maintained in the laboratory under ideal conditions of temperature, pH and oxygen (28 °C, pH 7.5–8.0) during four weeks until they reached stages 27–30 (Gosner, 1960). We collected both species under license n.18573-1, authorized by the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA).

2.2. Experimental design

2.2.1. Water heating

Aquariums (5 L) containing well water were immersed in three different tanks (100 L) in a "water bath" system. The tanks were partially filled until the water level reached the same level of the aquariums. Then, the tanks had their water heated to three different temperatures, 28, 32 or 36 °C, which were maintained by autoregulating thermostats. Temperature of the tanks and individual aquariums were recorded three times a day to observe possible variations during the experimental period. Temperature range selected in this study was based on field studies previously conducted by Freitas et al. (2016), which recorded water temperatures of natural ponds in which tadpoles of *P. nattereri* and *R. schneideri* are frequently found in Brazil.

2.2.2. Chemical exposure

Tadpoles of *P. nattereri* and *R. schneideri* were separately exposed to 0.01, 0.05 and 0.1 mg.L^{-1} of nominal concentrations of clomazone (2-[(2-chlorophenyl)methyl]-4,4-dimethyl-3-isoxazolidinone) from the commercial herbicide Gamit® 360 (Gamit 360 CS, 36% m:v, FMC Corporation, Philadelphia, EUA). Clomazone concentrations used in this experiment were based on those concentrations detected in areas of sugarcane and rice cultivation in Brazil (0.2–0.4 mg.L^{-1}) (Primel et al., 2005; Zanella et al., 2002). Tadpoles were exposed to each concentration at temperatures of 28, 32 and 36 °C during three and eight days. A control group containing no contaminants was provided for each temperature and experiment duration. Each experimental group consisted of five replicates (n = 5) containing eight tadpoles in each aquarium (n = 40 pseudoreplicates). The tadpoles were placed into the aquariums and the water temperature was gradually adjusted during the first 24 h to avoid thermal shock. Stock solutions were prepared by dissolving clomazone in water. Then, clomazone was added at nominal concentrations as described above. Aquariums were constantly aerated at a pH of 8.00 ± 0.26 and using a light-dark cycle of 12-12 h. Previous studies showed that clomazone concentrations are unchanged after four days in the water (Oliveira et al., 2016). Thus, the water and contaminant were renewed every two days to ensure the original concentrations of clomazone and also dissolved oxygen and ammonia. No significant variations on these parameters were observed among treatments during the experiments (dissolved oxygen 6.06–7.04 mg/L, pH 8.12–8.46). Animals were fed every two days with commercial food for tropical fish in minimum quantities (200 mg) avoiding accumulation in the aquariums. After three and eight days, the tadpoles were removed from the aquariums and euthanized by direct immersion in liquid nitrogen. All the procedures were performed in accordance with the Ethics Committee on Animal Use in Research of the Sao Paulo State University (CEUA IBILCE/UNESP No. 086/2013).

2.3. Analysis

2.3.1. Morphological analysis

Effects on growth, body mass gain (snout–vent length (SVL) and body weight) and development (Gosner, 1960) were evaluated individually in tadpoles of *P. nattereri* and *R. schneideri* after three and eight days of exposure at different temperatures.

2.3.2. Preparation of samples

For the enzymatic measurements, two tadpoles of each replicate (n = 10) were entirely and individually homogenized in a ratio of 1/4 (weight/volume) in a cold (4 °C) homogenization buffer (Tris-HCl 20 mM, EDTA 1 mM, DL-dithiothreitol 1 mM, sucrose 0.5 M, KCL 0.15 M, phenylmethylsulfonyl fluoride 1 mM), pH 7.4, and centrifuged for 30 min at 9000 g and 4 °C. The supernatant fraction was collected and centrifuged again for 1 h at 50.000 g. The second supernatant fraction was collected and stored at −80 °C for subsequent evaluations.

Lipid peroxidation was assessed by the measurements of MDA levels. For this analysis, other two tadpoles of each replicate (n = 10) were entirely homogenized in a ratio of 1:3 (w:v) in 0.1 M Tris-HCl buffer, pH 8.0. After homogenization, 300 μL of thiobarbituric acid (TBA, Sigma-Aldrich, Germany) diluted in 0.4% HCl were added to the sample. The mixture was heated at 90 °C for 40 min and the reaction product was extracted with 1.0 mL of n-butanol (Sigma-Aldrich, USA). After this procedure, the n-butanol extracted fraction was injected (10 μL) into a High-Performance Liquid Chromatography system (Shimadzu Corporation, Kyoto, Japan). Chromatogram was monitored during 10 min and peaks were identified and quantified using LAB Solutions 5.71 software (Shimadzu Corporation).

For *t*GSH analysis, two tadpoles of each replicate (n = 10) were entirely and individually homogenized in a ratio of 1/9 (weight/volume) in a cold (4 °C) solution of perchloric acid (PCA) 0.5 M and centrifuged during 5 min at 15.000 g and 4 °C. Supernatants were collected and neutralized with a solution of potassium phosphate 0.1 M, pH 7.0 and posteriorly centrifuged at 15.000 g during 2 min at 4 °C.

AChE and CbE enzymes were assessed in tadpoles entirely homogenized in a ratio 1:4 (weight/volume) in a cold (4 °C) solution of Tris HCl 0,1 mol.L^{-1} pH 8,0 and centrifuged at 10.000 g during 30 min at 4 °C. The enzymes were analyzed in the supernatant fraction.

2.3.3. Antioxidant enzymes

Analyses of CAT were performed in a Thermo Evolution 300 spectrophotometer with a dual beam and capacity for seven cuvettes. The activities of GST, G6PDH and SOD were performed on a Victor TM X3 microplate reader (Perkin ElmerR). The activity of all

enzymes was evaluated at the respective temperatures (28, 32 and 36 °C) at which the animals were subjected for the treatments.

CAT activity was measured using the method described by Beutler (1975), which monitors the rate of decomposition of hydrogen peroxide (H_2O_2) by the enzyme at 240 nm for 1 min. Specific activity was expressed as U mg of protein^{-1}. The assays were performed using Tris-HCl buffer (1 M, pH 8.0) with 5 mM EDTA and H_2O_2 as the substrate.

GST activity was measured using the method described by Keen et al. (1976), which monitors the formation of the conjugate of 1-chloride-2,4-dinitrobenzene (CDNB) with reduced glutathione (GSH) catalyzed by GST in the sample for 1 min at 340 nm. Specific activity was expressed as U.mg of protein^{-1}. The final volume of the assay was 110 μL, which contained a potassium phosphate buffer (0.2 M, pH 6.5), 1 mM CDNB and 1 mM GSH and the sample.

The enzymatic assay of G6PDH (Glock and McLean, 1953) measured the formation of nicotinamide adenine dinucleotide phosphate (NADPH) at 340 nm for 1 min. The assay consists of the reduction of $NADP^+$ to NADPH by the G6PDH using glucose-6-phosphate (G6P) as the substrate. The assay had a final volume of 205 μL, which contained Tris-HCl buffer (0.1 M, pH 7.4), MgCl2, NADP+, G6P and the sample. The reference blank did not contain G6P. Specific activity was expressed as mU.mg of protein^{-1}.

SOD activity was measured using SOD Assay Kit-WST (Sigma, Aldrich) by utilizing Dojindo's highly water-soluble tetrazolium salt, WST-1 (2-(4-Iodophenyl)- 3-(4-nitrophenyl)-5-(2,4-disulfophenyl)- 2Htetrazolium, monosodium salt) that produces a water-soluble formazan dye upon reduction with a superoxide anion. The rate of the reduction with O_2 is linearly related to the xanthine oxidase activity, and is inhibited by SOD. Since the absorbance at 440 nm is proportional to the amount of superoxide anion, the SOD activity as an inhibition activity was quantified by measuring the decrease in the color development at 440 nm. Specific activity was expressed as U.mg of protein^{-1}.

