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Ecotoxicological evaluation of water from the Sorocaba River using an integrated analysis of biochemical and morphological biomarkers in bullfrog tadpoles, *Lithobates catesbeianus* (Shaw, 1802)

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HIGHLIGHTS

- Metal concentrations were lower in water and sediment than that recommended by Brazilian resolution for aquatic communities.
- Metals caused oxidative stress even at low concentrations in bullfrog tadpoles.
- Significant biomarkers responses were found in summer and winter at tissues of the bullfrog tadpoles at Sorocaba river.
- *Lithobates catesbeianus* as a sensitive bioindicator of metals pollution.

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ABSTRACT

Lithobates catesbeianus tadpoles were exposed for 96 h to water from two sites of the Sorocaba River (summer and winter), Ibiúna (PI) and Itupararanga reservoir (PIR) that contained metals. In the liver, in PI, the glutathione peroxidase (GPx) decreased, and the glutathione S-transferase (GST) and carbonyl proteins (PCO) increased. In PIR, the glutathione reduced (GSH) increased, while there was a decrease in catalase (CAT), GPx, GST, PCO, and superoxide dismutase (SOD). In winter, GPx and GST increased in both points. Regarding the kidneys, lipoperoxidation (LPO) levels and GST decreased, while GSH increased in the summer. In the winter, LPO increased in PI. In the muscle, in the summer, there was an increase in GSH and GST and change in PCO. In the winter, the levels of PCO increased and CAT decreased in PIR. The area and volume of the hepatocyte and nucleus area increased in the summer and decreased in the winter. Hepatic melanin decreased in the summer after exposure to PIR water. There were the systemic effects of Sorocaba River water exposure at different times of the year with alterations in biomarkers at different levels, in which kidney shows highest Integrated Response of Biomarkers (IBR) value followed by liver and muscle. Biochemical biomarkers were more sensitive than morphological ones. The more

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sensitive biochemical markers were MT, PCO, GST and LPO. These effects confirm the hypothesis of metabolic alteration in bullfrog tadpoles by the Sorocaba River water.

1. Introduction

The presence of metals in aquatic ecosystems increases at an alarming rate and is considered to be a worldwide problem. Although some metals are trace elements necessary for various biological processes of all species (Uriu-Adams and Kleen, 2005), they become toxic at high levels due to an interference with various metabolic processes (De Boeck et al., 2003; Authman et al., 2012). The toxic effects of metals on aquatic organisms often depend on their ability to increase cell levels of oxygen-reactive species (ROS) (Van der Oost et al., 2003; Viarengo et al., 2007; Atli and Canli, 2010; Barhoumi et al., 2012), as demonstrated in fish (Ruas et al., 2008; Franco et al., 2009; Monteiro et al., 2010; Barhoumi et al., 2012; Carvalho et al., 2012, 2015; Sakuragui et al., 2013) and in amphibians (Veronez et al., 2016; Jayawardena et al., 2017; Boiarski et al., 2020; Carvalho et al., 2020). ROS and the reactive nitrogen species (RNS) are able to alter biomolecules, changing their structures and functions. In lipids, they can cause peroxidation or lipoperoxidation (LPO) (Palmer et al., 1987; Van der Oost et al., 2003; Vasconcelos et al., 2007) and in proteins they can cause oxidation and provide carbonyl derivatives which can be introduced into these molecules through reaction with aldehydes derived from LPO (Parvez and Raisuddin, 2005; Vasconcelos et al., 2007; Cattaneo et al., 2011; Machado et al., 2014). In nucleic acids, they can cause chromosomal fragmentation or damage to the mitotic apparatus (Heddle et al., 1983; Mouchet et al., 2006).

The main antioxidant and redox regulator in cells to combat oxidation of cellular constituents is reduced glutathione tripeptide (GSH). GSH plays a key role in detoxifying peroxides, RNS, and xenobiotic compounds (such as electrophilic reactive molecules) in cells (Han et al., 2006). Regarding antioxidant enzymes involved in combating ROS, we have catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione reductase (GR) in addition to the biotransformation enzyme of xenobiotics, glutathione S-transferase (GST) which have been used as biomarkers in biomonitoring programs around the world, using aquatic organisms as bioindicators (Carvalho et al., 2012; Abdel Rahman et al., 2019; Hinojosa-Garroa et al., 2020). These enzymes convert reactive radicals into non-reactive molecules, neutralizing ROS and maintaining the redox state in the tissues (Halliwell and Gutteridge, 2007).

Metals also alter the levels of metallothionein (MT) as demonstrated in fish (Hashemi et al., 2008; Werner et al., 2008; Barhoumi et al., 2012; Sakuragui et al., 2013; Yologlu and Ozmen, 2015) and in amphibians (Carvalho et al., 2017). This molecule binds strongly to metals because it contains about 30% cysteine (Berntssen et al., 2001) and is used as a biomarker for exposure to metals. Its cellular expression is induced by zinc (Zn), copper (Cu), cadmium (Cd), mercury (Hg), cobalt (Co), bismuth (Bi), nickel (Ni) and silver (Ag) (Werner et al., 2008; Barhoumi et al., 2012; Sakuragui et al., 2013; Yologlu and Ozmen, 2015).

Evaluated biomarkers at different levels are an important tool to elucidate systemic effects of aquatic contaminants in anurans (Pérez-Iglesias et al., 2019). Morphological biomarkers in the liver are used to test effects of xenobiotics in systemic physiology and metabolism. An important biomarker present in the liver is the melanomacrophages (MMs). These cells are widely used to describe effects of environmental stressors (De Oliveira et al., 2017). MMs are melanina-pigmented cells and possess catabolic functions (De Oliveira et al., 2017). Due to melanin presence in cytoplasm, MMs act as a non-enzymatic antioxidant system (Fenoglio et al., 2005). In addition, liver is an important organ for biotransformation of xenobiotics. This process involves hepatocytes and MM functions (Fenoglio et al., 2005). Therefore, liver alterations are an important response to test aquatic contamination exposure in anurans. Exposure to metals in aquatic environments is particularly harmful to amphibian species and can become an important factor in the decline of their population (Pérez-Iglesias et al., 2015; Soloneski et al., 2016; Carvalho et al., 2017; Carlsson and Tydén, 2018; Boiarski et al., 2020). Amphibians are vulnerable to the presence of environmental contaminants because they have low mobility, larval stage with periods of their lives in aquatic and terrestrial ecosystems, and permeable skin. Regarding tropical or subtropical species, there is little knowledge of the biology, and some of them are at risk of extinction. *Lithobates catesbeianus* is a species whose biology is well known among amphibians, it has great adaptive capacity to any environment, and it is easy to obtain specimens for laboratory tests. Several studies propose the use of *L. catesbeianus* as a model species with high potential in the evaluation of harmful effects of contaminated water (Boone et al., 2007; Ossana et al., 2013; Paetow et al., 2013; Veronez et al., 2016; Boiarski et al., 2020; Carvalho et al., 2020; Motta et al., 2020) and the results obtained may, to a certain extent, be considered as a subsidy for creating standards for the protection of endemic species in Brazil.

In the present study, we assumed that the Sorocaba River contains metals (Al, Cd, Cu, Mn, and Zn) and that these vary seasonally and cause changes in the different levels of biochemical, morphological and morphometric biomarkers in bullfrog tadpoles, *L. catesbeianus.* To test our hypotheses, lipoperoxidation biomarkers (LPO), reduced glutathione (GSH), catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione S-transferase (GST) and the concentrations of metallothionein (MT) and carbonyl proteins (PCO) and morphological alterations in the liver, kidney and muscle were selected as stress indicators in tadpoles after exposure to Sorocaba River water.

2. Materials and methods

2.1. Study area

The area belongs to one of the six sub-basins that make up the Sorocaba Médio Tietê basin (SMT), it has an area of 929 km^2 and is located in the southeastern portion of São Paulo State. This hydrographic basin is formed by the Una, Sorocabuçu and Sorocamirim rivers, whose headwaters are located in the municipalities of Ibiúna, Cotia, Vargem Grande Paulista and São Roque that form the Sorocaba River, a tributary of the Itupararanga Reservoir (Fig. 1). The choice of sampling points was based on the work of Conceição et al. (2011), selecting the point near the city of Ibiúna (PI) (23°27′27″ South latitude - 47°24′10.1″ West longitude) and the point located at the Itupararanga Reservoir (PIR) (23°38′11.3″ South latitude - 47°13′22.6″ West longitude). These sites were chosen due to the distance between them and the level