2.3.4. Determination of GSH levels

Total GSH (*t*GSH) contents were assessed by Tietze (1969) protocol, adapted by Sies (2003). Total amount of GSH was measured based on the reaction between GSH and DTNB (5,5′-Dithiobis(2-nitrobenzoic acid)), which produces the conjugate GS-TNB and the yellow TNB (5-thio-2-nitrobenzoic acid) anion that can be detected at 412 nm. The rate of TNB production is proportional to the GSH in the extract. Thus, the more GSH in the sample, the more TNB was formed, which was monitored at 412 nm during 2 min. A recycling reaction catalyzed by glutathione reductase in presence of NADPH was also coupled to the assay, regenerating 2GSH from the GSSG previously formed by the reaction with DTNB. This enables a continuous production of TNB, which is proportional to the total GSH (GSH + GSSG) in the sample. The μmol GSH g^{-1} of tissue in the sample was calculated based on a standard curve with known amounts of GSH, obtained in the assay by the change in absorbance per minute (ΔA). Analyses of GSH were performed in a Thermo Evolution 300 spectrophotometer with a dual beam and capacity for seven cuvettes.

2.3.5. Lipid peroxidation (MDA levels)

MDA levels were evaluated following the method described by Almeida (2003) using a high performance liquid chromatography (HPLC) system (ESA, USA) coupled to a UV–Vis detector (526, ESA, USA) to analyze the product formed between malonaldehyde (MDA) and TBA. The chromatographic column used in the analyzes was a Shimadzu C18 column (150 × 4.6 mm, 5 μm), using 50 mM potassium phosphate solution at pH 7.0 as the mobile phase, with 40% methanol, and at a flow rate of 1.0 mL/min.

2.3.6. Esterase activities

AChE and CbE activities were performed on a Victor TM X3 microplate reader (Perkin Elmer), following the methodology of Ellman et al. (1961). This method measures the formation of a thiol derivative produced by the enzyme action on the substrate, which reacts with the DTNB producing a yellow compound, which was monitored at 412 nm at 25 °C. We used acetylthiocholine as substrate for AChE analysis and the phenylthioacetate for CbE. Specific activities were expressed as U/mg protein^{-1}.

2.3.7. Protein quantification

Proteins were quantified using the Bradford (1976) with Coomassie Brilliant Blue G-250 in an acidic solution. The absorbance values were determined at 595 nm, and the results were compared to the analytical curve prepared with bovine serum albumin (BSA) as the standard.

2.3.8. Integrated biomarker response

We calculated the Integrated Biomarker Response (IBR) proposed by Beliaeff and Burgeot (2002) with modifications proposed by Sanchez et al. (2013), whose calculations are based on reference deviation concept (IBRv2, hereafter named as IBR). Tadpoles from the control group at 28 °C were considered as reference. IBR index is calculated for each sample and correspond to the sum of the standardized (z-score) absolute differences between the values of the individual biomarkers of a given sample in relation to the respective value found in the reference samples (Sanchez et al., 2013). Therefore, IBR (*sensu* Sanchez et al., 2013) correspond to changes (activation or inhibition) on the biomakers in relation to reference samples. IBR was calculated for each species at each exposure period, separately. As individual biomarkers were obtained for different individuals of each aquaria, our IBR calculation were done using aquaria as a sample ($n = 5$).

2.3.9. Data analysis

Statistical analyses were conducted using R version 2.11.1 (R Development Core Team, 2010) and STATISTICA (StatSoft). The normality and homogeneity of the data was checked by the Shapiro-Wilk and Levene's test, respectively. Logarithmic transformation [log (x+1)] was performed in those data that did not meet the assumptions of normality and homogeneity of variance. A two-way analysis of variance (ANOVA 2-way) followed by the Tukey post hoc test were used to identify statistical differences in antioxidant enzymes activity, MDA levels, esterase activities, IBR and effects on growth and weight gain. A Kruskal-Wallis of multiple comparisons test was used in those data not considered parametric even after the log-transformation. These tests were performed to evaluate the individual effects of temperature (28, 32 and 36 °C) and chemical treatments (clomazone - 0.01, 0.05 and 0.1 mg.L^{-1}) and the interaction effects between temperature and clomazone. Non-exposed tadpoles at 28 °C were assumed as the control group for comparison of interaction effects of temperature and chemical exposure, since the animals were acclimatized at this temperature and this group had no influence of either thermal stress or chemical exposure. The G-test (likelihood ratio test) with Yates's correction (Sokal, 1995) was performed to assess the effects of temperature and clomazone on tadpoles development. Values of $P < 0.05$ were considered as a reference to assign statistical significance (Zar, 1999).

3. Results

3.1. Effects on growth and development

We have not detected any effects of clomazone and temperature

treatments on *P. nattereri* and *R. schneideri* tadpoles' body weight and SVL for both three and eight days of exposure ($P > 0.05$) (Table S1 and S2). *R. schneideri* development was also unaffected by any treatment ($P > 0.05$) (Table S5 and S6). However, *P. nattereri* tadpoles exposed to 0.01 mg.L^{-1} of clomazone at 36 °C had their development accelerated when compared to the control group at 28 °C after eight days of exposure ($P = 0.048$) (Table S4). For *P. nattereri* tadpoles were classified between stages 27–39 (Gosner, 1960) and the amount of animals at each Gosner stage is summarized in Table S1. We also observed that in the end of the experiment, the control group at 28 °C presented tadpoles distribution at younger stages of development (Gs27-Gs33) when compared to those treated with 0.01 mg.L^{-1} at 36 °C, which presented 60% of them distributed between Gs35-Gs39. The frequency of tadpoles from both species classified at each Gosner (1960) stage is summarized at Tables S3–S6.

3.2. Antioxidant enzymes, lipid peroxidation and tGSH levels

3.2.1. Physalaemus nattereri

All studied enzymes had their activities changed by the isolated effect of temperature in *P. nattereri* tadpoles after three days (Fig. 1). Tadpoles unexposed to clomazone had CAT, SOD, G6PDH, and GST ($P < 0.004$) activities increased in animals maintained at higher temperatures (32 and 36 °C) compared to those at 28 °C (Fig. 1). After eight days, we observed the same pattern for SOD, with increased activity at 36 °C ($P = 0.019$) (Fig. 2B). CAT was increased only in the control group at 32 °C after eight days ($P = 0.033$) (Fig. 2A), and in the opposite way, GST was decreased in the control group at 36 °C when compared to the one at 28 °C ($P = 0.0002$) (Fig. 2D). G6PDH also presented a lower activity in tadpoles of the control group at 36 °C when compared to the one at 32 °C ($P = 0.006$).

At 28 °C, clomazone treatments only increased SOD activity at the highest concentrations (0.05 and 0.1mg/L^{-1}) after three days ($P < 0.05$) (Fig. 1 B). We have not observed any other effects on the enzymes activities at 28 °C. The combined effects of temperature and clomazone increased CAT, SOD, G6PDH and GST in tadpoles treated at 32 and 36 °C when compared to the control group at 28 °C after three days ($P < 0.05$) (Fig. 1). At 32 °C, animals exposed to 0.01 and 0.05 mg.L^{-1} had an increase on CAT activity when compared to animals at 28 °C ($P < 0.01$) (Fig. 1A). However, tadpoles exposed to 0.1 mg.L^{-1} at 32 °C showed a decrease on CAT activity compared to the control group at the same temperature ($P = 0.006$) (Fig. 1A). SOD and GST were increased in all groups treated at 32 and 36 °C after three days ($P < 0.05$) (Fig. 2A). G6PDH had its activity risen in all groups treated at 32 °C when compared to control group at 28 °C ($P = 0.01$, $P < 0.03$), in addition to an increased G6PDH activity in tadpoles exposed to 0.01 mg.L^{-1} at 36 °C ($P = 0.0004$) (Fig. 2C and D).

After eight days, no effects of clomazone were observed at 28 °C (Fig. 2). SOD had its activity increased by the interacted effects in tadpoles exposed to 0.1 mg.L^{-1} of clomazone at 32 °C ($P = 0.003$) and 0.05 mg.L^{-1} at 36 °C ($P < 0.001$), compared to the control group at 28 °C (Fig. 2B). We did not observe any effects of clomazone on G6PDH and GST activities at 28 and 32 °C after this period ($P > 0.05$) (Fig. 2C and D). However, animals treated with 0.01 mg.L^{-1} of clomazone at 36 °C decreased both G6PDH ($P = 0.017$) and GST ($P = 0.006$) activities when compared to the control group at 28 °C (Fig. 2C and D). GST activity also had a different response in animals exposed to 0.1 mg.L^{-1} at 36 °C, with an increased activity compared to the control group at the same temperature ($P = 0.005$) (Fig. 2D).

*t*GSH levels were not altered by clomazone exposure in *P. nattereri* tadpoles at any temperature for both experimental periods ($P > 0.05$) (Figs. 1 and 2). MDA levels were unaltered by treatments after three days in *P. nattereri* tadpoles (Fig. 1F). After eight days, we have not evidenced any lipid peroxidation effects in animals treated at 28 and 32 °C. However, MDA levels increased in non-exposed tadpoles at 36 °C ($P = 0.001$), also in those ones treated with 0.1 mg.L^{-1} at 36 °C ($P = 0.1$) after longer exposure (Fig. 2E).

3.2.2. Rhinella schneideri

Temperature rise only increased SOD and GST ($P < 0.05$) activity in non-exposed *R. schneideri* tadpoles after three days (Fig. 4B and C). No other changes were observed on antioxidant enzymes as result of isolated thermal stress after three and eight days (Fig. 4).

Clomazone caused no alterations in antioxidant enzymes activity in tadpoles treated at 28 and 32 °C after three days, excepted by SOD, which had its activity increased in animals exposed to 0.01 and 0.1 mg.L^{-1} at 32 °C ($P < 0.001$) when compared to the control group at 28 °C. At 36 °C, GST activity was enhanced in tadpoles exposed to 0.1 mg.L^{-1} when compared to the control group at the same temperature and at 28 °C ($P = 0.011$) (Fig. 3D). SOD also had its activity increased in all groups treated with clomazone at 36 °C compared to the control at 28 °C ($P < 0.01$), showing an interactive effect at higher temperatures (Fig. 3B). CAT and G6PDH were not changed following treatments with clomazone at any temperature and concentration after three days ($P > 0.05$) (Fig. 3A and B). After eight days of exposure, we did not observe any changes in the antioxidant enzymes response for any treatment, excepted by G6PDH which had its activity decreased in tadpoles exposed to 0.01 mg.L^{-1} at 28 and 32 °C ($P < 0.05$) when compared to the control group at 28 °C (Fig. 4).

*t*GSH levels were lower in all groups treated with clomazone at 32 °C and 36 °C in comparison to those at 28 °C, after three and eight days of exposure ($P < 0.05$) (Figs. 3E and 4E). After eight days, *R. schneideri* tadpoles treated with 0.01 and 0.05 mg.L^{-1} at 32 °C also had lower *t*GSH levels than those ones from the control group at the same temperature ($P = 0.003$, $P = 0.005$, respectively) (Fig. 4E).

Similarly to *P. nattereri*, we have not observed any changes on MDA after three days of exposure in *R. schneideri* ($P > 0.05$) (Fig. 3E). After eight days, tadpoles exposed to 0.05 mg.L^{-1} of clomazone at 32 °C had its MDA increased when compared to the control group at 32 ($P = 0.003$) and 28 °C ($P = 0.006$) (Fig. 4E). We observed no alterations on MDA levels at 28 and 36 °C after the longest exposure.

3.3. AChE and CbE activities

AChE activity was unchanged following clomazone exposures in *P. nattereri* tadpoles after both periods ($P > 0.05$) (Fig. 5). CbE was lower in animals exposed to 0.1 mg.L^{-1} at 36 °C than at 28 °C after three days ($P = 0.019$), but we did not observe any differences comparing to the control group. After eight days, CbE was increased in *P. nattereri* exposed to 0.01 mg.L^{-1} of clomazone at 28 °C ($P = 0.007$) and to 0.1 mg.L^{-1} at 32 °C ($P = 0.005$) compared to their respective controls (Fig. 5). In *R. schneideri*, tadpoles exposed to 0.1 mg.L^{-1} at 36 °C presented lower AChE activity than those exposed to the same concentration at 32 °C ($P = 0.033$) after three days. We observed no differences on AChE in *R. schneideri* after eight days ($P > 0.05$). CbE was higher in *R. schneideri* tadpoles exposed to 0.1 mg.L^{-1} at 28 °C when compared to the control group after three days ($P = 0.0003$), and we noticed no relevant differences after eight days (Fig. 6).

3.4. Integrated biomarker response (IBR)

IBR of *P. nattereri* was influenced by clomazone and temperature

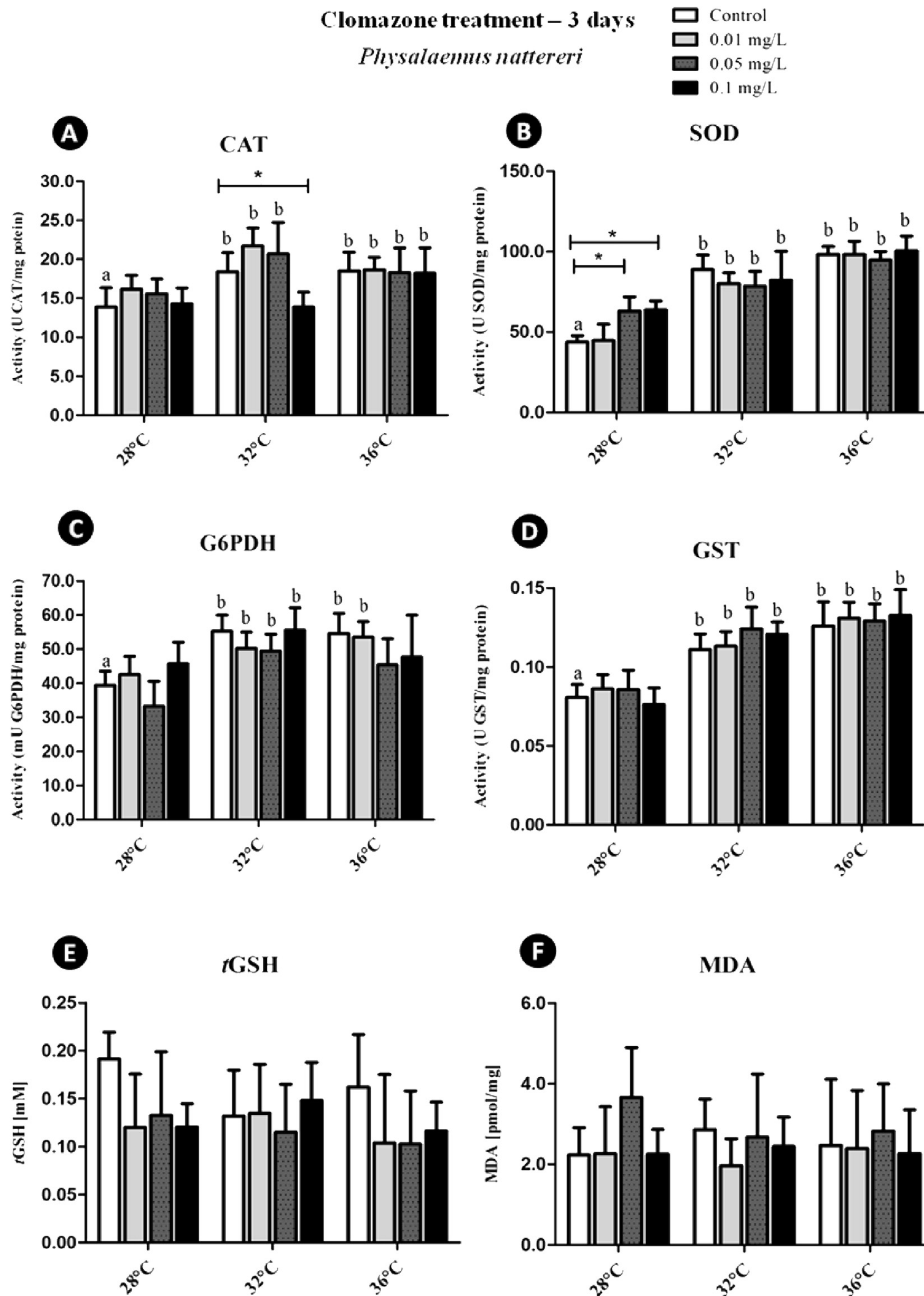

Fig. 1. Activity of the antioxidant enzymes CAT, SOD and G6PDH, the biotransformation enzyme GST and levels of *t*GSH and MDA in tadpoles of *P. nattereri* exposed to clomazone (Gamit® 500) at different temperatures (28, 32 and 36 °C) after three days. Control groups represent animals maintained at different temperatures without chemical treatment. Asterisk (*) indicates significant difference between chemical treatments (0.01, 0.05 and 0.1 mg/L) at the same temperature. Different letters indicate statistical significance among relevant groups treated at different temperatures ($P < 0.05$).