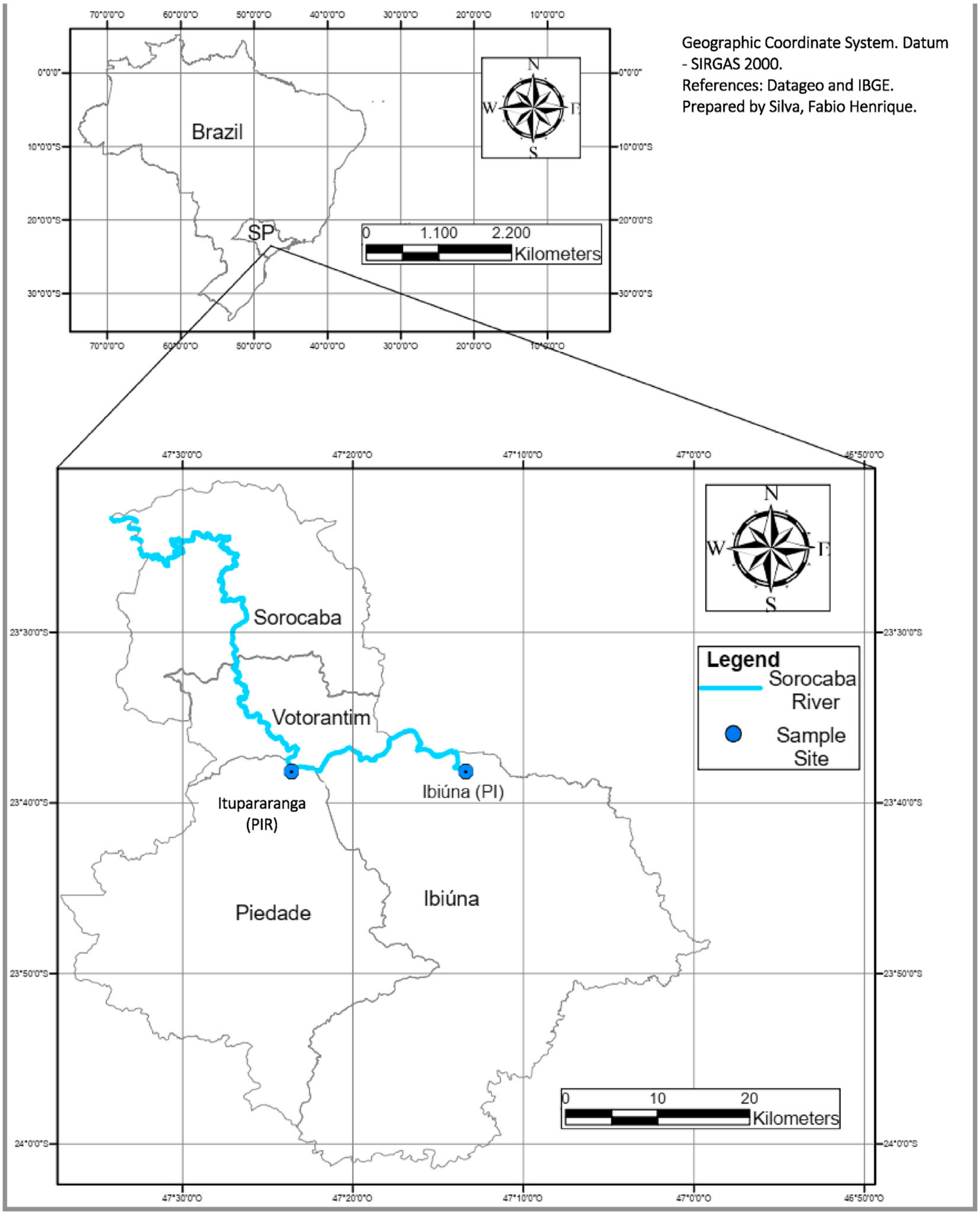

Fig. 1. Localization map of the sampling sites on the Sorocaba River.

of anthropogenic influence (such as mining and cement factories). Therefore, four collections were made: two collections for each chosen point (PI and PRI) comprising one in the summer and one in the winter.

2.2. Experimental protocol

Samples of surface water and also sediment were performed during two periods in March 2019, referring to the summer, and in August 2019, referring to the winter. In the field, the pH and water temperature were measured. Surface water samples at each point were manually collected from the river and packed in 50L gallons totaling 120L. Sediment samples were collected from each location under the water depth using a shovel from the river's convex curve areas. The amount of sediment samples was approximately 500 g. The samples were stored in labeled plastic bags and kept under ice until arriving at the laboratory, where they were stored in a freezer at −20 °C until the moment of analysis according to guidance from the *Laboratório Ambiental - Plantec Laboratórios* (Iracemápolis, SP). Total hardness was determined following the methodology described by American Public Health Association (APHA, 1992). Ammoniacal nitrogen, nitrite, nitrate and phosphate were determined using the colorimetric method (Mackereth et al., 1978). The metals concentrations in water and sediment were determined by mass spectroscopy with inductively coupled plasma (ICP-MS) according to the quality assurance and quality control (QA/QC) requirements and the SRM 3114 NIST (USA) reference standards. The mean values and standard deviations of the reference material were within 10% the ranges. The limits of quantification (LQ) were 10 μg/L for Al and 1 μg/L for Cd, Cu, Mn and Zn in water and in sediment the LQ was 2500 μg/kg for Al and 250 μg/kg for Cd, Cu, Mn and Zn. These LQ values were 10-fold higher than the limit of detection (LD).

2.3. Animal collection and laboratory maintenance

L. catesbeianus tadpoles (syn. *Rana catesbeiana*) (Shaw, 1802) (Body mass = 2.09 ± 0.20 g) were obtained from the Santa Rosa Frog Farm (Santa Barbara d'Oeste, SP, Brazil) (22°46′53.0″S/47°24′17.7″W). Tadpoles (~216 animals), in stage 25 of Gosner (1960), were kept at densities of one specimen per liter of water and acclimated in 80 L tanks containing tap water dechlorinated with continuous water flow (1.2 L h^{-1}) and aeration, a controlled temperature (25 ± 1 °C) under natural photoperiod (~12 h light, 12 h dark) for at least 10 days before the experiments. Tadpoles were fed ad libitum with commercial pellets (40% of protein) and to preserve the water quality, food was withheld for 24 h preceding the start of the tests. Thereafter, the tadpoles were randomly transferred to 16 L aquariums and remained for 2 days before experiments to recover from transference stress and acclimatization. During the collections and exposure, the water was monitored daily, and the physical and chemical parameters were kept nearly constant: pH 7.2–7.6, dissolved oxygen 7.0–7.5 mg/L, hardness 50–58 mg/L (as $CaCO_3$), conductivity 56–97 ± 0.02 μS/cm and ammonia concentrations remained at < 1 mg/L.

Tadpoles were divided into three experimental groups: the control group (n = 12), that corresponded to the group exposed to water free from contaminants; group exposed to water of the city of Ibiúna (PI, n = 12) and to the water from the Itupararanga Reservoir (PIR, n = 12) for 96h under static conditions. The experiment was conducted in triplicate. Two exposures were made for each location, in the summer and one in the winter. The aquariums (control, PI and PIR groups) used during the tests had a capacity of 16 L, and were maintained at a controlled temperature (25 ± 1 °C) and constant aeration (>6.0 mg O_2/L).

During the exposure experiments (96 h), the animals' behavior was observed regularly. At the end of the experimental period, the animals were anesthetized with 0.1% benzocaine, and euthanized by cranial concussion according to the recommendations of the American Veterinary Medical Association (AVMA, 2001). Afterwards, the liver, kidney and muscle were removed and frozen at −80 °C until the biochemical assays and tissue samples were fixed for morphological analysis. The Animal Ethics Committee at the Federal University of São Carlos (CEUA-UFSCar) approved all the procedures carried out in this study (Protocol#2578040219/2019); all the procedures were followed in accordance with the standards established by the American Society for Testing and Materials (ASTM, 2000).

2.4. Biochemical analyses

The liver, kidney and muscle (N = 8 per group) were homogenized in a saline-phosphate buffer PBS (containing 137 mM NaCl, 2.7 mM KCl, 5.4 mM Na_2HPO_4 ($7H_2O$) and 1.8 mM KH_2PO_4, pH 7.2 in a Teflon tissue homogenizer (IKA 10®) at −4 °C. Homogenate was centrifuged at 10,500 g (HERMLE Z 323 K) for 30 min at 4 °C as described by Carvalho et al. (2020). After centrifugation, the supernatant was collected and immediately stored at −80 °C for biochemical determinations. All biochemical assays were carried out in a Synergy HTX, with the exception of the CAT that was performed on the Biochrom spectrophotometer at 25 °C, in triplicate.

The determination of protein concentration was according to Bradford (1976), using bovine serum albumin (BSA) as the standard at 595 nm. LPO was measured according to methods described by Jiang (1991) and the Cumenehydroperoxide (CHP) was used as a standard. The LPO was expressed in nmol of CHP per mg of protein. GSH was determined according to Beutler et al. (1963) using 0.01 M DTNB (5,5′-dithio-bis (2-nitrobenzoic acid)) (Ellman, 1959) with thiolate anion formation at 515 nm. CAT activity was determined according to Aebi (1974) by decomposing H_2O_2 in nmol per mg of protein per minute. SOD activity was determined by the method proposed by McCord and Fridovich (1969) and expressed in units per mg of protein per minute after SOD inhibited 50% of the cytochrome *c* reduction rate. GPx activity was determined according to Flohé and Gunzler (1984) and expressed in units per mg of protein per minute using the molar extinction coefficient of nicotinamide adenine dinucleotide phosphate (NADPH), 6,22 mM^{-1} cm^{-1}; GST activity was determined according to Keen et al. (1976) using GSH as a substrate and 1-chloro-2,4-dinitrobenzene (CDNB). The activity was expressed such as nmol of CDNB per mg of protein per minute at 340 nm, using the molar extinction coefficient, 9.6 mM^{-1} cm^{-1}.

The content of metallothionein (MT) was determined by concentration of sulfhydryl groups (SH), using reduced glutathione (GSH) according to the method described by Viarengo et al. (1997) using Ellman's reagent (DTNB-5,5-dithiobis-2-nitrobenzoic acid) at 412 nm. MT levels were expressed as moles-SH per mg of protein. Protein carbonylation (PCO) was determined using the method described by Levine et al. (1994), which is based on the reaction of carbonylated proteins using 2,4-dinitrophenylhydrazine (DNPH) and the molar extinction coefficient, 22 mM^{-1} cm^{-1} at 370 nm. The PCO concentration was expressed as nmol per mg of protein.