after three days of exposure ($P < 0.01$), but there was no interaction between these variables ($P = 0.24$). Tadpoles from control group and those treated with clomazone (0.01 and 0.05 $mg.L^{-1}$) at higher temperatures (32 and 36 °C) showed an increased trend of IBR compared to 28 °C ($P < 0.001$). Similar trend was observed in tadpoles exposed to 0.1 $mg.L^{-1}$ of clomazone, but statistical significance was only observed between 36 °C and 28 °C ($P < 0.001$). No differences were observed among groups at 32 and 36 °C ($P > 0.95$) (Fig. 7). There was interaction between temperature and clomazone on IBR after eight days of exposure ($P = 0.001$). Tadpoles

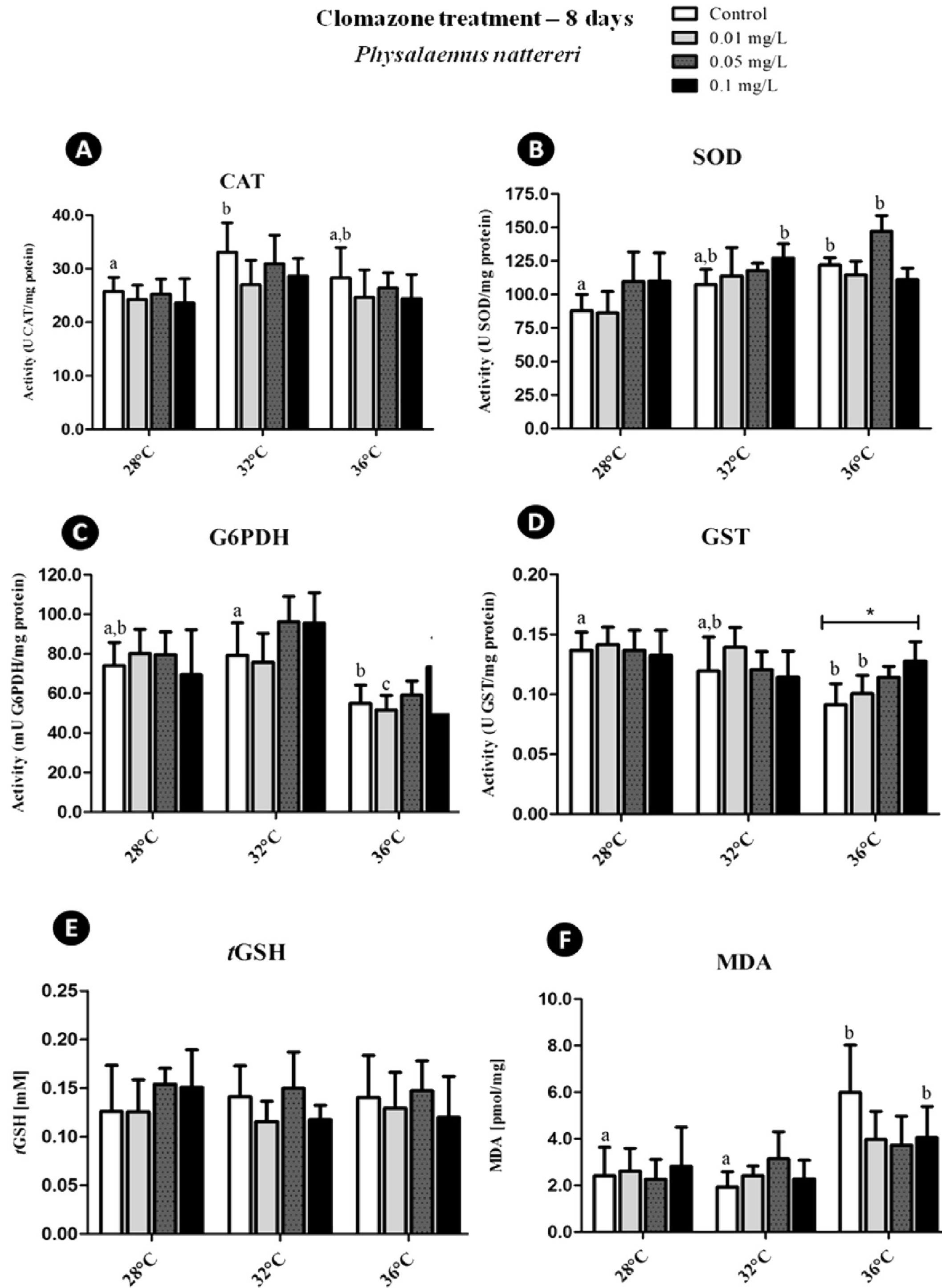

Fig. 2. Activity of the antioxidant enzymes CAT, SOD and G6PDH, the biotransformation enzyme GST and levels of *t*GSH and MDA in tadpoles of *P. nattereri* exposed to clomazone (Gamit® 500) at different temperatures (28, 32 and 36 °C) after eight days. Control groups represent animals maintained at different temperatures without chemical treatment. Asterisk (*) indicates significant difference between chemical treatments (0.01, 0.05 and 0.1 mg/L) at the same temperature. Different letters indicate statistical significance among relevant groups treated at different temperatures ($P < 0.05$).

exposed to 0.05 mg.L^{-1} of clomazone at 32 °C showed higher IBR value than the control group at 28 °C ($P = 0.04$). IBR of tadpoles unexposed to clomazone was increased at 36 °C compared to the same group at 28 °C. Similar increased trend also occurred in tadpoles exposed to 0.01 mg.L^{-1} of clomazone at 36 °C ($P = 0.006$), but not at higher concentrations ($P > 0.08$) (Fig. 7).

In *R. schneideri*, IBR was influenced by temperature and clomazone only after three days of exposure (Fig. 7). In this case, there was isolated effect of temperature and clomazone ($P < 0.001$), but no interaction between these variables was observed ($P = 0.06$). IBR showed a trend of increase with the rising of temperature in all groups. Particularly, IBR of tadpoles from control group and those

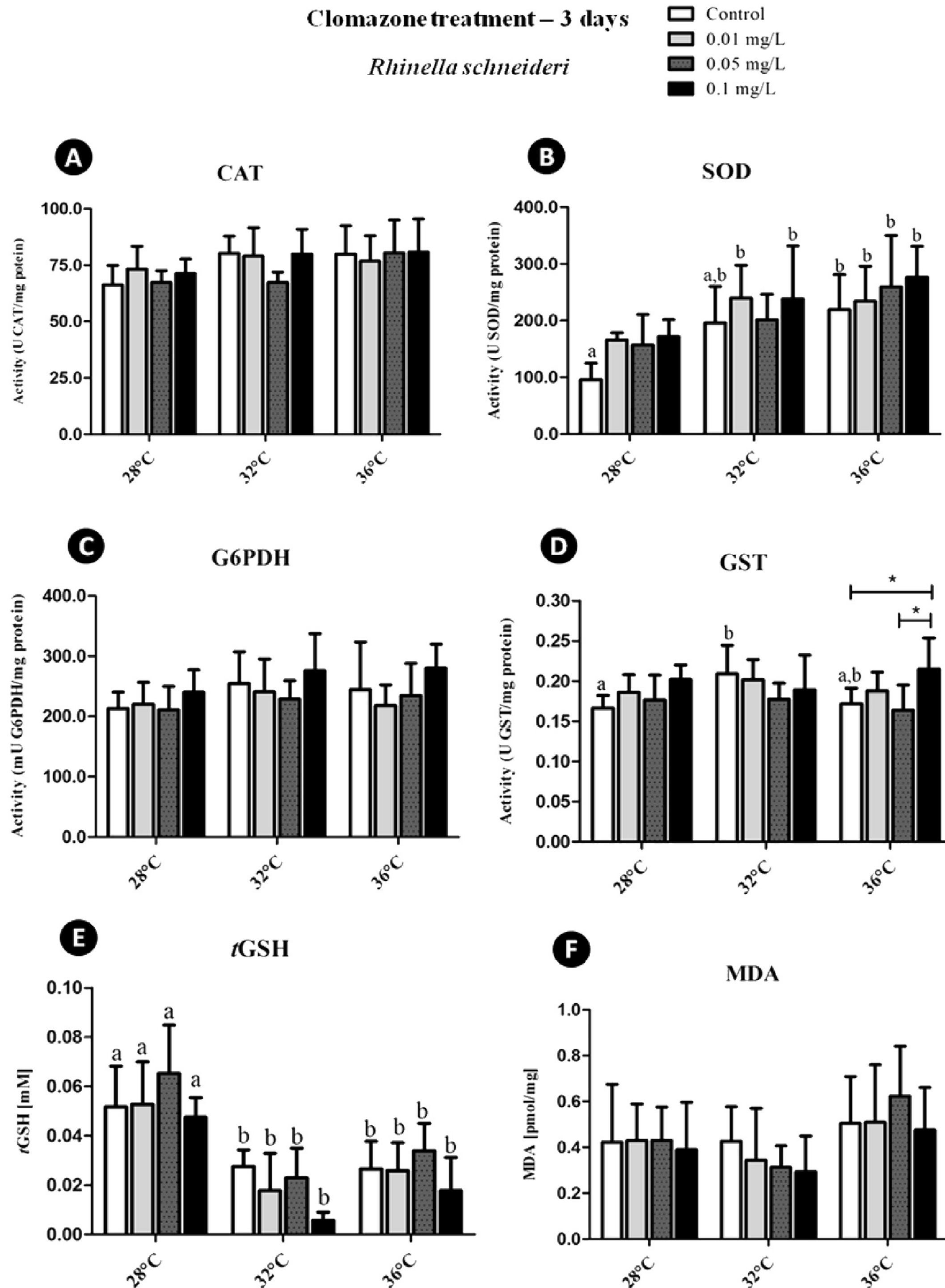

Fig. 3. Activity of the antioxidant enzymes CAT, SOD and G6PDH, the biotransformation enzyme GST and levels of *t*GSH and MDA in tadpoles of *R. shneideri* exposed to clomazone (Gamit®500) at different temperatures (28, 32 and 36 °C) after three days. Control groups represent animals maintained at different temperatures without chemical treatment. Asterisk (*) indicates significant difference between chemical treatments (0.01, 0.05 and 0.1 mg/L) at the same temperature. Different letters indicate statistical significance among relevant groups treated at different temperatures (P < 0.05).

treated with the highest concentration of clomazone (0.1 mg.L^{-1}) was increased at 32 °C and 36 °C compared to the 28 °C (P = 0.01) (Fig. 7). Similar trend was observed for tadpoles treated with 0.05 mg.L^{-1}, but in this case IBR was only increased at 36 °C compared to the 28 °C (P < 0001). Furthermore, IBR of tadpoles exposed to 0.1 mg.L^{-1} at 36 °C was higher compared to the ones exposed to 0.01 mg.L^{-1} at the same temperature (P = 0.05) (Fig. 7).

4. Discussion

Understanding how different species deal with contaminants in environments influenced by thermal stress is extremely urgent in a context where the toxicity of most agrochemicals used in crops in Brazil is still unknown for most native species. In fact, the use of pesticides in agricultural areas has been pointed as one of the main

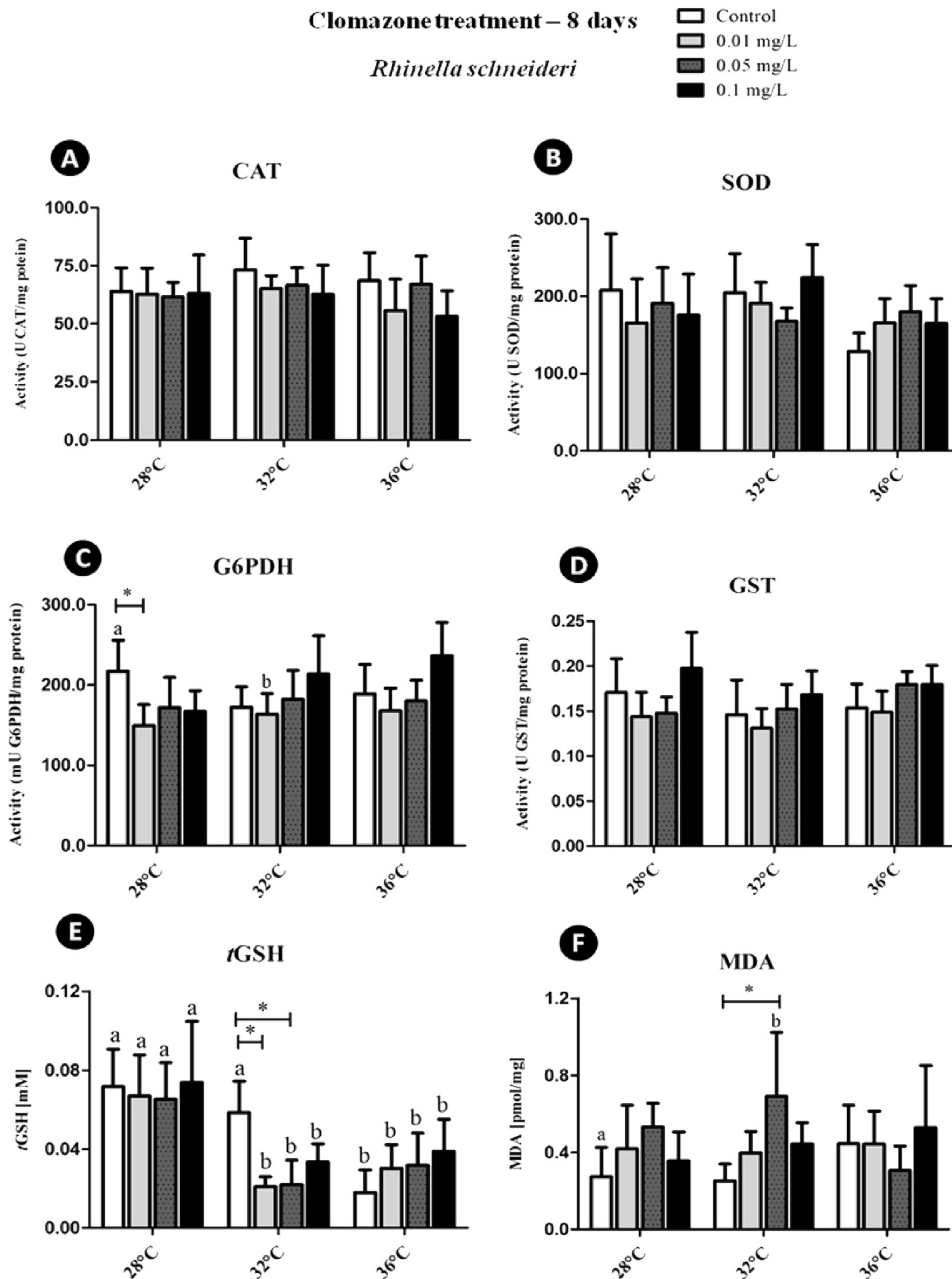

Fig. 4. Activity of the antioxidant enzymes CAT, SOD and G6PDH, the biotransformation enzyme GST and levels of *t*GSH and MDA in tadpoles of *R. schneideri* exposed to clomazone (Gamit® 500) at different temperatures (28, 32 and 36 °C) after eight days. Control groups represent animals maintained at different temperatures without chemical treatment. Asterisk (*) indicates significant difference between chemical treatments (0.01, 0.05 and 0.1 mg/L) at the same temperature. Different letters indicate statistical significance among relevant groups treated at different temperatures ($P < 0.05$).

factors contributing to decline of local amphibian populations (Hayes et al., 2010). In this context, our study focused on evaluating the isolated and interactive effects of the herbicide clomazone on biochemical and physiological aspects of two species of neotropical tadpoles. Our results indicated that temperature is an important factor affecting the herbicide clomazone toxicity in tadpoles at field concentrations, evidenced by alterations in the antioxidant defense enzymes, esterase activities and lipid peroxidation status with temperature-associated responses.