2.5. Morphological analysis of the liver

To analyze the liver parameters, the liver of each animal (N = 5 per group) was fixed in a Methacarn fixing solution (60% methanol, 30% chloroform 10% acetic acid), for 3 h, at 4 °C. Subsequently, the excess fixer was removed, dehydrated in an alcoholic series and embedded in historesin (Leica-historesin embedding kit). Sections of the 2 μm were obtained from a microtome (RM 2265, Leica, Switzerland) and stained with Hematoxylin-eosin, for morphological description and quantification of the melanin area, under a microscope (Leica DM4B) using an image capture system (Leica DMC 4500). Stereological analysis was performed using the Image Pro-Plus program, Media-Cybernetics Inc. (version 6.0) to determine the area occupied by melanin, according to the methodology proposed by Santos et al. (2014). For this quantification, 25 histological fields were analyzed for each animal, totaling 125 analyzed regions, per experimental group.

For morphometric analysis, random images (N = 10 images per animal; 1000x magnitude) captured in the same image capture system were used. Direct measurements of the hepatocyte perimeter (μm) and area (μm^2), perimeter (μm), area (μm^2), larger and smaller diameter (μm) of the 5-cell nucleus per image (n = 50/animal) were obtained. Additionally, the following indirect measures were taken: nucleus area/cytoplasm ratio (Ranc = nucleus area/cytoplasm area x 100). The nuclear volume of the hepatocyte was estimated by the formula: Vn (μm^3) = (4/3 x $\pi.r^3$), where r is the radius of the nuclear diameter (Freere and Weibel, 1966). The volume of the hepatocyte was calculated indirectly using the formula (VHep = (AHep.VNuc)/ANuc), where AHep = area of the hepatocyte (μm^2); VNuc = nuclear volume (μm^3) and ANuc = nuclear area (μm^2). Motic® 2.0 software (Motic Asia, Hong Kong) was used for this analysis.

2.6. Integrated Response of Biomarkers (IBR)

The mean values of each of the biomarkers were used to calculate the IBR, a methodology described by Beliaeff and Burgeot (2002) and modified by Sanchez et al. (2013) (IBRv2). Initially, the individual average of each biomarker (Xi) was divided by the respective control (Xo), followed by the transformation to log [$Yi = \log(Xi/Xo)$]. The mean (μ) and standard deviation (s) of Yi was calculated, followed by normalization of Yi [$Zi = (Yi\text{-}\mu)/s$]. The standardized response of the biomarker (Zi) and standardized response of the control (Zo) are used to define the deviation index of the biomarker (S) [$S = Zi\text{-}Zo$], used to create the graph in radar. The sum of the absolute value of S (A) represents the IBR value.

The IBR was calculated in different ways to determine: (1) the most sensitive biomarker in each organ/tissue (Individual IBR) - sum of the scores in each organ/tissue. (2) the most sensitive biomarker (general) (Total IBR) - sum of the scores in each analysis (biochemical or morphological) with subsequent standardization of the number of organs/tissues analyzed (three in the biochemical analysis and one in the morphological). (3) the organ/tissue most sensitive to treatment (Organ/Tissue IBR) - sum of all scores with standardization of the number of biomarkers analyzed. For the liver, the IBR average of the biochemical and morphological analysis was performed. (4) which treatment promoted the greatest response from biomarkers (Group IBR) - average of scores in each group. (5) which season (summer or winter) and which point (PI or PIR) promoted the greatest responses - sum of the IBR for each corresponding group (IBR value not shown in the table).

2.7. Statistical analysis

The concentration of metals (water and sediment) and the biomarkers were expressed as mean ± standard deviation (SD). The Kolmogorov and Levene tests were used to evaluate the normality and homogeneity of data, respectively. Two-way analysis of variance (ANOVA) with the post-hoc Tukey HSD test were used to determine whether there were differences in the individual biochemical variables among the sites (PI and PIR) and the seasons (Summer and Winter) and the interaction between these two factors. All tests were performed using SigmaStat 3.5 for Windows (Copyright © 2006, Systat Software, Inc.). $P < 0.05$ values were considered significant.

3. Results

3.1. Metals in water and sediment

The analyses of the water and sediment samples, from both periods, are shown in Tables 1 and 2, respectively. The water temperature and pH did not show significant changes during the exposure period; however, the temperature was higher in the summer (22–26 °C) and with a lower pH (6.7) compared to the winter (13–18 °C and pH 8.0). The physical-chemical parameters were within the values provided by the Brazilian Environmental Council Resolution (CONAMA, 357/2005) at the beginning of the exposure, except for ammonium, nitrite and phosphate in PI and nitrite in PIR that were significantly higher in winter. After 96 h of exposure, all parameters increased significantly compared to the control. The presence of Al, Cu, Mn and Zn in the water samples was found in concentrations below the limits established as safe by the CONAMA (2005) (Table 1). The prevalence of metals at the beginning and end of the exposure (0 and 96h, respectively) in the summer in PI_{0h} was Mn > Al > Zn > Cu and in PI_{96h} as Zn > Mn, in PIR_{0h} as Mn > Al > Zn > Cu and Al > Zn > Mn > Cu in PIR_{96h}. Al was detected at PI_{0h} and PIR_{0h}, with 29.10 μg/L and 27.50 μg/L, respectively, while in PIR_{96h}, the maximum mean value recorded was 116.5 μg/L, with a significant increase of about 4 times compared with the control and with PIR_{0h}. Cd was not detected in the water samples. Cu was recorded in all groups at the beginning and at the end of the exposure, except in PI_{96h}. There was a significant increase in Cu in the PIR_{96h} (1.6 μg/L). Mn was recorded in all groups, except for the control at the beginning of the exposure. PI and PIR showed the highest concentrations of this metal at the beginning of the exposure, with 31.3 μg/L and 32.4 μg/L, respectively, compared to the control. Zn was recorded in all groups but compared to the control (79.6 μg/L), the concentrations were lower in PI_{0h} and PIR_{0h} with 6.5 μg/L and 5.2 μg/L, respectively. At the end of the exposure, there was a significant increase in Zn concentration in PI (38.3 μg/L) and PIR (32.6 μg/L). However, the concentration of Zn in the two points was significantly lower than that observed in the control at the beginning and at the end of the exposure.

In the winter, the prevalence of metals in PI_{0h} was Al > Mn and in PI_{96h} as Al > Mn > Zn > Cu, in PIR_{0h} as Mn > Al and Mn > Zn > Cu in PIR_{96h} (Table 1). Al was detected in all groups, except at the beginning of the exposure in the control group and in PIR_{96h}. The concentration of Al increased significantly in PI_{0h} compared to the control and decreased significantly 80% after 96h compared to PI_{0h}. Cd was also not detected in any group. Cu was recorded in the control group and there was no significant difference between concentrations during the experiment. In PI_{96h} and $PIR_{96h,}$ there was a significant increase in this metal in relation to the control and compared to their respective groups at the beginning of the exposure. Mn was detected in all groups, except in the control at the beginning of the exposure. Mn concentrations were significantly higher (about 3–5 times) in PI_{0h} (93.64 μg/L) and PIR_{0h} (86.78 μg/L), compared to PI_{96h} and PIR_{96h}, respectively. The Zn concentration was higher in the control group and increased significantly after 96h. Zn was not recorded in PI_{0h} and PIR_{0h}, however, after 96h the concentrations were PI_{0h} and PIR_{0h}, 11.56 μg/L and 4.20 μg/L, respectively. In PIR_{96h}, the mean value of Zn was lower in comparison with the control and with PI_{96h}. Comparing the results obtained in the two periods, the concentrations of Al and Mn were higher in the winter compared to the summer, followed by Zn and Cu (Al = Mn > Zn > Cu).

The results of the analysis of metals in the sediment are shown in Table 2. In the summer, the concentrations of Al, Cu and Mn were significantly higher (about 1–5 times) in the PIR in relation to the PI. In the winter, the concentrations of Cu and Mn were significantly higher (about 1–3.5 times) in the PIR compared to PI, and Al and Zn were the metals with the lowest concentrations in relation to PI. The prevalence of metals in the summer in PI was Al > Zn > Mn = Cu and in PIR it was Al > Mn > Cu > Zn while in the winter, the ratio in PI was Al > Zn > Mn = Cu and in PIR it was Al > Cu > Mn > Zn. Comparing the different periods, the concentrations of metals in the winter were significantly lower, around 20–50% in relation to the values registered in the summer.

3.2. Biochemical parameters

After the exposure to Sorocaba river water, there was a significant difference in biochemical parameters in the liver of the tadpoles, and interactions between the two variables (groups and periods) was found for all enzymes and in GSH and PCO (Fig. 2). In the summer, GSH increased about 2.5 times in PIR in relation to the control and was higher compared to PI. CAT, SOD, GPx and GST decreased activity in PIR compared to the control and also their activities were significantly lower compared to PI, except GPx. The PCO levels decreased in PIR by about 30–60% relative to the control and PIR compared to PI. In the winter, CAT increased significantly (about 1.5x higher) in PIR compared to the PI. GPx (both PI and PIR)

Table 1

Physico-chemical parameters and metal concentrations (μg/L) in water samples collected in the summer and winter from Ibiúna (PI) and the Itupararanga Reservoir sites (PIR) of Sorocaba River and the values established by the Brazilian Environmental Council (CONAMA), resolution 357/2005. The significant difference is shown in bold compared to the control in the same period; # indicates a significant difference compared to PI and PIR. LQ = limit of quantification; ↑ ↓ indicates a significant difference in relation to the initial (0h) and end of exposure (96h) time.