Tadpole's development was unaltered in *R. schneideri* after exposure to clomazone at different temperatures. In *P. nattereri*, tadpoles exposed to 0.01 $mg.L^{-1}$ at 36 °C presented more advanced stages than those from the control group at 28 °C after eight days. It is already known that changes of temperature (Morand et al., 1997) and the presence of chemicals (Brande-lavridsen et al., 2010; Freitas et al., 2016; Peltzer et al., 2013b) in the environment can alter the

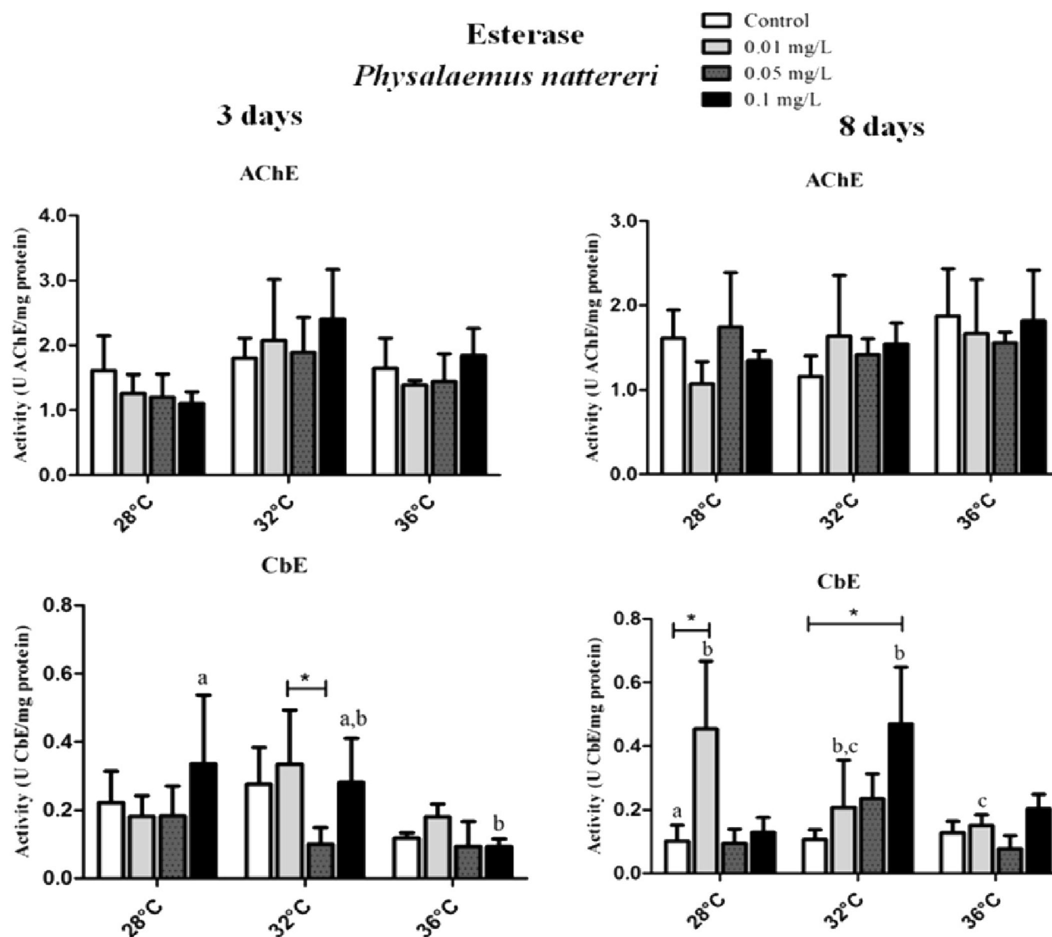

Fig. 5. Acetylcholinesterase (AChE) and carboxylesterase (CbE) activities in tadpoles of *P. nattereri* exposed to clomazone (Gamit® 500) at different temperatures (28, 32 and 36 °C) after three and eight days. Asterisk (*) indicates significant difference between chemical treatments (0.01, 0.05 and 0.1 mg/L) at the same temperature. Different letters indicate statistical significance among relevant groups treated at different temperatures (P < 0.05).

development of frogs. Acceleration on metamorphosis process is considered an acclimation mechanism used by different species for dealing with adverse conditions in stressed environments (Hooper et al., 2013). Although this may bring benefits, accelerated metamorphosis compromise many associated traits, such as body size, age of reproduction and fecundity (Semlitsch, 1988), which result in adults with altered physiological performance and not competitive enough to survive in nature (Denver, 1997). Our study suggests that combined exposure to clomazone and higher temperatures accelerate *P. nattereri* metamorphosis, however we recommend more studies with longer exposures to clarify the effects of clomazone on tadpoles metamorphosis.

Results showed that the clomazone formulation affected oxidative stress and esterase activities in *P. nattereri* and *R. schneideri* tadpoles, and that temperature had a strong influence on these parameters for both species. GST activity was changed by both temperature and clomazone, but the interaction of higher temperature and clomazone exposure mostly increased GST. Enzymes of the GSTs family act in phase II of the detoxification process, which involves the conjugation of xenobiotics with endogenous compounds (Di Giulio et al., 1989). The sensibility of GST to environmental temperatures have been already reported in aquatic organisms exposed to thermal stress, such as in saltwater fish (*Diplodus vulgaris*, *Diplodus sargus*, *Dicentrarchus labrax*, *Gobius niger* and *Liza ramada*) (Madeira et al., 2013; Vinagre et al., 2012) and *P. nattereri* tadpoles (Freitas and Almeida, 2016). Previous studies with the herbicide clomazone have also reported an increased GST activity in fish (*P. lineatus*) exposed to 5 and 10 mg.L^{-1} after 96 h (Pereira et al., 2013). However, the interactive influence of temperature and clomazone we observed in both studied species suggests a synergic influence of both factors on GST activity, probably associated with an adaptation response of the organisms to stressful conditions in the environment (van der Oost et al., 2003).

SOD was the only enzyme changed by exposure to clomazone at 28 °C in *P. nattereri*, with an increased activity in tadpoles exposed to the highest concentration. SOD was also influenced by heat stress in both species, an effect that was particularly pronounced after shorter exposure. After eight days, SOD activity returned to control levels for all treatments in *R. schneideri*. SOD is already known to be altered by exposure to some pesticides, such as methyl parathion, atrazine and DDC (diethyldithiocarbamate), in aquatic organisms (Abhijith et al., 2016; Lushchak, 2011; Nwani et al., 2010), but its activity can also be enhanced by changes on environmental temperature (Vinagre et al., 2014; Walters et al., 2016). Studies with salmon (*Oncorhynchus kisutch*) showed that SOD was significantly increased in thermal stressed fish after 17.5 h, but the activity had returned to the basal levels after 48 h (Nakano et al., 2014). Otherwise, SOD activity was observed to decrease in fish of *Notothenia coriiceps* and *Notothenia rossii* exposed to heat stress after three and six days compared to those exposed for one day (Machado et al., 2014). In agreement with the literature data, our

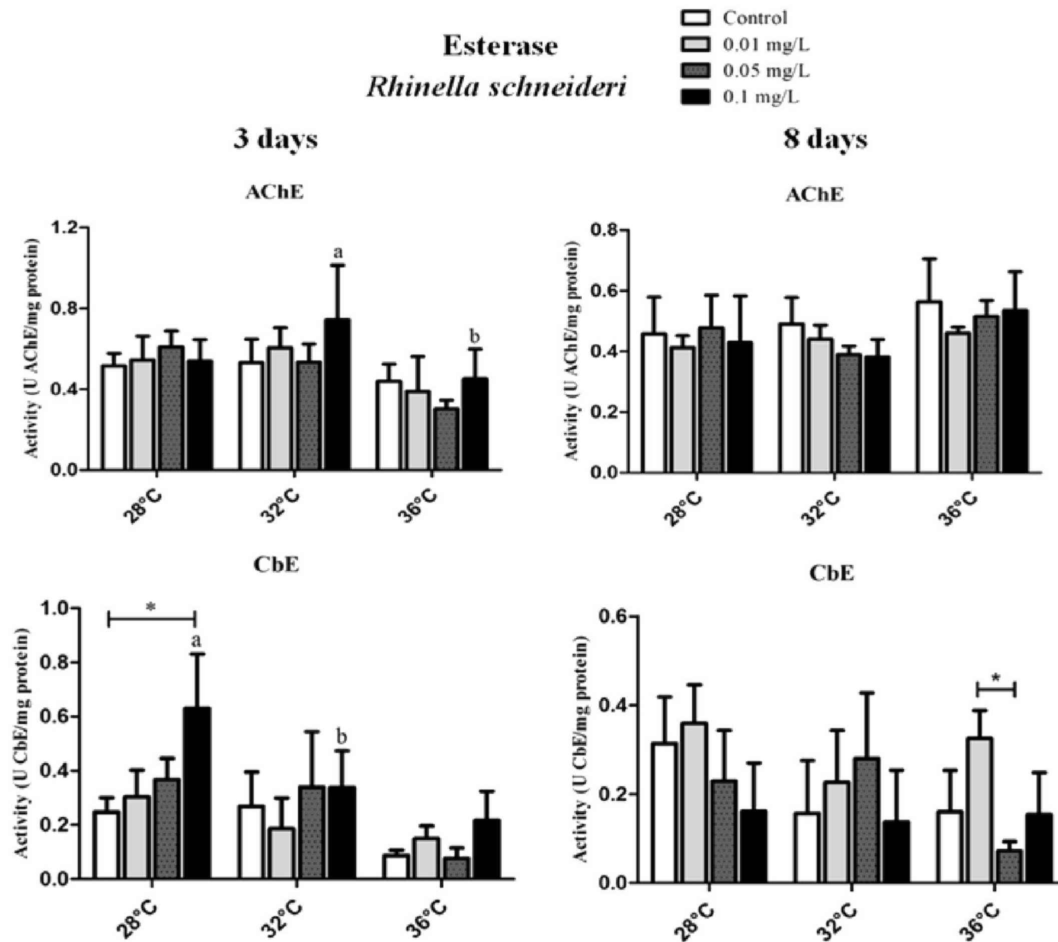

Fig. 6. Acetylcholinesterase (AChE) and carboxylesterase (CbE) activities in tadpoles of *R. schneideri* exposed to clomazone (Gamit® 500) at different temperatures (28, 32 and 36 °C) after three and eight days. Asterisk (*) indicates significant difference between chemical treatments (0.01, 0.05 and 0.1 mg/L) at the same temperature. Different letters indicate statistical significance among relevant groups treated at different temperatures ($P < 0.05$).

findings suggest that increased SOD activity might neutralize the harmful effects of superoxide radicals generated as result of thermal stress, also by the combined effect of heat stress and clomazone exposure; however, adaption mechanisms used by *R. schneideri* after longer periods may compensate its activity.

Heat treatments induced CAT activity in all groups treated at 32 and 36 °C in *P. nattereri* after three days, while its activity was unchanged in *R. schneideri* for any treatment, showing that clomazone affected CAT activity depending on the water temperature, but also on the species studied. Previous studies have shown opposite results for CAT in different species of fish exposed to the combined effects of temperature and presence of pesticides (Kaur et al., 2011; Tu et al., 2012). For example, CAT activity was decreased in the saltwater fish *Channa punctata* and in the black tiger shrimp (*Penaeus monodon*) exposed to the insecticide deltamethrin in combination with heat stress (Kaur et al., 2011; Tu et al., 2012). Similarly to CAT, G6PDH activity was also increased in tadpoles of *P. nattereri* exposed to clomazone at higher temperatures after three days. However, some decreases in G6PDH were also observed in *P. nattereri* and *R. schneideri* tadpoles exposed to clomazone after eight days. G6PDH and CAT are important enzymes for redox metabolism. Altered CAT activity may impair the capacity of cells to catalyze the decomposition of hydrogen peroxide (H_2O_2). Increased CAT activity observed in tadpoles exposed to the combination of clomazone and high temperatures may be a consequence of increased H_2O_2 production in organisms exposed to stressful conditions, in an attempt to neutralize the injuries caused by the excess of this compound in the cells. Otherwise, decreased CAT activity should compromise the ability of the organisms to act against ROS damages. Changes on G6PDH activity are also important to organisms because this enzyme participates of the pentose phosphate pathway recycling NADPH, an important reducing agent used to maintain GSH levels in the cells. Decreased G6PDH activity can result in an diminished supply of GSH, which is an important tripeptide that protects cells against oxidative damage (Almeida et al., 2011). Therefore, balanced activities of both CAT and G6PDH are required for a good cellular performance linked to antioxidant response, and alterations in their functioning can make the animals more vulnerable to ROS effects and consequently increasing chances of oxidative stress.

*t*GSH levels were unchanged in *P. nattereri* tadpoles following the treatments with clomazone and temperature. In *R. schneideri*, we observed higher levels of *t*GSH in the groups exposed to 28 °C than at 32 and 36 °C for both exposure periods, indicating an increased GSH demand as a consequence of temperature increase. Endogenous GSH plays a role in cells as an antioxidant, as a cofactor of glutathione peroxidase (GPx), and participates in the reduction of peroxides, with concomitant formation of oxidized glutathione disulfide (GSSG) (Almeida et al., 2011). GSH is central to the detoxification of ROS and has been mentioned as one of the protective mechanisms that aquatic organisms adopt in the initial phases of exposure to xenobiotics, being also an important co-

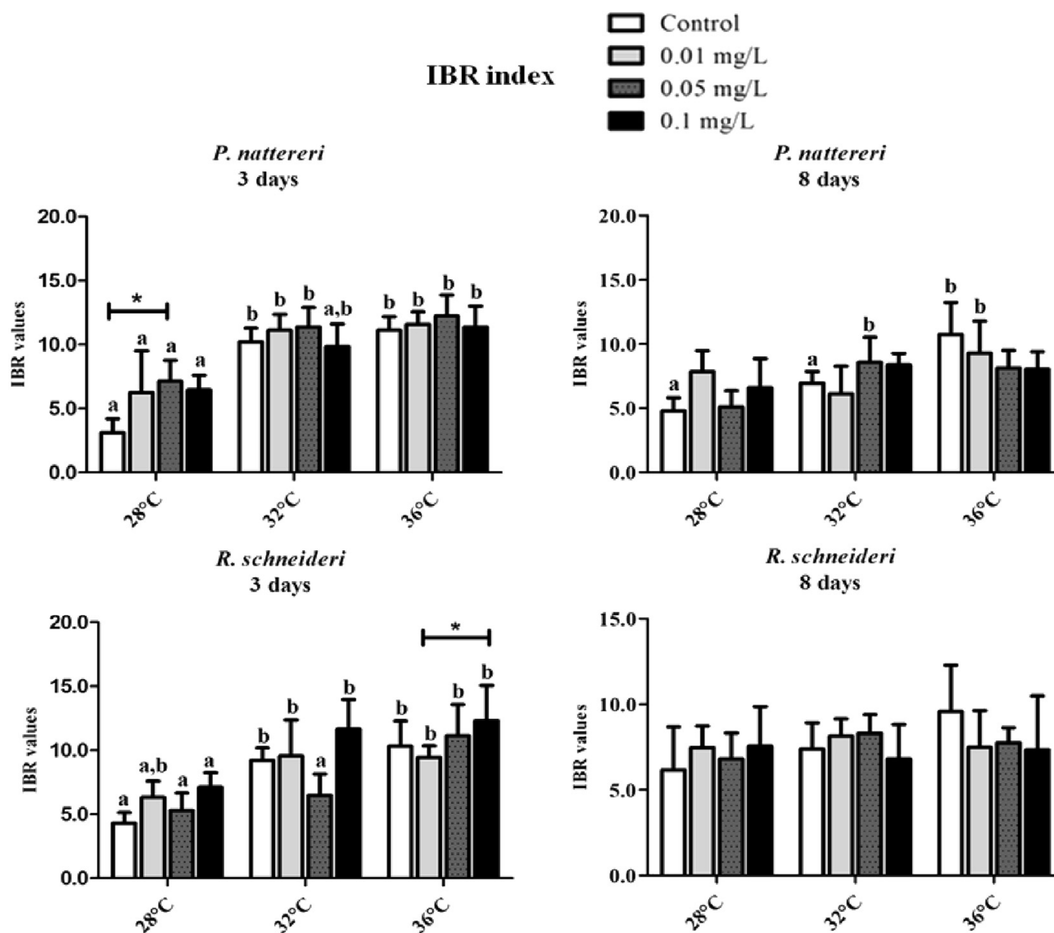

Fig. 7. Integrated biomarker response (IBR) index summarizing the responses of biochemical biomarkers to different concentrations of clomazone (0.01, 0.05 and 0.1 mg/L) and temperatures (28°, 32° and 36 °C) for both periods of exposure (three and eight days). Asterisk (*) indicates significant difference between chemical treatments (control, 0.01, 0.05 and 0.1 mg/L) at the same temperature. Different letters indicate statistical significance ($P < 0.05$) among relevant groups treated at different temperatures considering both factors, temperature and sulfentrazone.