Variables	CONAMA	Control		PI		PIR	
Summer	Class I and II waters	Time 0h	Time 96h	Time 0h	Time 96h	Time 0h	Time 96h
Temperature (^{o}C)	–	25.0 ± 1.0	23.6 ± 1.4	26.5 ± 2.3	24.1 ± 1.5	24.4 ± 1.0	22.7 ± 1.5
pH	6.0–9.0	6.8	6.6	7.7	6.7	6.7	6.7
Hardness (mg/L)	–	50	50	50	50	50	57
Ammonium (mg/L)	0.02	<0	4.5 ± 0.3↑	<0	4.5 ± 0.00↑	<0	6.5 ± 0.5↑
Nitrate (mg/L)	10	<0	6.67 ± 0.03↑	5.0 ± 0.02	**10.0 ± 0.02**↑	<0	**20 ± 0.03**$^{\#}$↑
Nitrite (mg/L)	1	<0	0.25 ± 0.00	<0	0.5 ± 0.02	<0	**1.25 ± 0.32**$^{\#}$↑
Phophate (mg/L)	0.15	1.5 ± 0.03	1.17 ± 0.06	**0.1 ± 0.00**	1.67 ± 0.32↑	**0.1 ± 0.00**	1.67 ± 0.29↑
Aluminium (μg/L)	100	<LQ	<LQ	29.10 ± 1.15	<LQ	27.50 ± 0.98	116.5 ± 7.1↑$^{\#}$
Cadmium (μg/L)	1	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ
Copper (μg/L)	9	1.3 ± 0.03	2.2 ± 0.15↑	1.5 ± 0.05	<LQ	1.0 ± 0.10	1.6 ± 0.09$^{\#}$↑
Manganese (μg/L)	100	<LQ	25.1 ± 1.45↑	**31.3 ± 2.01**	28.2 ± 1.52	**32.4 ± 2.62**	28.2 ± 1.89
Zinc (μg/L)	180	79.6 ± 3.35	71.8 ± 2.34	**6.5 ± 0.45**	38.3 ± 2.81↑	**5.2 ± 0.89**	**32.6 ± 1.9**↑
Winter	Class I and II waters	Time 0h	Time 96h	Time 0h	Time 96h	Time 0h	Time 96h
Temperature (^{o}C)	–	24.0 ± 1.0	23.3 ± 1.0	**13.7 ± 1.0**	23.7 ± 0.6↑	**16.5 ± 1.5**	21.0 ± 1.5↑
pH	6.0–9.0	6.7	6.8	7.3	6.9	**8.0**	6.9↓
Hardness (mg/L)	–	45	46	**28**	**32**	41$^{\#}$	46$^{\#}$↑
Ammonium (mg/L)	0.02	0.29 ± 0.04	1.32 ± 0.04	**0.04 ± 0.00**	1.65 ± 0.24↑	**0.02 ± 0.00**	1.20 ± 0.42↑
Nitrate (mg/L)	10	0.20 ± 0.05	0.44 ± 0.03↑	0.10 ± 0.01	**0.32 ± 0.02**↑	**1.01 ± 0.23**	**1.92 ± 0.03**$^{\#}$↑
Nitrite (mg/L)	1	9.86 ± 1.30	4.46 ± 1.37↓	7.79 ± 2.05	2.96 ± 0.87$^{\#}$↓	**2.08 ± 0.36**$^{\#}$	4.84 ± 0.61↑
Phophate (mg/L)	0.15	0.06 ± 0.02	0.56 ± 0.01↑	**0.20 ± 0.00**	**0.24 ± 0.03**	0.07 ± 0.00	0.48 ± 0.06$^{\#}$↓
Aluminium (μg/L)	100	<LQ	3.42 ± 5.92↑	**111.79 ± 0.59**	**20.47 ± 3.93**↓	**54.32 ± 0.59**$^{\#}$↓	<LQ
Cadmium (μg/L)	1	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ
Copper (μg/L)	9	4.97 ± 0.90	4.72 ± 1.0	<LQ	**2.45 ± 0.66**↑	<LQ	**2.27 ± 0.23**↑
Manganese (μg/L)	100	<LQ	27.72 ± 7.67↑	**93.64 ± 9.75**	18.27 ± 8.37↓	**86.78 ± 6.65**	28.47 ± 1.41
Zinc (μg/L)	180	15.97 ± 1.29	23.85 ± 2.83↑	<LQ	11.56 ± 0.59$^{\#}$↓	<LQ	**4.20 ± 0.26**$^{\#}$↓

Table 2

Metal concentrations (mg/Kg) in sediment samples collected in the summer and the winter from Ibiúna (PI) and the Itupararanga Reservoir sites (PIR) of Sorocaba River and the values established by the Brazilian Environmental Council (CONAMA), resolution 357/2005. The significant difference is shown in bold compared to the control in the same period; # indicates a significant difference compared to PI and PIR. LQ = limit of quantification.

Sediment	CONAMA	Summer		Winter	
		PI	PIR	PI	PIR
Aluminium (mg/kg)	–	105.49 ± 0.60	**182.81 ± 1.58**	78.05 ± 4.6$^{\#}$	**66.41 ± 3.52**$^{\#}$
Cadmium (mg/kg)	0.6	<LQ	<LQ	<LQ	<LQ
Copper (mg/kg)	35.7	0.61 ± 0.08	**2.03 ± 0.31**	0.49 ± 0.03$^{\#}$	**1.70 ± 0.23**
Manganese (mg/kg)	–	0.84 ± 0.04	**4.01 ± 0.47**	0.55 ± 0.03$^{\#}$	**0.95 ± 0.10**$^{\#}$
Zinc (mg/kg)	123	1.02 ± 0.05	**0.83 ± 0.10**	0.71 ± 0.04$^{\#}$	**0.57 ± 0.05**$^{\#}$

and GST (only in PI) increased in relation to the control and GST was lower in PIR than PI. The significant differences in the biochemical parameters GSH, LPO, CAT (only in the control and PI), SOD, GPx, GST, MT and PCO levels in the control, PI and PIR, evidenced the seasonal influence between summer and winter in the reduction for most these parameters in winter, except for GPx (in PI and PIR) and GST (only in PIR), which increased in winter.

There was a significant difference in the biochemical parameters in the kidney of tadpoles exposed to Sorocaba river water, and there was also an interaction only between LPO, GSH and GST between the points and the periods (Fig. 2). In the summer, LPO levels were significantly lower at both points and GSH was higher in PI than in control and PIR while GST in PI and PIR were lower than control. In winter, LPO increased in PI versus control and PIR. The bidirectional analysis showed an influence between the different groups and the periods with decreased LPO, GSH, SOD and GST in almost all groups.

The tadpole muscle exposed to the water of the Sorocaba river showed a significant difference in the biochemical parameters as well as interaction in LPO, GSH, CAT, GST and PCO between the two points and two periods (Fig. 2). In the summer, GSH and the GST enzymes increased (about 2-5x higher) in PI and PIR, while PCO decreased compared to the control. In the winter, CAT activity decreased about twice in PIR compared to the control and PCO was significantly higher in PIR in comparison with the control and PI. Exposure in different types of water and seasonality clearly influenced LPO, GSH and PCO and the enzymes CAT, SOD, GPx and GST.

3.3. Liver morphological parameters

Liver parameters are altered after Sorocaba river water exposure and the effects of these parameters were shown to be dependent on exposure to water of PI and PIR and the periods. In the summer, the hepatocyte area and volume, nucleus area and volume (only in PIR) increased in PI and PIR, and PIR was bigger than PI (Figs. 3 and 4) and the hepatic melanin decreased only in PIR (Figs. 3 and 4). In the winter, the area and volume of the hepatocytes and their nucleus decreased in PI and PIR; in all hepatic morphometric variables were there a difference between both points (summer and winter).

3.4. Integrated Response of Biomarkers - IBR

The IBR values are observed in Tables 3 and 4 and Fig. S1 (supplementary material). The kidneys have the highest IBR value (4.23) followed by the liver (3.81) and muscle (3.79). In general,