factor for GST during phase II biotransformation (Stephensen et al., 2002). Thermal stress can potentially alter GSH contents in fish (Machado et al., 2014; Nakano et al., 2014), but also the presence of chemicals combined to temperature effects (Kaur et al., 2011; Min et al., 2014). For example, GSH levels were higher in the Antarctic fish *N. rossii* exposed to 8 °C than to 0 °C (control temperature) (Machado et al., 2014). GSH levels were also higher in the black rockfish *Sebastes schlegeli* exposed to different concentrations of copper at 23 and 28 °C than at 18 °C (Min et al., 2014). Furthermore, some studies have shown that fluctuations in GSH in organisms exposed to some chemicals can be accompanied by variations in GST, which conjugates GSH to various xenobiotic compounds (De Almeida et al., 2011). In our study, contents of *t*GSH had a temperature-associated response in *R. schneideri* tadpoles, with differential response after three and eight days of exposure, however alterations on GSH contents were not associated with GST activity for any of the species. This lack of association may be related to the method used in our study, which measured the total contents of both oxidized and reduced glutathione in the cells. However, we observed that temperature was an important factor controlling GSH levels in tadpoles, with differential responses for different species probably due to different pathways using GSH as the co-factor in the antioxidant response.

Peroxidation of membrane components are caused when enzyme activity is not compensated by the excessive amount of ROS produced in response to stress. Pesticides may induce oxidative stress, leading to the generation of free radicals and causing lipid peroxidation (LPO) (Sevgiler et al., 2004). In agreement to this, clomazone exposure has been previously mentioned to induce LPO in liver, brain and muscle tissues of the piava *Leporinus obtusidens* (Miron et al., 2008). In *R. quelen,* exposure to clomazone (0.5 or 1.0 mg.L^{-1}) also increased TBARS production in liver after exposures of 12–192 h, with differential responses in brain, where these levels were decreased after 96 and 192 h (Crestani et al., 2007). Increases on environmental temperature were also associated with increased (Nakano et al., 2014) or decreased (Machado et al., 2014) LPO in fish. Our study showed that MDA levels were more influenced by temperature than by exposure to clomazone in *P. nattereri*, which had their MDA increased for both control and clomazone-treated tadpoles at 36 °C after three days. For *R. schneideri*, we observed no changes after three days, but LPO was induced in tadpoles exposed to clomazone at 32 °C after longer exposure. The lack of variation on MDA in the other experimental groups can be related to the activities of antioxidant enzymes, which were mostly upregulated in animals exposed to combination of higher temperatures and clomazone exposure, potentially protecting the cells against lipid peroxidation.

Cholinesterases such as AChE and CbE are known to be inhibited by organophosphates and carbamates pesticides (van der Oost et al., 2003). However, AChE activity was also reported to be

inhibited by other types of pesticides, such as the herbicide clomazone (Miron et al., 2008) and the insecticide endosulfan (Dutta and Arends, 2003). Previous studies have shown that clomazone act as a potent brain-inhibitor in *P. lineatus* fish (5.0 and 10.0 mg.L^{-1}) and *R. quelen* (5.0, 10.0 and 20.0 mg.L^{-1}) after 96 h of exposure (Miron et al., 2008; Pereira et al., 2013). Subsequent studies also showed that *R. quelen* was sensitive to lower concentrations of clomazone, presenting inhibition of brain and muscle AChE when exposed to 0.5 and 1.0 mg.L^{-1} (Crestani et al., 2007). In the opposite way, muscle tissue of *Leporinus obtusidens* fish showed AChE activity increased until 65% after exposure to clomazone (0.5 mg.L^{-1}), indicating this herbicide can inhibit or activate AChE; however, the mechanisms that lead to activation are still unknown. In the present study, we observed that AChE was unchanged following clomazone treatments in *P. nattereri.* In *R. schneideri*, AChE was lower only in tadpoles exposed to 0.1 mg.L^{-1} at 36 °C compared to the ones exposed to the same concentration at 32 °C after three days. CbE activity is also mentioned to be altered by different pesticides in tadpoles (Lajmanovich et al., 2010; Leite et al., 2010). This enzyme is involved in the metabolism of xenobiotics and endogenous or exogenous short-chain fatty acids (Wheelock et al., 2008). Ours results showed that CbE was sensitive to clomazone at tested conditions, with diminished activity in animals exposed to the combined effects of the chemical at higher temperatures for both species after three days. After eight days, CbE changes were more pronounced in *P. nattereri* tadpoles. B-esterase enzymes have been recommended as important biomarkers evaluating amphibians exposure to pesticides in contaminated areas (e.g. Lajmanovich et al., 2010; Leite et al., 2010; Peltzer et al., 2013a). However, studies relating the effects of pesticides rather than organophosphate and carbamates in tadpoles are still scarce, especially considering endemic species from Brazil and its environmental temperatures.

Evaluation of tadpole's response through IBR provides a useful approach to reveal the biological consequences of environmental stressors (Devin et al., 2016). By integrating the response of individual biomarkers, IBR provided an overall picture of the influence of temperature and clomazone on tadpoles. In this study, effects of temperature on IBR were particularly evident for *P. nattereri* and *R. schneirei*, highlighting the thermal-induced response on oxidative stress and esterase activities. Otherwise, responses to clomazone were less consistent in relation to the period of exposure. IBR detected the influence of clomazone only after three days of exposure for both species, probably due the action of antioxidant enzymes after longer periods. These results reinforce that IBR can be used as a reliable tool for early-warning of contaminants and thermal stress. This is especially important considering that amphibians inhabit or temporarily occur in ponds immersed in agricultural environments, which are often exposed to pesticides (Mann et al., 2009).

5. Conclusion

In summary, we showed that temperature is an important factor inducing changes on biochemical responses in *P. nattereri* and *R. schneideri* tadpoles exposed to the herbicide clomazone. For the first time, we reported the negative effects of clomazone on the biochemical stress response in amphibians. Antioxidant enzymes are important integrants of the antioxidant defense system, acting into the organisms to minimize the effects of ROS, which are generated during the biotransformation of the xenobiotics (van der Oost et al., 2003). Therefore, disturbance in the activity of these enzymes can interfere in the redox homeostasis of tadpoles, resulting in oxidative damage of cellular components. Different from results observed in fish studies, clomazone had not pronounced effects on AChE at the studied concentrations and temperatures. However, clomazone itself or in combination with higher temperature impaired CbE activity in *P. nattereri* and *R. schneideri*, which has not previously been reported for aquatic organisms exposed to this compound. Based on the studied biomarkers, *R. schneideri* showed better adaptability to the tested conditions, since most of the enzymes had their activities returned to the control levels after longer exposure. The discrepancies in physiological responses of *P. nattereri* and *R. schneideri* also support that the susceptibility and ecological risk of amphibians populations to exposure to environmental contaminants and temperatures may particularly vary among species. It should be reinforced that a formulation of the clomazone herbicide was used (Gamit® 360CS), which contains 36% (m:v) of clomazone only, in addition to unknown inert ingredients (not disclosed by the manufacturer); thus, possible effects due to inert ingredients of the formulation should be not excluded. Also, we strongly suggest that fluctuations on environmental temperature should be taken into account in further studies assessing environmental risks of pesticides for amphibian communities, especially considering tropical species.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.chemosphere.2017.07.061.

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