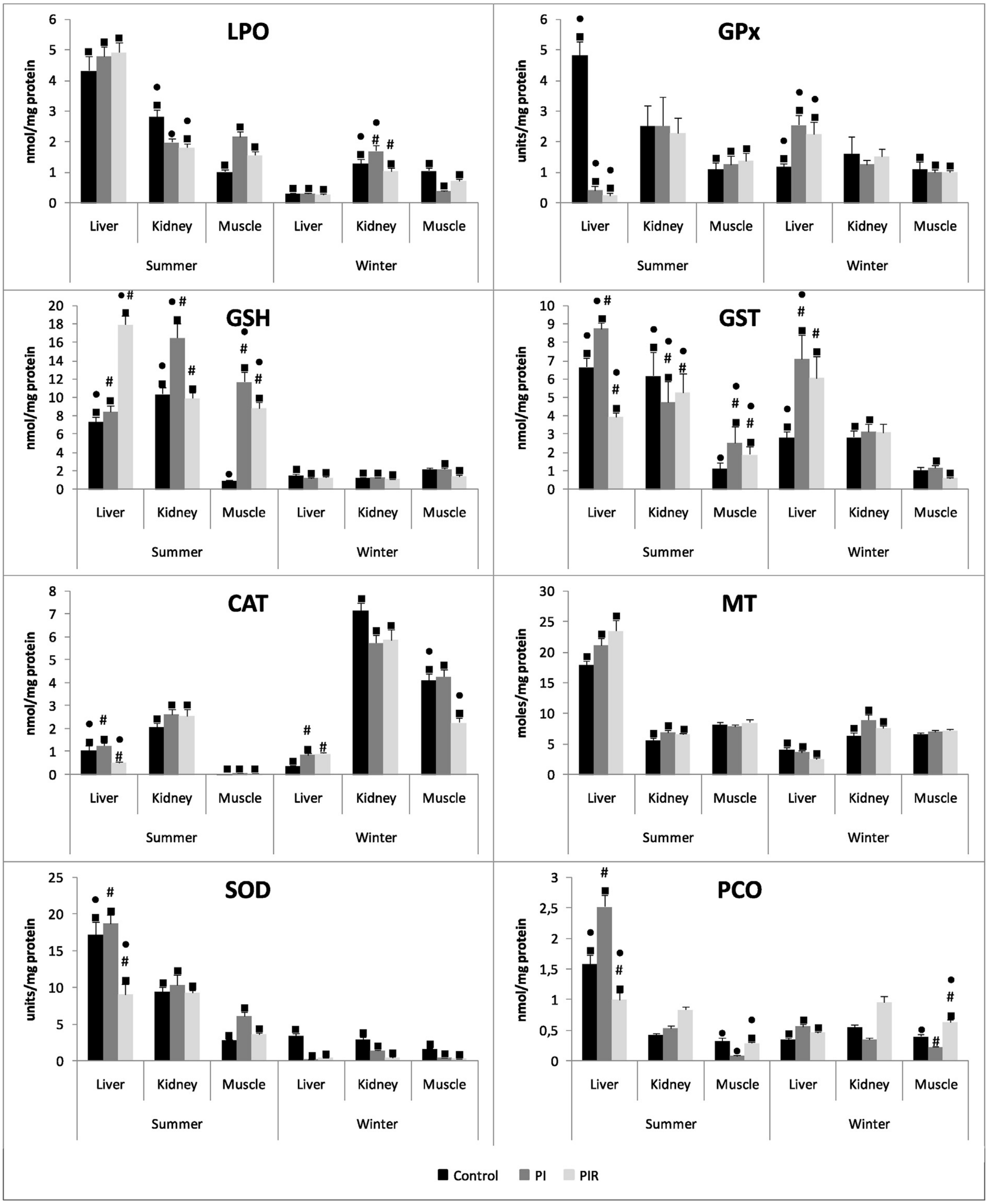

Fig. 2. Oxidative stress parameters in *L. catesbeianus* tadpole organs exposed to water from the Sorocaba river: PI = Ibiúna point and PIR = Itupararanga Reservoir point in summer and winter for 96h. Values are represented as the mean standard error. Groups that share the same symbol present a significant difference ($p < 0.05$): ● represents a difference compared with their respective controls; # represents a difference compared with PI and PIR; ■ represents a difference compared with the summer and the winter; (n = 8).

biomarkers were more sensitive to water exposure in the PIR (31.33) than in PI (27.48), with biochemical biomarkers (3.97) being more sensitive than morphological ones (3.73). Individually MT was the most sensitive biomarker (5.01), followed by PCO (4.05), GST (3.98), LPO (3.97), hepatocyte area (3.88), GPx (3.87), nucleus area (3.85), SOD (3.80), hepatocyte volume (3.78), CAT (3.77), nucleus volume (3.66), melanin area (3.38) and GSH (3.29).

4. Discussion

The evaluation of the physical and chemical parameters of the Sorocaba River revealed a growing condition of anthropic

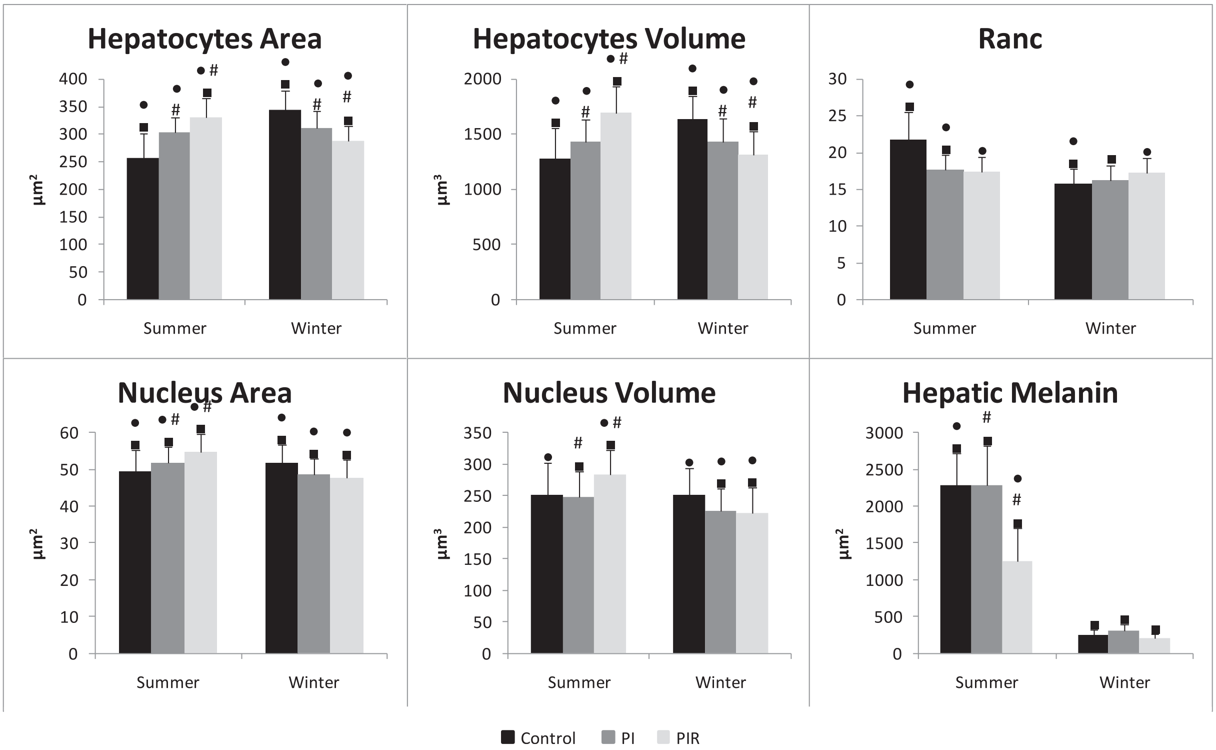

Fig. 3. Hepatic morphometric variables in *L. catesbeianus* tadpole exposed to water from the Sorocaba river: PI = Ibiúna point and PIR = Itupararanga Reservoir point in the summer and the winter for 96h. Values are represented as the mean standard error. Groups that share the same symbol present a significant difference ($p < 0.05$): ● represents a difference compared with their respective controls; # represents a difference compared with PI and PIR; ■ represents a difference compared with the summer and the winter; (n = 5). Ranc = nucleus cytoplasm ratio.

interference and the changes in these factors can influence the bioavailability of metals and, therefore, their toxicity. The available forms of nitrogen in water (for example, nitrate or nitrite), have consequences for the environment and living beings since the occurrence of diseases or toxicity of free ammonia such as the reduction of dissolved oxygen. Thus, according to Sardinha et al. (2008) the large amount of ammonia and phosphate in the Sorocaba River can be considered as a triggering factor in the eutrophication process of these waters. The increase in ammonia and phosphate is of anthropic origin (Sardinha et al., 2008; Conceição et al., 2011, 2015), therefore, the difference in these factors between PI and PIR may be due to the release of domestic and industrial effluents, detergents, excrement of animals and use of fertilizers.

Metals are considered very toxic substances due to their distribution, persistence, accumulation and biomagnification in the food chains with their concentrations varying in water, sediments and aquatic organisms (Atli and Canli, 2010; Authman et al., 2012; Kelepertzis et al., 2012; Javed and Usmani, 2019). Observed levels of Cd, Pb, Cu, Cr, Fe, Mg, Mn and Zn were above those allowed by the CONAMA Resolution, in the water of the Salto Grande reservoirs (SP) (Espíndola et al., 2005), and Rocha and Azevedo (2015) who verified the presence of As, Cd, Cu, Pb, Hg and Zn in the São Córrego basin, attributed to the dumping of toxic materials by industries and household waste. The metals investigated in the water and sediment of the Sorocaba River presented concentrations below the limit established as safe by resolution CONAMA 357/05, however the presence of these metals was harmful to bullfrog tadpoles.

In the Summer, the levels of metals in the PI and PIR sites, at the beginning of the exposure, was similar such as Mn > Al > Zn > Cu, but at the end of the exposure, after 96h, were different with Zn > Mn in PI and Mn > Al > Zn > Cu in PIR. In contrast, in the Winter the Al and Mn prevailed in PI and PIR at the beginning and, after 96h, the Al > Mn > Zn > Cu were detected in PI and Mn > Zn > Cu in PIR. The decreased concentrations of Al, Cu and Mn, after 96h of exposure, detected in both places and in the summer and winter, reflects seasonal environmental changes associated with the bioavailability of contaminants and abiotic factors. Although the presence of metals in tadpoles has not been determined, the changes in biomarkers in the different organs of these animals may indicate that these were absorbed by the tadpoles. Al can have harmful effects on the aquatic environment, as well as on humans who can consume fish containing high levels of this metal (Casarini et al., 2001; Camargo et al., 2009) and its inorganic form has been reported as the most harmful to several species of fish (Camargo et al., 2009). Cu and Mn are essential in several physiological process and play important functions for living organisms (Crossgrove and Zheng, 2004; Carvalho et al., 2015; O'Neall and Zheng, 2015; Carvalho et al., 2017; Anni et al., 2019). However, in high concentrations, Cu promotes oxidative stress, with increased LPO (Carvalho et al., 2015; Tesser et al., 2020) and Mn may cause progressive and permanent neurodegenerative damage (Crossgrove and Zheng, 2004; O'Neall and Zheng, 2015).

Various studies report the ability of these aquatic organisms to concentrate metals in their tissues, such as the studies by Stolyar et al. (2008), who compared the bioavailability of metals in frogs, *Pelophylax ridibundus* (syn. *Rana ridibunda*), from urban and rural locations in western Ukraine and found that animals from both locations accumulated high concentrations of metals despite the metal concentrations in the water being below the detection limit. Kelepertzis et al. (2012), who verified in tadpoles of the *Pelophylax kurtmuelleri* accumulated significant levels of Cu, lead (Pb), Zn, magnesium (Mn), Cd, nickel (Ni), and chromium (Cr) inhabiting the metalliferous streams of the Asprolakkas River basin (northeast

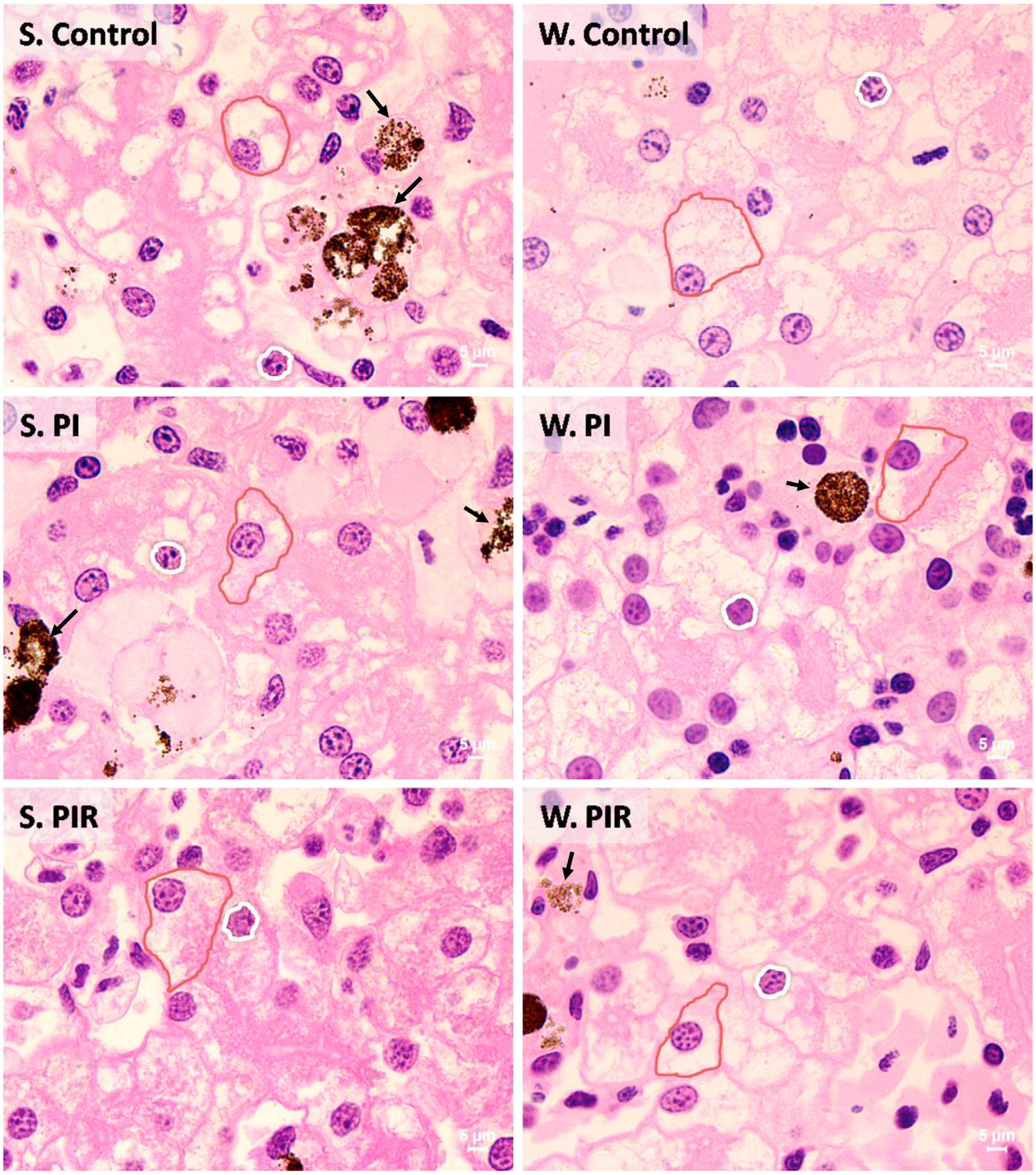

Fig. 4. Liver sections of *L. catesbeianus* stained in HE. S and W: Summer and Winter; PI = Ibiúna point and PIR = Itupararanga Reservoir point. In tadpoles exposed to S. PI and S. PIR. Morphometric analysis of the liver showing the area of the hepatocyte (red) and the nucleus (white). The black arrows indicate the melanomacrophage. Bars - 5 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Chalkidiki peninsula, Greece) (Kelepertzis et al., 2012) and Carvalho et al. (2017) who exposed tadpole bullfrogs, *L. catesbeianus* in Zn, Cu and Cd and found that these metals accumulated in the liver, kidney and muscle.

Amphibians can absorb metals through the skin directly from water and also through the digestive system from ingested food and accidentally from soil and sediments (Papadimitriou and Loumbourdis, 2002; Kelepertzis et al., 2012). Therefore, although the concentration of metals is below the limit allowed by the CONAMA Resolution, their presence in the environment can negatively impact the animals that live there, as verified by Abdel-Moneim et al. (2012), who determined the presence of metals such as Pb, Cd, Hg, Cu and Cr lead in various river locations in Al-Hassa, Saudi Arabia and also accumulation of these metals in the liver, kidney and gills of Nile tilapia, *Oreochromis niloticus*, as well as abnormalities in the gills and livers of these animals. In addition, higher concentrations of metal in the water at the end of the exposure (96 h) indicate that bullfrog tadpoles have accumulated metals from some other source, probably from organic matter contained in the water and did not detect at the beginning of the exposure and/or that they were intermittently exposed to the metals previously. According to Stolyar et al. (2008), in metal-free water, amphibians can take longer to purify metals in liver and kidneys and, their increase in water can be due to the elimination of other organs such as skin, intestine and muscle.

Regarding seasonal variations, for Al, the maximum mean value was measured in PIR_{96h} (116.5 μg/L) and the minimum mean value was recorded in PI_{96h} (20.47 μg/L) in the Summer and Winter, respectively. The minimum average concentration of Cu was recorded in the summer at PIR_{0h} (1.0 μg/L), while the maximum was observed in the winter in the $control_{0h}$ (4.97 μg/L). The highest mean value of Mn was recorded during winter in PI_{0h} (93.64 μg/L),

Table 3
Standardized biochemical biomarker responses and integrated biomarker response (IBR) values in water samples collected in the summer and winter from Ibiúna (PI) and the Itupararanga Reservoir sites (PIR) of Sorocaba River. In bold are the values of IBR observed for each site, and the highest contributing biomarker scores for the IBR value. * mean liver IBR between biochemical and morphological analyzes.

Season/site	Organ/Tissue	Biomarkers score (Biochemical analyses)								**Group IBR**
		LPO	GSH	CAT	SOD	GPx	GST	MT	PCO	
Summer.PI	Liver	1.42	0.31	0.25	0.07	1.38	0.46	0.58	1.20	0.71
Summer.PIR		1.80	1.92	1.06	0.46	1.67	0.89	0.94	1.17	1.24
Winter.PI		0.00	0.57	1.27	2.17	0.43	1.58	0.37	1.20	0.95
Winter.PIR		0.48	0.45	1.37	1.60	0.36	1.31	1.65	0.70	0.99
Individual IBR		3.71	3.24	3.94	4.29	3.85	4.24	3.52	4.27	
Organ IBR		3.88 (3.81*)								
Summer.PI	Kidney	1.22	2.04	1.10	0.12	0.00	1.65	1.68	0.55	1.04
Summer.PIR		1.53	0.20	0.96	0.02	1.08	1.02	1.24	1.51	0.95
Winter.PI		0.95	0.18	1.01	0.90	2.39	0.65	2.79	0.99	1.23
Winter.PIR		0.77	0.49	0.92	2.24	0.46	0.51	1.45	1.22	1.01
Individual IBR		4.47	2.91	3.99	3.28	3.93	3.84	7.16	4.27	4.23
Organ IBR		4.23								
Summer.PI	Muscle	1.11	1.80	1.08	0.77	0.93	1.57	0.86	2.01	1.27
Summer.PIR		0.63	1.60	0.60	0.23	1.60	1.03	0.61	0.18	0.81
Winter.PI		1.45	0.00	0.11	1.26	0.62	0.23	1.20	0.78	0.71
Winter.PIR		0.53	0.31	1.57	1.56	0.69	1.03	1.68	0.64	1.00
Individual IBR		3.72	3.72	3.37	3.82	3.85	3.86	4.35	3.62	3.79
Tissue IBR		3.79								
Total IBR		3.97	3.29	3.77	3.80	3.87	3.98	5.01	4.05	

Table 4
Standardized biomarker, morphological, responses and integrated biomarker response (IBR) values in water samples collected in the summer and winter from Ibiúna (PI) and the Itupararanga Reservoir sites (PIR) of Sorocaba River. In bold are the values of IBR observed for each site, and the highest contributing biomarker scores for the IBR value. * mean liver IBR between biochemical and morphological analyzes.

Season/site	Organ	Biomarkers score (Morphological analyses)						**Group IBR**
		Hepatocyte area	Nucleus area	Hepatocyte volume	Nucleus volume	Ranc	Melanin area	
Summer.PI	Liver	0.93	0.63	0.60	0.09	1.43	0.00	0.61
Summer.PIR		1.40	1.36	1.43	1.25	1.55	1.96	1.49
Winter.PI		0.57	0.81	0.67	1.07	0.25	0.65	0.67
Winter.PIR		0.99	1.05	1.08	1.24	0.62	0.76	0.96
Individual IBR		3.88	3.85	3.78	3.66	3.85	3.38	
Organ IBR		3.73 (3.81*)						
Total IBR		3.88	3.85	3.78	3.66	3.85	3.38	

and the lowest in PI_{96h} (18.27 μg/L) in the same period. The minimum value of the Zn concentration was recorded during the winter in PIR_{96h} (4.20 μg/L) and the maximum value during the summer in the $control_{0h}$ (79.60 μg/L). Contrary to what was observed, the study by Chiba et al. (2011) in a sub-basin of São Carlos-SP, Southeastern Brazil, found a higher concentration of Cd, Mn, Zn and Fe associated with greater rainfall, resulting in the accumulation of metals and also due to the presence of industries close to the collection site. Souza et al. (2016), registered the presence of Cd, Cr, Cu, Fe, Mn, Ni, Pb and Zn in the water of the São Francisco River lower basin, with Cd, Cr, Ni and Cu at concentrations above the permitted CONAMA during periods of highest rainfall of the hydrographic network. On the Sorocaba River, the studies by Pedrazzi et al. (2014), who verified the presence of Al, Mn, Ni, Pb and Zn in concentrations below the maximum values allowed for Class 2 waters by CONAMA Resolution 357 (2005) in the summer and that of Conceição et al. (2015), who found that the concentrations of these metals were above the values established by legislation in both the winter and summer. The higher concentrations of metals in winter compared to summer may be due to fluctuations in the amounts of water discharged from agricultural drainage, untreated illegal domestic sewage and industrial waste (Authman et al., 2012; Javed and Usmani, 2019). These differences can also be attributed to the rains in autumn and winter that occur most frequently and the metals are washed away by rainwater to the adjacent aquatic systems (Khallaf et al., 1994; Dixit and Tiwari, 2008; Authman et al., 2012; Javed and Usmani, 2019). The Cd was not detected at any point and period, and was below the limit of quantification in all the analyses, however this metal is toxic and has agricultural origin and it can be emitted to soil and water by using phosphate fertilizers (Tokatli, 2019). A possible explanation for its non-detection is the seasonal rainfall variation, which can decrease the amount of contaminants and/or close to the collection sites there is no presence of electronic companies which requires Cd for the production of its components (Idowu et al., 2014).

Metals can be distributed in different compartments of aquatic ecosystems, water, sediment and the trophic chain through a process of bioaccumulation resulting from assimilation and elimination rates. Once inside the animal, the metal can interfere with the normal physiology of the organism causing damage. Our results show the presence of Al, Cu, Mn and Zn in higher concentrations in the sediment compared to those found in the water of the Sorocaba river at the analyzed points and periods, however the PIR sediment presented the highest concentrations of metals in the summer and indicate that sediments can provided the bulk of these metals for tadpoles and/or other organisms. Variations in metal concentrations can also be attributed to sedimentary origin in addition to the influence of industrialization. Metals can be concentrated in the sediments of reservoirs, rivers and lakes and are considered as a compartment for the accumulation of contaminants and indicators of aquatic contamination (Bonai et al., 2009; Kelepertzis et al., 2012; Wu et al., 2014; Guan et al., 2018). Oliveira et al. (2018) recorded the presence of Cr and Ni in the sediment of Lake Água Preta (PA), in concentrations that characterize contamination, while Cd, Co and Zn were in concentrations below what was considered toxic. Rabello et al. (2018) found metals in the superficial sediments of the Rodrigo de Freitas lagoon (RJ) such as Hg, Zn, Cu, Pb, Ni, Cr, As and Cd. In the present study, the concentrations of Cu, Mn and Zn detected in the Sorocaba river sediment were higher than those reported by Espíndola et al. (2005) in the sediment of the Salto Grande reservoir (SP) and by Bonai et al. (2009) in the Itá reserve (SC). In this case, although the metals in the water are in concentrations below that allowed by CONAMA, the animals that live in this environment are continuously exposed to these chemical compounds mainly via sediment. In addition, the toxicity of metals in the sediment can be affected by the connections between the metal ions, the constituents of the sediment and their availability (Bonai et al., 2009). Thus, metals with a weak association linked to the sediment can be easily broken by biota, becoming bioavailable (Bonai et al., 2009; Kelepertzis et al., 2012). Therefore, despite the fact that the bioassays were in the water of the Sorocaba River, analyzing the presence of metals in the sediment is essential and important in the assessment of aquatic ecosystems. Thus, although metals are in low concentrations, both in water and in sediment, their presence is evidence of population and industrial growth in the region.

The variation in the biochemical parameters in PI and PIR, in the summer and winter, shows that metals caused toxic effects and seasonal variation, even at low concentrations. The liver is the organ responsible for detoxification and excretion and, therefore, is the target of several contaminants in water, including metals. In PI-summer, the decrease in GPx and induction of increased GST in the liver shows a possible change to a detoxification mechanism. While in PIR the effect of metals seems to have been greater with an increase in GSH and a decrease in enzymes and PCO. These results can be attributed to the presence of metals in the water and by their connection to the sulfhydryl groups (-SH) in proteins and/or enzymes with an increase in radicals such as superoxide and H_2O_2 in cells. The reduction in CAT and SOD activities may be due to an increase in superoxide radicals associated with the interruption of antioxidant defenses, as described in fish by Falfushynska and Stolyar (2009). CAT is a heme-enzyme that contains iron (Van der Oost et al., 2003) and catalyzes H_2O_2 into molecular oxygen (O_2) and water (H_2O). SOD facilitates the breakdown of superoxide anions ($O_2^-\bullet$) by converting it to H_2O_2, which is subsequently catalyzed in water and O_2 by CAT in high concentration and/or GPx in low concentration. In the presence of transition metals, such as iron and Cu, $O_2^-\bullet$ and H_2O_2 can generate hydroxyl radical ($\bullet$OH) through the Fenton reaction (Storey, 1996). In addition, other metal ions may also be involved in the generation of ROS (Stohs et al., 2000) and which were not analyzed in this study but which were recorded in Sorocaba River (Pedrazzi et al., 2014; Conceição et al., 2015), indicating its persistence in aquatic environments. GPx and GST represent enzymes dependent on reduced glutathione (GSH) because they use this tripeptide as a cofactor for enzymatic reactions (Halliwell and Gutteridge, 2007). GST catalyzes the conjugation of GSH with various substances in phase II detoxifying and plays a role in preventing oxidative damage by conjugating the decomposition products of lipid peroxides to GSH (Van der Oost et al., 2003; Carvalho et al., 2015). GST decrease is correlated to lipid oxidative damage and suggests extensive oxidative damage affecting antioxidant defenses in tadpoles exposed in water of the PIR during the summer.

Exposure to water from the PIR showed a decrease in PCO. Studies by Grune et al. (2003) showed that at moderate concentrations of oxidant, the degradation of damaged proteins increases, while higher concentrations of oxidant can inhibit proteolytic degradation. According to Almroth et al. (2005) exposure to the contaminant can increase the rate of PCO formation and inhibit, at the same time, the activity of the proteasome (decrease in proteolysis) resulting in the accumulation of insoluble aggregates. Therefore, changes in PCO levels can serve as biomarkers of environmental contamination, however, an increase can be the more serious of the responses.

In the winter, the effects of metals were similar in the liver, both in PI and in PIR, with increased GPx and GST. The induction of GPx and GST of tadpoles liver indicates detoxification processes and can also lead to an inefficient regeneration of GSH by glutathione reductase (GR), increased levels of cell alterations and persistence oxidative stress caused by exposure to water from the Sorocaba River. Studies also attribute the induction of GST to the presence of xenobiotics, such as polycyclic aromatic hydrocarbons (PAHs) (Ahmad et al., 2005) and metals (Monteiro et al., 2010; Carvalho et al., 2012). The activation of this enzyme can confer resistance to the tadpoles to the toxicity of metals and can also be important in the fight against ROS because it has peroxidase activity. Similar results for GST were found by Gabriel et al. (2013) in the fish *Colossoma macropomum* exposed to Mn and Veronez et al. (2016) that addressed the effect of iron ore, Fe and Mn, on the liver of tadpoles, *L. catesbeianus*.

Metals caused oxidative damage to proteins in the liver of bullfrog tadpoles. PCO is formed by the direct oxidation of amino acid side chains by metals or ROS and also by the action of lipid peroxidation products (Stadtman and Oliver, 1991; Vasconcelos et al., 2007) and, our results show a scenario of oxidative stress (antioxidant enzymes, PCO and GSH) in the liver of animals exposed to water from the Sorocaba River. However, the non-change in the LPO can be attributed to the action of GPx (Veronez et al., 2016; Boiarski et al., 2020) or other antioxidant defense, while the increase in PCO indicates the action of metals in proteins, reduced ability to protect against H_2O_2 and other non-radicals being eliminated by antioxidant enzymes. In addition, the formation of PCO is irreversible leading to changes in the conformation of the protein, causing a loss and/or decrease in the catalytic activity of the enzymes causing proteolytic degradation of the protein. Requena et al. (2003) demonstrated that 55–100% of PCO is derived from specific chemical structures resulting from, for example, reactions catalyzed by metal or other biochemical pathways such as adduction of oxidized lipids or sugars containing carbonyls.

Tadpoles' kidneys can serve as valuable indicators of environmental pollution (Medina et al., 2016; Carvalho et al., 2017, 2020; Chagas et al., 2020) and suggests that this organ is an important target for metals. The metals caused a decrease in LPO and GST and an increase in GSH in the summer, both in PI and in PIR, demonstrating that they are suitable biomarkers, and are capable of providing early identification of the presence of contaminants in the water. While in the winter, the effects were different with an increase in LPO indicating a scenario of oxidative stress caused by metals. Differences for the same parameter can occur in environmental studies. This may be due to specific functions of the organs and must consider that the water both in PI and in PIR, after 96h, had high concentrations of Al, Cu and Zn indicating that these metals were previously absorbed by the tadpoles showing intense activity of the kidney to excrete them. However, these results can lead to injury and renal dysfunction due to the toxic effects of these metals, as shown by Hadi and Alwan (2012) who demonstrated in fish, *Tilapia zillii* histopathological changes in the kidneys caused by

Al.

The significant increase, in muscle, of GSH accompanied by the increase of GST enzyme and changes of PCO, show a picture of oxidative stress in PI and PIR in the summer. The maintenance of high constitutive levels of these biomarkers is essential to prevent the action of radicals mediated by LPO. In the winter, exposure to water from PIR decreased CAT and increased PCO which may be related to the action of metals present in water in these molecules and can induce severe disturbances of CAT activity in the muscle by binding these metal ions to −SH groups of the enzyme. The decrease in CAT activity may also be due to the flow of superoxide radicals, which inhibit the activity of this enzyme. Atli et al. (2006) found in *O. niloticus* exposed to various concentrations of Ag, Cd, Cr, Cu and Zn different responses in CAT activity depending on tissues, metals and their concentrations. Different results were reported on the CAT activity in amphibians exposed to metals (Peltzer et al., 2013; Prokić et al., 2016; Carvalho et al., 2020) and exposed to water from urbanized stretches and rural areas of the Cascavel River (Boiarski et al., 2020). According Hermes-Lima (2005) and Carvalho et al. (2012) CAT is more effective in controlling oxidative stress when intracellular concentrations of H_2O_2 are high and is considered a sensitive biomarker of exposure to metals in fish (Atli et al., 2006).

GSH is important in cell protection against reactive oxygen metabolites, serving as a substrate for GPx and GST. The increased demand for cysteine residues, such as GSH synthesis, during the detoxification of contaminants present in the Sorocaba River suggests a reduction (and/or maintenance) in the synthesis of MT. The significantly higher levels of PCO in PIR during the winter indicate that the tadpoles were suffering from oxidative stress as a result of oxidation catalyzed by metals. The resulting damaged protein is more susceptible to degradation and may lose some or all of its functions. Interestingly, there was no induction of MT in the tadpole muscle in both periods and PI and PIR, this result may be associated with increased GSH. According to Almroth et al. (2008) MT concentrations vary in field studies, as levels can be influenced by biotic factors (age, organ, reproductive status, season) and abiotic factors (temperature, pH, salinity). Thus, MT levels may reflect the bioavailability and pharmacokinetics of the metal uptake organ or MT synthesis. The tadpoles are particularly sensitive to chemical contamination, and also show the susceptibility of muscle due in part to stress during metamorphosis (Sorensen, 1991; Boiarski et al., 2020; Carvalho et al., 2017; Chagas et al., 2020).

Morphological changes observed in the liver of tadpoles may be related to the direct effects of ROS induced by metal in these hepatocytes and in their plasma membrane, which can generate tissue changes compromising tissue physiology. The amount of melanin decreased in PIR in the summer, but it was the lowest in the liver of tadpoles exposed in the winter. According to Franco-Belussi et al. (2020a, 2020b) melanin absorbs and neutralizes free radicals, participates in the innate immune response and protects tissues in ectotherms. Thus, tadpoles are more sensitive and vulnerable to environmental contaminants. The liver is the main metabolic organ where detoxification occurs and the intensity of the effects can impair its functioning as well as other organs, such as kidneys and muscles. Histological lesions, due to metals, have also been reported previously in fish liver of *Channa punctatus* exposed to hexavalent chromium (Mishra and Mohanty, 2008), sugar mill effluents (Javed et al., 2016) and *Tilapia zilli* exposed to Al (Hadi and Alwan, 2012) and amphibians such as *Rhinella arenarum* exposed to Cd (Medina et al., 2016) and *L. catesbeianus* exposed to water from an urban stream (Boiarski et al., 2020). Morphometrics parameters of hepatocytes (i.e., hepatocytes and nucleus area) varies in exposed group. These alterations can reflect cytotoxic effects water compounds in hepatic. Presence of microplastics in water promotes morphological alteration in hepatocytes nucleus size and shape of *Physalaemus cuvieri* tadpoles (Araújo et al., 2020) demonstrating that morphometrics parameters are a good tool to evaluate hepatotoxicity (Franco-Belussi et al., 2020b). Then, morphological changes can cause metabolic effects due tissue damage. Thus, the liver may thus be expected to be the primary targets of xenobiotics, showing an excellent biomarker of aquatic pollution in bullfrog tadpoles.

The IBR allowed to identify the specific sensitivity of each organ/tissue and the individual sensitivity of each biomarker. In the present study, the kidney was the most sensitive organ, with the highest IBR value, followed by the liver and muscles. Among the biochemical biomarkers, the non-enzymatic antioxidant system (MT and PCO) showed greater sensitivity to treatment, followed by GST > LPO > GPx > SOD > CAT > GSH. Individually, each organ/tissue presents a variation in the sensitivity of the antioxidant system (Liver: SOD > PCO > GST > CAT > GPx > LPO > MT ≫ GSH; Kidney: MT > LPO > PCO > CAT > GPx > GST > SOD > GSH; Muscle: MT > GST > GPx > SOD > GSH > LPO > PCO > CAT), as well as in response to treatment. In the liver, the sensitivity is greater for the water in the PIR (Summer.PIR > Winter.PIR > Winter.PI > Summer > PI), whereas the kidney has an opposite sensitivity (I.PI > V.PI > I.PIR > V.PIR), while the muscle presents intermediate sensitivity between the two organs (V.PI > I.PIR > V.PIR > I.PI). This demonstrates that each organ has a different sensitivity to treatment, with each biochemical biomarker responding differently according to the organ in which it is found.

Considering the morphological analyzes in the liver, we observed that the morphometric analyzes of hepatocytes are more sensitive than quantification of the melanin area (hepatocyte area > nucleus area > NCR > hepatocyte volume > nucleus volume > melanin area), in addition, the organ sensitivity in these analyzes is greater for the PIR (Summer.PIR > Winter.PIR > Winter.PI > Summer.PI). It is noteworthy that the liver's sensitivity pattern was the same for biochemical and morphological biomarkers, which indicates that despite being different analyzes, the organ presents the same response behavior when exposed to metals.

Finally, most biochemical parameters were lower in the winter in the three organs of bullfrog tadpoles, showing seasonal variations related to increasing metabolic rates during the summer and did not only correlate with seasonal variations in the metal concentrations in water or sediment. Biomarkers should be a useful tool to predict and prevent the risk of toxicity, oxidative stress induction and the mechanisms of toxicity, as well as limit exposure and prevent health risks in exposed populations.

5. Conclusion

The tadpoles were highly susceptible to water from the Sorocaba River, in both places (PI and PIR) and periods (i.e summer and winter) even in low concentrations of metals, thus could be a pollution sensitive bioindicator. Antioxidant enzyme and molecule measurements, as well as oxidative damage and morphology, showed that these responses were triggered after tadpoles' exposure to contaminated water. Finally, determining the extent and severity of water contamination by pollutants is difficult, but the results observed in tadpoles suggest monitoring the Sorocaba river.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2021.130000.

Credit author statement

Isabela Ferreira Fernandes, performed the experiments and analyzed the data. Heidi Samantha Moraes Utsunomiya, proposed and planned the experiments, performed the experiments and analyzed the data. Bruno Serra de Lacerda Valverde, prepared figures and/or tables, wrote, reviewed and approved the final draft of the manuscript. João Victor Cassiel Ferraz, performed the experiments and analyzed the data. Gabriel Hiroshi Fujiwara, performed the experiments and analyzed the data. Davi Marques Gutierres, performed the experiments and analyzed the data. Classius de Oliveira, prepared figures and/or tables, wrote, reviewed and approved the final draft of the manuscript. Lilian Franco Belussi, proposed and planned the experiments, prepared figures and/or tables, wrote, reviewed and approved the final draft of the manuscript. Marisa Narciso Fernandes, proposed and planned the experiments, prepared figures and/or tables, wrote, reviewed and approved the final draft of the manuscript. Cleoni dos Santos Carvalho, proposed and planned the experiments, prepared figures and/or tables, wrote, reviewed and approved the final draft of the manuscript. All the authors participated in discussion of the research.

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